Maize Alpha-Amylase: Purification and Properties and Induction by Gibberellic Acid

by

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Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
Master of Science
in
Botany

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May, 1987

Blacksburg, Virginia

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(ABSTRACT)

Alpha-amylase synthesis can be induced in wheat and barley half-seeds by addition of gibberellic acid (GA) to the incubation medium. In maize, induction in de-embryonated kernels by exogenous GA has been reported in some studies but not others.

Alpha-amylase induction was investigated in maize by measuring activity in extracts from whole and de-embryonated kernels incubated with and without GA during germination. Alpha-amylase activity was first detected on the 3rd day of germination in whole kernels and GA-treated endosperms and on the 4th day in the controls. Thereafter both whole kernels and GA-treated endosperms followed approximately the same time course in α-amylase activity with the control lagging a day behind. Studies indicated that maximum α-amylase activity occurred on the 7th day in whole kernels and GA-treated endosperms and the 8th in control endosperms.

Maize α-amylase was purified using differential solubility, column chromatography, glycogen precipitation and polyacrylamide gel electrophoresis, of these, the best purification method was glycogen precipitation.

Maize α-amylase exhibited isozymes. The isozyme patterns were qualitatively similar in all samples and throughout incubation. Wheat and barley α-amylase isozymes have been divided into two groups on the basis of a number of characteristics. Genetics

analysis revealed these isozymes to be the result of two multigene families. To shed light on the genetic basis of the maize α-amylase isozymes, physicochemical characterization was initiated. Studies of pH and temperature profiles and optima showed no differences between maize isozymes. The pH optima was pH 5 and the temperature optima was about 37°C.

Acknowledgements

There are many people who have helped me complete this work. I would first like to express my appreciation to my advisor, Asim Esen, for giving me this question to study and for his help in writing and revising this work. I would also like to acknowledge the Biology Department for giving me financial support throughout my studies.

I thank my committee for their assistance and advice. I thank Dr. Stout for reading and revising this work, Dr. Bates for his technical assistance and good straightforward advice, and Dr. Johnson who gave me good technical advice and moral support.

I would especially like to mention Dr Shokraii who gave me a good deal of technical and moral support and Drs. Benfield and Webster for the use of their photographic equipment and chemicals. I would also like to thank Dr. Lynn Lewis, who without her moral and technical support the isoelectric focusing would not be done and Adrien Malick for his advice and help with the immunological aspects of this project.

To my labmates Kamaruzaman Mohammad and Mahmoud Rifaat I am very grateful for their help and friendship. I would also like to thank Carrie, Marolyn and all my fellow graduate students for good ideas, stimulating conversations and for their support and friendship.

I am especially grateful to my husband, Mark, who stuck with me during difficult times and understood as only one who has been there can understand. I am also grateful to my parents, Bob and Ann Kane, who gave me an intense curiosity for the world around me and believed in me.

I finally would like to thank all who gave me words of encouragement and a sympathetic ear. God bless you.

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Introduction

In the initial stages of seed germination, seedlings depend upon nutrient reserves stored within the seed. Germination rates and seedling viability can be increased by fast and efficient hydrolysis and mobilization of carbohydrate and protein reserves in the endosperm. An increase in germination success and seedling viability may improve crop yields as well as lower the amount of seed to be sown.

To improve the speed and efficiency of carbohydrate reserve mobilization, the enzymes which are involved in hydrolysis must be understood. In a wide range of organisms, α-amylase (E.C.3.2.1.1) catalyzes the hydrolysis of starch into smaller glucose polymers. In plants, α-amylase is responsible for the first breakthrough of an intact starch granule (Hopkins, 1946). Alpha-amylase is especially important in cereal grains such as maize, because starch is the major component (80%) of the endosperm.

In plant physiology, α-amylase is important because of its induction by the plant hormone gibberellic acid. In fact the gibberellic acid induction of α-amylase in barley aleurone layers is well established and has served as a model for the study of hormonally-controlled gene expression (Chrispeels and Varner, 1967; Pagel, 1960; Yomo, 1960). Gibberellic acid induction has been reported in rice, wild oats, and wheat

(Miyata and Akazawa, 1982; Zwar and Hooley, 1986; Gale and Ainsworth, 1979). In maize, hormonal induction of α-amylase is still a matter of controversy (Ingle and Hageman, 1965; Goldstein and Jennings, 1975). One aspect of the present study is to assess the involvement of gibberellic acid in inducing maize α-amylase synthesis.

Alpha-amylase exists in multiple molecular forms, or isozymes in maize (Goldstein and Jennings, 1975). Isozymes are enzymes which catalyze the same reaction but differ structurally. Isozymes of an enzyme differ from one another in amino acid composition and in isoelectric points (Lehninger, 1975). Isozymes exist in different cellular organelles or tissues and may play a role in metabolic control (Goodwin and Mercer, 1983).

In barley and wheat the isozymes of α-amylase can be divided into two groups on the basis of a number of physiocochemical characteristics. Major antigenic, proteolytic fingerprint, time lag of appearance and requirement of gibberellic acid differences have been found between the two isozyme groups. In addition, the isoelectric points and pH optima differ among the isozyme groups of wheat and barley (Jacobsen and Higgins, 1982; Bog-Hensen and Daussant, 1974; Jacobsen et al., 1970; Tanaka and Akazawa, 1970).

Genetic analysis revealed two different sets of structural genes that encode barley and wheat α-amylase. Comparisons between the barley and wheat gene families have produced interesting conclusions. The genes encoding the barley and wheat low pI isozyme group are more similar to each other in several features than to the genes encoding the high pI isozyme group within the same species (Baulcombe et al., 1985).

These cross-species comparisons indicate that the two α-amylase gene families are old, dating back to before the evolutionary divergence of the two cereals (Baulcombe, 1985). This raises questions about when the two gene families originated and whether other less closely related species also have these families. This study addresses this question at the protein level by isolating and characterizing α-amylase and determining

the heterogeneity of α-amylase in a related species; that is, maize. The results should provide further insight into the evolution of the α-amylase multiple gene families in cereal grains.

Little is known about the maize α-amylase isozymes and their genetic basis. Scandalios (1966) found that maize α-amylase has isozymes. In later studies, Goldstein and Jennings (1978) were able to isolate 3 isozymes using ion-exchange chromatrography and isoelectric focusing. Further characterization of maize α-amylase isozymes and their genetic basis have not been done. Another aspect of this study was to purify maize α-amylase and to characterize some of its physiocochemical properties as a prelude to genetic analysis.

My hypotheses were:

1. Maize α-amylase activity can be induced in de-embryonated kernels and this activity is the same as found in whole kernels.

2. The properties and characteristics of maize α-amylase are similar to other cereal α-amylases.

Research was undertaken with the following specific objectives in mind:

1. To determine the time-course of α-amylase synthesis during seed germination and the relationship of gibberellic acid to the induction of maize α-amylase synthesis.

2. To perform comparative electrophoretic studies on whole kernel endosperms and gibberelic acid-treated and control endosperms to determine possible isozymic variations.

3. To determine possible tissue-specificity differences among the isozymes.

4. To purify α-amylase from germinating maize seeds and characterize it with respect to various physicochemical properties, and to obtain antibodies monospecific for α-amylase and use them in immunological characterization of the different isozymes.

Literature Review

The study of α-amylase has always been closely associated with the production of alcoholic beverages. Brewers discovered that when more dextrins were broken down into fermentable sugars, a more complete fermentation and a higher yield of alcohol were achieved (Hopkins, 1946). This finding stimulated early research into starch-degrading enzymes.

In 1833, Pazyen and Persoz gave the name diastase to the starch-degrading enzyme found in germinating barley. The term amylase is now preferred since it conforms to modern enzyme nomenclature. Marker (1879) concluded from thermal stability and acid inactivation studies that two distinct amylase enzymes exist in barley. This observation was confirmed by Lintner (1887) who identified the enzymes on the basis of how they degrade starch. He called the forms starch-saccharifying and starch-liquifying. The two forms have since been renamed, α-amylase for the starch-liquifying form and β-amylase for the starch-saccharifying form. In addition, Lintner found that the only form of enzyme present in ungerminated barley was β-amylase (Geddes, 1946).

Alpha-amylase is characterized by the hydrolysis of α-1,4 linked glucose polymers at most internal bonds. The cleavage is random and produces maltose, a mixture of

oligosaccharides and glucose (Bayer, 1972). The random breakdown of starch rapidly decreases the viscosity of starch pastes leaving products which do not react colorimetrically with iodine (Geddes, 1946).

Alpha-amylase is a metalloenzyme requiring calcium for stability and activity (Barman, 1969). In cereals, calcium is required for maximal enzyme synthesis and secretion. A concentration of 10 mM to 20 mM causes a four-fold increase in α-amylase secretion in germinating barley (Chrispeel and Varner, 1967). However, little is known about when calcium is needed in the synthesis and secretion process. An early hypothesis linked the need for calcium to diffusion of the enzyme through the cell wall (Varner and Mense, 1972). Other studies concluded that calcium stimulated secretion and release of amylase through the plasma membrane as well as facilitate diffusion through the cell wall (Moll and Jones, 1982). The most recent work shows that the cell wall is not involved in calcium-stimulated secretion (Bush et al., 1986). Other divalent ions can be substituted for calcium but with varying success (Chrispeels and Varner, 1967). High concentrations of strontium can substitute for calcium, but magnesium, barium and monovalent cations have no effect, while cadmium is inhibitory (Jones and Carbonell, 1984).

In barley and wheat, α-amylase can be divided into two groups on the basis of a number of characteristics. One of these characteristics is the requirement for calcium. In barley one α-amylase group is more affected by the addition of calcium than the other. Bush et al. (1986) found that calcium stimulated the release of one α-amylase group by only 3-fold, but causes a 30-fold enhancement in the other group. This enhancement does not occur during transcription, but acts during translation and secretion of the enzyme (Deikman and Jones, 1985 1986; Bush et al., 1986).

Alpha-amylase is very heat stable in the presence of calcium ions and withstands temperatures of up to 70°C for 15 min. (Kneed et al., 1943). Although α-amylase is

thermostable, it is sensitive to acidic conditions. It was inactivated if held at pH 3.3 for 15 min. (Gedded, 1946). Alpha-amylase from maize was completely inactived at pH 3.4 (Dure, 1960).

Beta-amylase does not reduce starch paste viscosity like α-amylase, but catalyzes the release of maltose from starch. This difference led to the early names starch-liquifying and starch-saccharifying enzyme for α-amylase and β-amylase, respectively (Geddes, 1946). Beta-amylase also hydrolyses α-1,4 glucan polymers like α-amylase but removes maltose units from the non-reducing ends of the chain (Barman, 1969). It successively hydrolyses the chain until there remains a non-reducing, high molecular weight dextrin. Residual dextrin reacts with iodine, producing a blue-violet or violet color (Geddes, 1946).

Cereal β-amylase differs from α-amylase in calcium requirement, thermal stability and acid inactivation (Kneed et al., 1946). These and other differences such as organic solvent solubility, ammonium sulfate solubility and heavy metal inactivation were used by Ohlsson (1930) to separate the two amylases (Caldwell and Doebbleing, 1935; Kneed et al., 1946).

In 1887, Lintner found that only β-amylase is present in ungerminated barley. Beta-amylase occurs in an inactive or latent form in wheat and barley (Rowsell and Goad, 1962; Daussant and Corvazier, 1970; Tronier and Ory, 1970). It is activated by disulfide reducing agents such as β-mercaptoethanol or proteases such as papain (Tronier and Ory, 1970). In rice, β-amylase is tightly bound to the periphery of starch granules in an inactive form (Okamoto and Akazawa, 1979). By contrast wheat, β-amylase is bound by disulfide bridges to the wheat protein glutenin (Daussant and Corvazier, 1970). As for α-amylase, its activity in germinating cereal grains is the result of *de novo* synthesis (Filner and Varner, 1967).

The molecular weight of mammalian, fungal, bacterial and plant α-amylase is between 45,000 and 60,000 daltons (Fischer and Stein, 1960). Among cereal grains the molecular weight is very consistent ranging from 42,000 daltons in barley to 50,000 daltons in sorghum (Rodaway, 1978; Botes et al., 1967). Maize α-amylase molecular weight is about 44,000 daltons, between the high and low molecular weights reported (Scandalios et al., 1978)

A putative precursor of α-amylase has been found in wheat, barley and rice (Okita et al., 1979; Higgins et al., 1976; Miyata et al, 1981). Okita et al. (1979) suggested that the 1,500 - 2,500 dalton fragment represents a signal sequence to facilitate the transport of of α-amylase from the endoplasmic reticulum of the aleurone layer cells to the endosperm. Support for this interpretation has come from studies of rice scutellar α-amylase biosynthesis (Miyata and Akazawa, 1982). Miyata and Akazawa (1982) found that a signal sequence was translated and then was cleaved from the growing protein by the time the polypeptide chain is about thirty percent (30%) completed. More recent studies of the endoplasmic reticulum and the Golgi apparatus have confirmed their roles in the synthesis and transport of α-amylase (Jones and Jacobsen, 1982). These studies further support the signal sequence hypothesis in α-amylase as has been the case with a large number of secreted proteins.

Many secreted proteins are also glycosylated. Glycosylation of α-amylase has been found in barley, rice, and sorghum (Rodaway, 1978; Miyata and Akazawa, 1982; Botes et al., 1967). Although glycosylation of α-amylase has been reported in many cereal grains, but differences in the degree of glycosylation have been reported.

The amino acid compositions and sequences of α-amylase from various sources are known. Comparisons of the amino acid compositions between organisms reveal few similarities (Fischer and Stein, 1960). Three sequence domains found in α-amylase are common to all organisms (Ihara et al., 1985). These sequence domains are similar in

conformation, surface charge and appear at similar intervals along the polypeptide (Rogers, 1985). Ihara et al., (1985) suggested that these regions represent active centers and hypothesized that the prokaryotic and eukaryotic active centers were the same. One of these domains appears to be a calcium binding site in barley (Rogers, 1985).

The gibberellic acid induction of α-amylase in barley aleurone layers is well established serving as a model for hormonally-regulated gene expression (Chrispeels and Varner, 1967; Pagel, 1960; Yomo, 1960). Gibberellic acid induction has been reported in rice, wild oats, and wheat (Miyata and Akazawa, 1982; Zwar and Hooley, 1986; Gale and Ainsworth, 1979). In maize, hormonal induction of α-amylase has been found in some studies but not in others (Ingle and Hageman, 1965; Goldstein and Jennings, 1975).

As currently hypothesized, induction begins when the embryo produces and secretes gibberellic acid into the endosperm. The gibberellic acid diffuses through the endosperm to the outermost layer of endosperm cells called the aleurone (Chrispeels and Varner, 1967). Many cellular changes occur here including extensive proliferation of the rough endoplasmic reticulum, distention of the cisternae and development of the intracellular membrane system (Jones, 1969a, 1969b, 1969c; Jones and Price, 1970). An increase in membrane-bound polysomes, total messenger RNA and α-amylase messenger RNA have also been found in gibberellic acid stimulated aleurone cells (Evins, 1971; Chandler et al, 1984).

The initiation site of α-amylase synthesis has been debated for over 100 years (Akazawa and Miyata, 1982). This controversy centers on whether the scutellum or the aleurone layer is the site of α-amylase synthesis initiation. Tangl (1866) reported that endosperm breakdown in rye begins in the scutellum, and only later is found in the aleurone layer. This was confirmed by Haberlandt (1890) and refuted by Brown and Morris (1890). Although Dure (1960) and Briggs (1964, 1972) have re-emphasized the

importance of the scutellum in α-amylase synthesis, most researchers have focused on the aleurone layer as the site of α-amylase synthesis (Akazawa and Miyata, 1982).

After addition of gibberellic acid to the medium, α-amylase is first detected at 6 to 8 hours (Chrispeels and Varner, 1967). There is a linear increase in α-amylase from 16 to 24 hours, after which synthesis levels off (Chrispeels and Varner, 1967). Greater than 50% of the protein synthesized during this time is α-amylase (Moser, 1980). Gibberellic acid is continuously required during the period of α-amylase synthesis and secretion (Chrispeels and Varner, 1967). Removal of gibberellic acid during the course of incubation results in a substantial reduction of α-amylase synthesis (Gregerson and Taiz, 1985).

The plant hormone, abscisic acid (ABA) reportedly inhibits the gibberellic acid-enhanced synthesis of α-amylase (Chrispeels and Varner, 1966). This inhibition can be partially overcome by the addition of a greater amount of gibberellic acid (Chrispeels and Varner, 1966). This antagonistic interaction between gibberellic acid and abscisic acid has been noted in other systems (Thomas et al., 1965; Nitsan and Lang, 1966). But the complexity of this interaction makes the reaction kinetics very difficult to interpret (Chrispeels and Varner, 1967).

The gibberellic acid enhanced induction of α-amylase synthesis increases with greater amounts of hormone until the maximal level is reached. A gibberellic acid concentration of 10^{-5} M produces maximal induction of α-amylase synthesis (Chrispeels and Varner, 1967). Higher amounts of gibberellic acid cause no further increase in α-amylase synthesis (Chrispeels and Varner, 1967).

The α-amylase activity detected in germinating cereal grains is the result of *de novo* synthesis (Briggs, 1963; Varner, 1964; Filner and Varner, 1967). In addition, the the messenger RNA that is specific for α-amylase is also synthesized *de novo* (Ho and Varner, 1974). Mozer (1980) reported that α-amylase messenger RNA accounts for

more than fifty percent (50%) of the proteins synthesized *in vivo* following 10 to 12 hours of hormone treatment.

Electrophoretic variants of α-amylase have been identified in barley, wheat, sorghum and maize (Tanaka and Akazawa, 1970; Ockelen and Verbeek, 1969; Callis and Ho, 1983; Frydenberg and Nielson, 1960; Marchylo et al., 1980; Gale and Ainsworth, 1984; Botes et al., 1967; Chao and Scandalios, 1969; Goldstein and Jennings, 1975). Electrophoretic variants or isozymes catalyze the same reaction but differ structurally. They differ in their amino acid composition and, thus, in their isoelectric points (pI). They are thought to exist in different cellular organelles or tissues and may play a role in metabolic control (Goodwin and Mercer, 1982). Isozymes can result from multiple alleles, multiple genes or may be post-translational modifications of a single polypeptide (Scandalios, 1968).

Of the cereal grains wheat, barley and maize, the maize isozymes and their genetic basis are the least understood. Scandalios (1966) found that maize α-amylase has isozyme polymorphisms and began to characterize them. Using gel electrophoresis, Scandalios (1969) separated two anodic bands which he identified as α-amylase. More recently, Goldstein and Jennings, (1978), isolated three maize α-amylase isozymes using ion-exchange chromatography and isoelectrofocusing. The isoelectric points (pI) reported for the α-amylase forms are 4.3, 4.95, and 5.05. All three forms exhibit broad pH activity profiles (Goldstein and Jennings, 1978). Maize α-amylase is a monomeric enzyme encoded by two co-dominant alleles and is genetically independent of β-amylase (Scandalios, 1969)

Barley α-amylase has four major isozymes which fall into two groups (A and B). Major antigenic differences and proteolytic fingerprint differences are found between group A and group B (Jacobsen and Higgins, 1982). The time lag of appearance for group B isozymes is longer than for group A and requires higher levels of calcium and

gibberellic acid (Jacobsen and Higgins, 1982). In addition, the isoelectric point and pH optima differ among the isozymes of barley α-amylase (Bod-Hansen and Daussant, 1974; Jacobsen et al., 1970; Tanaka and Akazawa, 1970). There are two different sets of structural genes that encode barley α-amylase. These are located at different loci on separate chromosomes (Brown and Jacobsen, 1982). Genes encoding the group A isozymes for barley α-amylase are located on chromosome 1 while those encoding group B isozymes are located on chromosome 6 (Muthukrishnan et al., 1984).

Wheat contains up to 30 isozymes of α-amylase which can also be divided into two distinct groups (I and II) (Nishikawa and Nobuhara, 1971). Group I isozymes are the higher isoelectric point group with pI's in the range of 6.5 to 7.5. These isozymes are the dominant forms during early incubation and then later decline as incubation proceeds (Nishikawa and Nobuhara, 1971). This group of isozymes is controlled by the α-AMY 1 locus located on chromosome 6 (Baulcombe, 1983). Group II isozymes of wheat α-amylase are the low isoelectric point isozymes with pI's in the range of 4.9 to 6.0 (Nishikawa and Nobuhara, 1971). They are found in small quantities in the beginning of incubation, but become the dominant form in later stages (Baulcombe, 1983). This group is coded for by the α-AMY 2 locus located on chromosome 1 (Nishikawa and Nobuhara, 1971).

In order to determine the mechanism for isozyme formation in cereal α-amylase, much work has centered on defining the heterogeneity of the wheat and barley α-amylase messenger RNA population. Muthukrishnan et al., (1984) showed that there is heterogeneity in the barley α-amylase messenger RNA although not enough heterogeneity to account for all the isozymes.

Recently, complementary DNA (cDNA) has been used to further probe the genetic basis of isozymes. Muthukrishnan et al. (1984) found that two mechanisms of isozymes formation are involved in barley; post-translational modifications and multiple genes.

Baulcombe (1983) using restriction digests and complementary DNA probes, located 24 regions which hybridize with the α-amylase probe in wheat. This is greater than the number of translation products found but still less than the actual number of isozymes. The genetic basis of isozyme formation in wheat probably involves both multiple gene families and post-translational modifications.

Comparisons between the barley α-amylase and the wheat α-amylase gene families have produced interesting conclusions. The genes encoding the barley and wheat low pI isozyme group are more similar to each other in sequence, N-termini and in the 5′ and 3′ noncoding regions than to the genes encoding the high pI isozyme group within the same species (Baulcombe, 1985). These cross species comparisons indicate that the two α-amylase gene families are old, dating back to before the evolutionary divergence of the two cereals (Baulcombe, 1985). To date, no data on the molecular basis of the maize α-amylase isozymes has been published.

Chapter One

The Role of Gibberellic Acid in the Induction of Maize Alpha-Amylase

Introduction

Alpha-amylase is a key enzyme in carbohydrate metabolism in plants and is important in the hydrolysis and mobilization of starch reserves in the seed during germination. This is especially true of maize in which starch is the major component of the endosperm. Alpha-amylase is produced in the aleurone layer in the cereal grains and secreted to the endosperm during germination (Chrispeels and Varner, 1967).

In barley, induction of alpha-amylase by gibberellic acid is well established and has served as a model system for the study of hormonally controlled gene expression (Chrispeels and Varner, 1967; Higgins et al, 1982). Gibberellic acid also induced the synthesis of alpha-amylase in wheat (Okita et al, 1979) and in rice (Miyata and Akazawa, 1982). In maize, hormonal induction of alpha-amylase synthesis has been reported by some researchers but not by other researchers. Induction of alpha-amylase

by gibberellic acid in maize has been observed in a study by Ingle and Hageman (1965). In contrast, work by Goldstein and Jennings (1975) indicates that exogenous gibberellic acid is not necessary for the induction of maize alpha-amylase. However, exogenous gibberellic acid induces or promotes earlier development of alpha-amylase activity. Goldstein and Jennings (1975) suggested that the endosperms of certain maize strains may possess an endogenous source of gibberellic acid.

Alpha-amylase exists in multiple molecular forms, or isozymes, in maize. Isozymes are enzymes which catalyze the same reaction but differ structurally. Isozymes exist in different cellular organelles or tissues and may play a role in metabolic control (Goodwin and Mercer, 1983). This study addresses the question of gibberellic acid induction of alpha-amylase in the maize inbred K55, the changes that occur in the isozyme pattern during induction and the tissue specific differences in the isozyme pattern. If isozyme pattern differences are detected over the course of induction this might indicate differential regulation of the α-amylase genes in maize. Two isozyme groups are found in wheat and barley and are due to two sets of structural genes. Determining differences in the maize α-amylase isozymes may give clues to their genetic basis.

Materials and Methods

Extraction of Alpha-Amylase

Lyophilized endosperms were ground to a fine powder in a Wiley Mill. The powder was dispersed using a 10 to 1 volume to weight ratio in a buffer containing 10 mM Tris-HCl, 1 mM $CaCl_2$ and 2 mM Phenylmethylsulfonyl fluoride (PMSF) for the α-amylase extraction. Extraction was performed at 4°C for a period of 14 to 18 hrs.

Extracts were then centrifuged at 10,000xg for 15 min. to remove the debris. The supernatant was removed and heated to 70°C for 16 min. to inactivate the β-amylase (Kneed, 1946). After heating, the extract was quickly cooled and centrifuged at 10,000xg for 15 min. This supernatant fraction was used as an α-amylase source in further studies.

Measurement of Alpha-Amylase Activity

The α-amylase activity was measured using a modification of the Varner and Mense (1972) spectrophotometric assay. The modifications involved scaling down the procedure to perform it in microtiter plates and measuring the absorbance at 600 nm. Use of the microtiter plates allowed replicates to be performed at the same time and read almost simultaneously on an ELISA reader (a modified spectrophotometer, (Dynatech MR600). Enzyme activity was measured in units defined as: ΔA600 nm/min. x ml. To convert the readings to arbitrary units the following formula was applied:

$$\text{unit} = \frac{1000}{\text{vol. of enzyme soln.}(\mu l)} \times \frac{1}{\text{length of incubatn.}} \times (\text{initial A600nm - final A600nm})$$

*based on Varner and Mense (1972) as revised by R. Hammerton (unpublished)

Time Course of Alpha-Amylase Activity in Kernels

Kernels were surface sterilized using 1.5% hydrogen peroxide for 10 min., followed by commercial strength bleach (5.25%) for 20 min. and Fungizone (20 ug/ml) for 10 min. The sterilized seeds were then rinsed in sterile distilled water five times and germinated in a covered tray of vermiculite and water. Each day ten seeds were removed from the tray; the endosperm was separated from the remainder of the seed and then freeze-dried. Alpha-amylase was extracted from the lyophilized endosperms.

Gibberellic Acid Induction

Whole seeds were surface sterilized as previously described and the germ asceptically excised. The de-embryonated kernels were again surface sterilized as previously decribed and placed on 1% agar plates containing chloramphenicol (20 ug/ml), Genomysin (50 ug/ml) and Fungizone (20 ug/ml). The gibberellic acid plates additionally contained 10^{-6} M gibberellic acid in the form of gibberellic acid 3 (Sigma). Plates were sealed with parafilm and incubated at room temperature. At specific time intervals endosperms were harvested and lyophilized; α-amylase was extracted from the freeze-dried and ground endosperms.

Protein Determination

The protein content of the samples was determined using a modification of the Bradford (1976) procedure. This procedure determines the protein content on the basis

of Coomassie Blue dye binding in free solution. The modification involved scaling down the amount of sample and reagent for use in microtiter plates. *Bacillus subtilis* alpha-amylase was used as a standard.

Fixed pH Electrophoresis

Native and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed according to the Ornstein and Davis (1964) and Laemmli (1970) procedures, respectively. Gels (7-14%, nongradient) were run using a vertical Protean Cell (BioRad) electrophoresis unit. Protein bands on the SDS-PAGE gels were visualized using a Commassie Blue stain dissolved in isopropanol in lieu of ethanol.

Native gels were stained for α-amylase activity using starch and iodine solutions. Upon completion of a run the gels were immersed in a 2 - 5% starch solution, depending upon the thickness and density of the gel for one-half to two hours. The gel was rinsed and placed in an iodine solution (0.1% iodine and 1% potassium iodide) to visualize the clear α-amylase bands against the dark-blue background formed by the starch-iodine complexes.

Isoelectric Focusing

Isoelectric focusing was carried out on a flat bed apparatus (Pharmacia) using polyacrylamide gels. The pH ranges of the gradients used were either 3-10 or 3.5-6.5. Ampholytes were purchased from LKB.

Visualization of α-amylase bands was carried out by incubating the gel in 2% starch solutions for one-half to one hour. The gels were then rinsed and placed in an

iodine solution (0.1% iodine, and 1% potassium iodide). The pH gradient was determined using a surface electrode prior to staining for α-amylase activity.

Results

Gibberellic acid induction of maize alpha-amylase

Alpha-amylase activity was monitored in de-embryonated maize kernels incubated with and without gibberellic acid to determine the effects of this plant hormone on the synthesis of α-amylase. The time course profile of α-amylase activity in the de-embryonated kernels is summarized in Figure 1.

There was no α-amylase activity in the endosperm of the dry kernels. Alpha-amylase activity was first detected in the gibberellic acid treated endosperms after 3 days of incubation. Activity was not detected in the controls until the 4th day. In both treated and control endosperms there was a slow initial rise in the level of activity. In the treated endosperms the level of α-amylase activity dramatically increased between the 4th and the 7th day while in the control the increase began about the 6th day and continued to the 8th day. In both cases, the increase in activity leveled off and even slightly decreased beyond the 8th day.

The greatest difference in activity between the gibberellic acid-treated and the control endosperms was observed in the period from the 5th to the 8th day of incubation. During this time the gibberellic acid-treated endosperms show a 2 to 3 fold greater activity than the control endosperms. The activity levels were similar before the 5th day and after the 8th day of incubation.

Time course of alpha-amylase synthesis in whole kernels

Injury of plant tissues can often bring about synthesis of stress and injury related proteins and enzymes. In this study comparisons were made between the whole, intact kernels and the de-embryonated endosperms to determine if the time course of α-amylase was similar between the two systems. Consequently, alpha-amylase was monitored in developing maize kernels. The time course pattern is summarized in Figure 2. Activity was first detected on the third day of incubation as was found in the gibberellic acid treated endosperms. The activity rose slowly initially but dramatically increased from the 5th to the 7th day. The increase in activity leveled off and dropped after the 7th day. This pattern of development essentially parallels that found in the gibberellic acid treated endosperms.

Electrophoretic analysis of induction

Buffer extracted α-amylase was electrophoresed under native conditions to reveal starch-degrading bands upon activity staining. Concomitant with the development of α-amylase activity was the appearence of a set of starch-degrading bands in electrophoretic profiles. Three bands were detected in native polyacrylamide gels, both fixed pH and isoelectric focusing gels, when stained with an activity stain (Figure 3). Their relative mobility rates are about 0.56, 0.58

and 0.60. The two more anodic of the 3 bands are usually more intense than the least anodic band but all increase in intensity as incubation time increases.

The starch-degrading bands were first detected on the 3rd day of incubation in the gibberellic acid treated endosperms (Figure 3) and whole kernels endosperms (Figure 4) and on the 4th day in the control endosperms (Figure 3). The full complement of three bands was observed in the initial detection. Further incubation did not produce more bands but did produce increased intensity of the existing bands. Electrophoretic analysis showed no differences in the banding patterns of the samples from the control, the gibberellic acid-treated and the whole kernels.

Beta-amylase activity band

Earlier studies of starch-degrading bands did not focus on identification of α-amylase and β-amylase bands from each other. Electrophoretic analysis of un-heated samples and heated samples allowed easy identification of the β-amylase band in native fixed pH gels. Figure 5 (arrow) clearly shows the β-amylase band before heating and its disappearance after heating.

Tissue-specificity of the maize alpha-amylase isozyme bands

Electrophoretic variants or isozymes are thought to exist in different cellular organelles or tissues. To determine the tissue specificity of maize α-amylase

isozymes, the enzyme was extracted from various tissues and subjected to native gel electrophoresis. These studies revealed that the isozyme pattern found in the endosperms was also found in roots, shoots and embryos of 7-day old seedlings (Figure 5). The three α-amylase bands are present in all tissues. There were differences in intenstiy levels between the tissues with the embryo having the greatest intensity and the endosperm and shoot having less activity but the isozyme pattern was identical in all tissues. Note that there was an inactivation of α-amylase in shoots after heating the sample. Before heating all three bands are present as well as the β-amylase band and after heating they are absent. This inactivation was found only in the shoots and not in any other tissues.

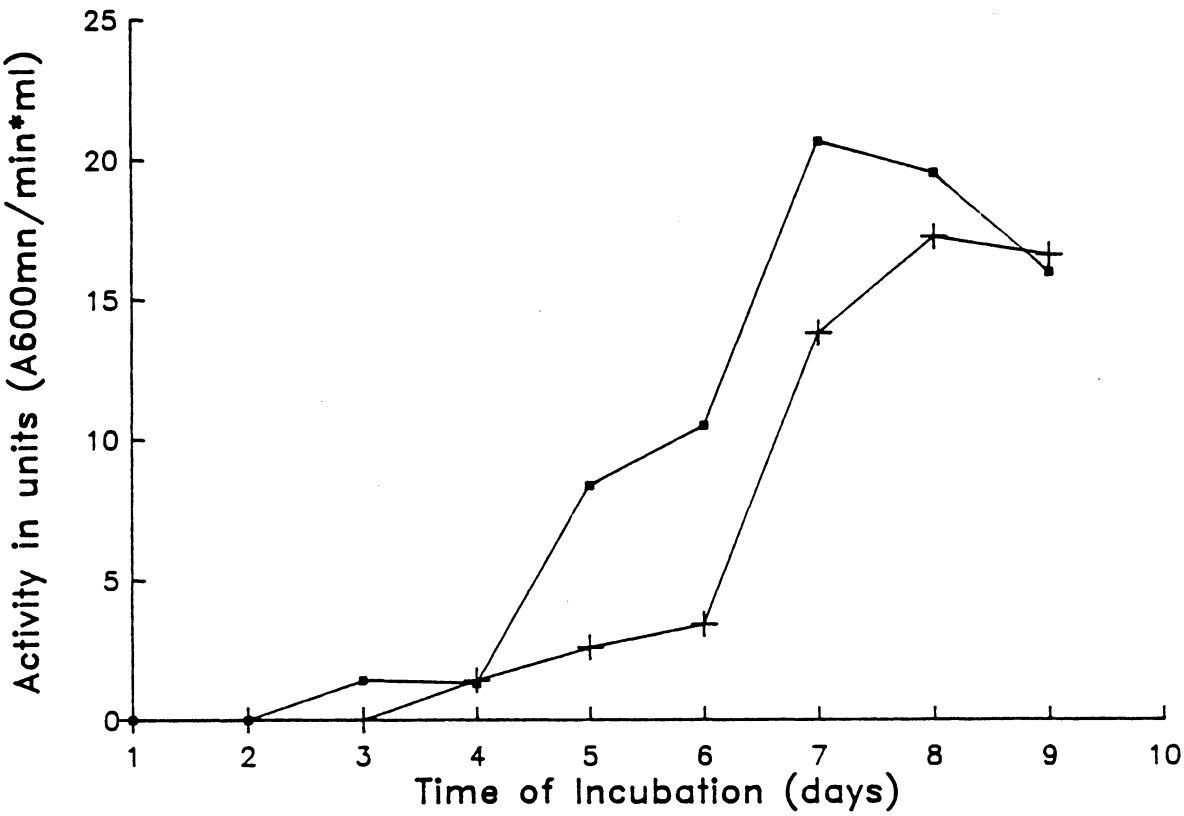

Figure 1. Gibberellic Acid Induction of Maize Alpha-Amylase: Alpha-amylase activity for de-embryonated maize endosperms incubated with gibberellic acid (•) and without gibberellic acid (+). Activity was first detected in the gibberellic acid treated endosperms on the 3rd day and in the control on the 4th day. Alpha-amylase activity dramatically increases in the treated endosperms from the 4th to the 7th day and from the 6th to the 8th day in the control. Note the 2 to 3 fold difference in activity levels on the 5th day between the gibberellic acid endosperms and the control endosperms. This difference decreases as incubation time increases.

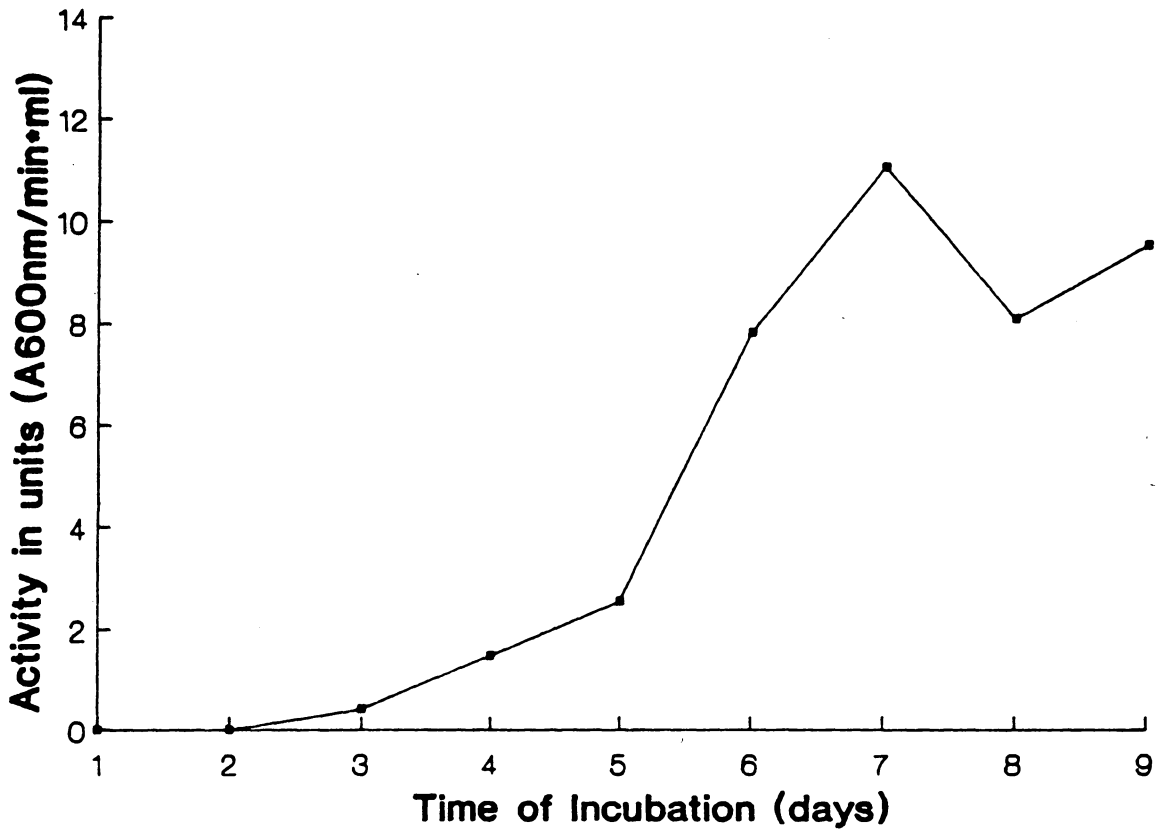

Figure 2. Time Course of Alpha-Amylase Synthesis in Maize Kernels: Alpha-amylase activity in whole maize kernels was followed during germination and was first detected on the 3rd day, increased until the 7th day of germination and decreased thereafter.

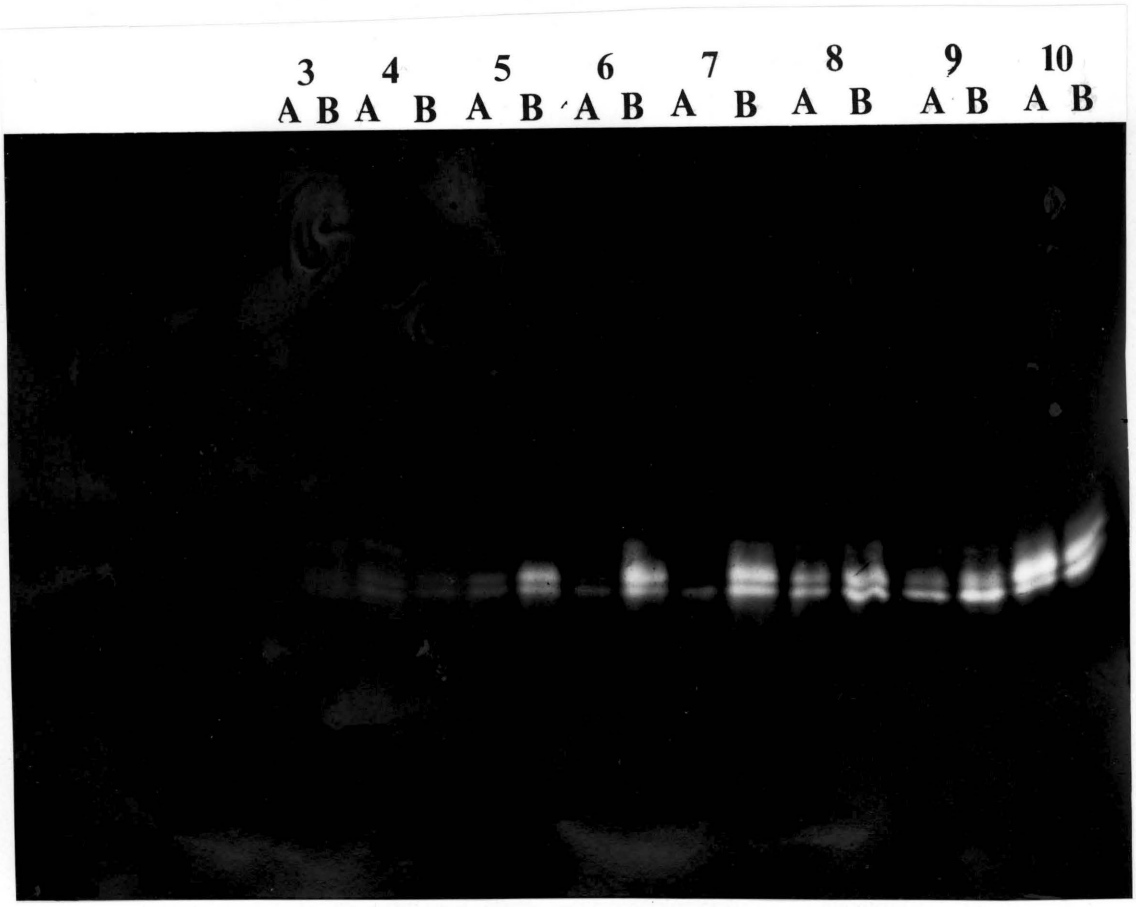

Figure 3. Native gel of α-amylase activity in de-embryonated kernels: The starch-degrading bands of α-amylase are found on an alkaline gel (pH 8.8). (A) Control endosperms and the (B) gibberellic acid treated endosperms are shown. The numbers indicate the amount of time the samples were incubated in days. Note that the starch-degrading bands first appear in the gibberellic acid treated sample on the 3rd day and that the full complement of bands was observed in the initial detection. Further incubation increased the intensity of the existing bands but produced no new bands.

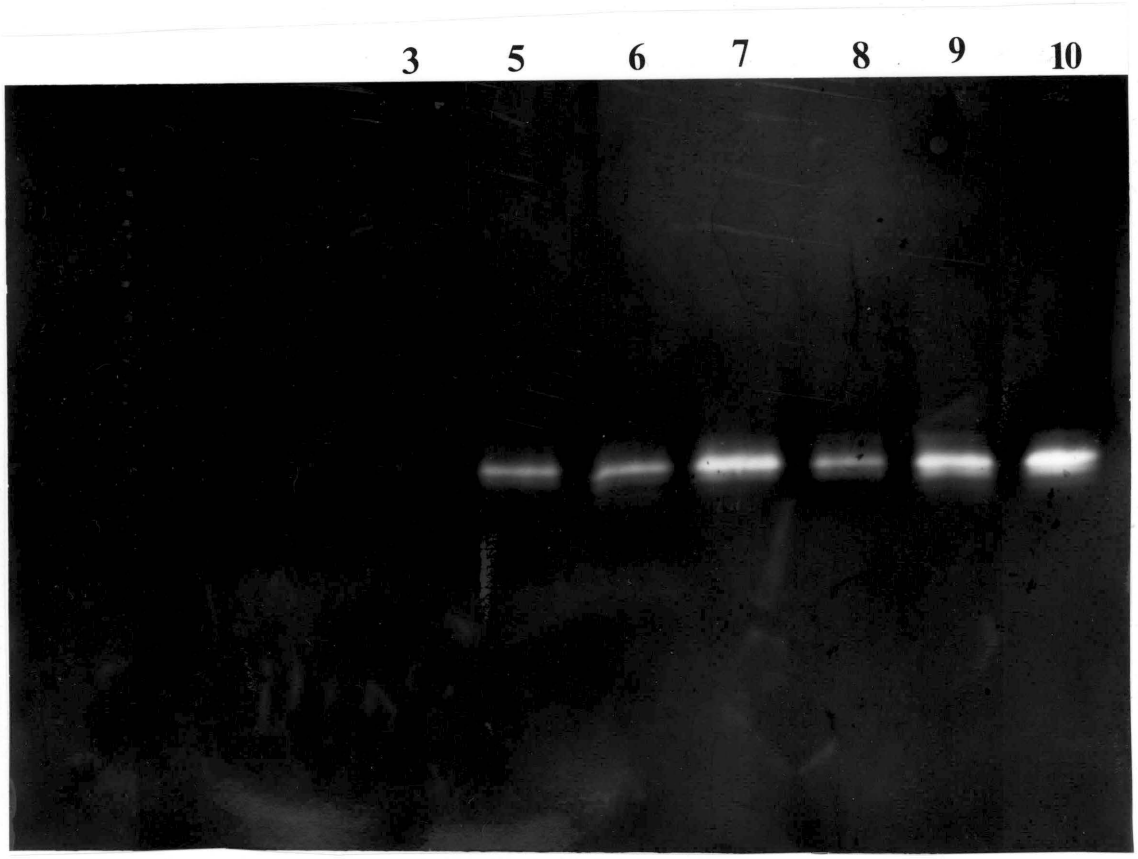

Figure 4. Native gel showing α-amylase activity bands in whole kernels: The numbers indicate the amount of time the samples were incubated in days. Electrophoretic analysis reveal no differences between the whole kernel endosperms, the gibberellic acid treated endosperms and the control endosperms.

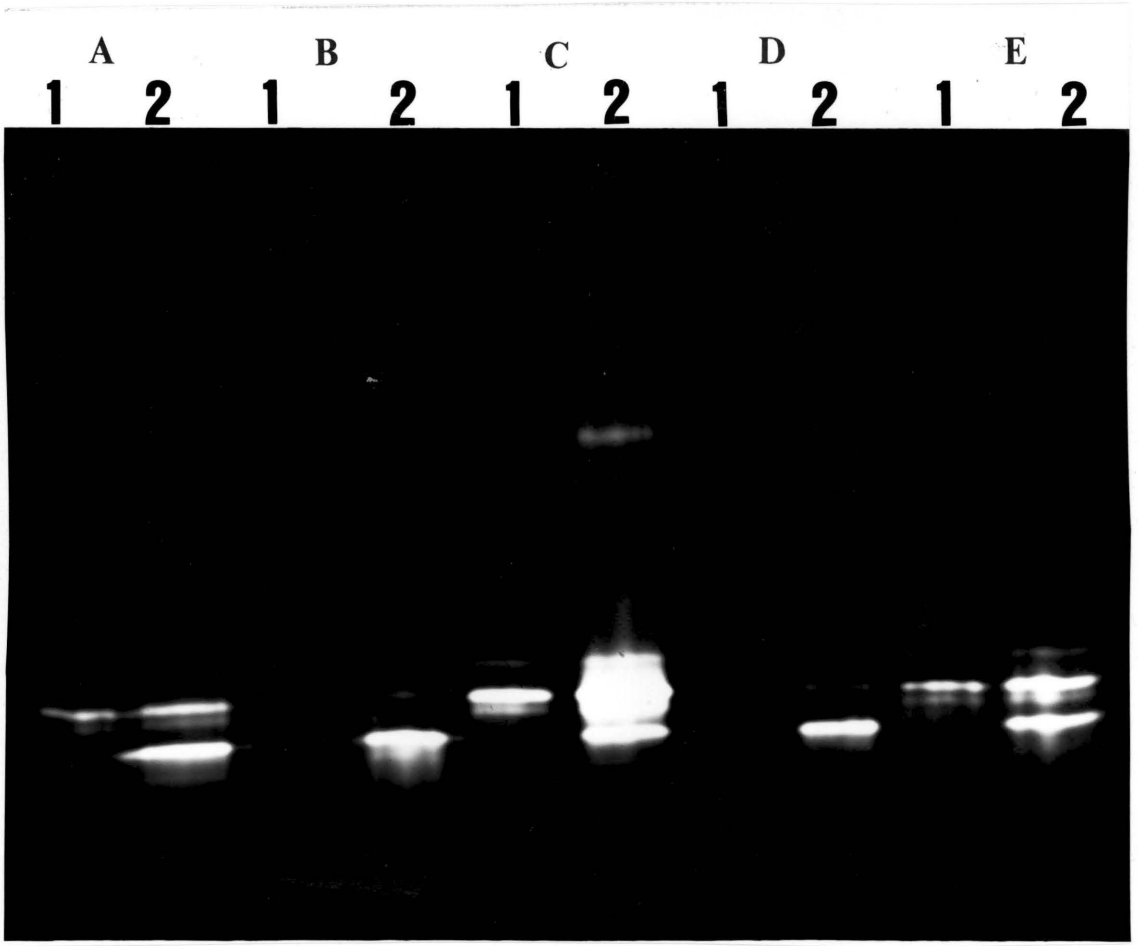

Figure 5. Tissue Specificity of Maize Alpha-Amylase: The alpha-amylase banding pattern is shown in the root (A), shoot (B), embryo (C), endosperm (D) and roots and shoots grown in no light (E). Note the disappearance of the lower β-amylase band in the heated samples (1) as opposed to the unheated samples (2) in every tissue. The banding pattern in all tissues is qualitatively the same but differ in intensities.

Discussion

An increase in alpha-amylase activity was found in de-embryonated maize seeds (Goldstein and Jennings, 1975; Dure, 1960; Ingle and Hageman, 1965). The question is whether the increase in activity is induced by the plant hormone gibberellic acid. Studies by Dure (1960) and Ingle and Hageman (1967) suggest that exogenous gibberellic acid is necessary for the induction of alpha-amylase. Goldstein and Jennings (1975) and Harvey and Oaks (1974) refuted these findings stating that the development of activity is independent of an exogenous source of gibberellic acid. The data from my studies are in agreement with the findings of Goldsetin and Jenning (1975) and Harvey and Oaks (1974). In other words, an exogenous source of gibberellic acid is not necessary for the development of alpha-amylase activity in maize.

Goldstein and Jennings found that alpha-amylase activity was first detected on the second day of incubation, which was confirmed by Harvey and Oaks (1974). Dure's (1960) study showed that activity was first detected on the third day of incubation. In this study activity was also first detected on the third day. This difference may be due to differences in the incubation temperatures. Goldstein and Jennings (1975) incubated at 30°C and Harvey and Oaks (1974) at 28°C while Dure's work and this study were

carried out at room temperature (23°C). The difference in detection times may also be a reflection of the cultivars used in the studies.

Assuming hormonal induction of α-amylase is a requisite, Goldstein and Jennings (1975) postulated the existence of an endogenous source of gibberellic acid. They suggested that the amount of exogenous gibberellic acid may be the critical factor in hastening the development of alpha-amylase activity. This was supported in their study by the delayed onset and slower development of activity observed in the untreated endosperms. This hypothesis was also supported by the results of this study where the effect of exogenous gibberellic acid appears to be quantitative rather than qualitative. Exogenous gibberellic acid appeared to cause earlier onset of alpha-amylase activity in the earlier stages of incubation. This enhancing effect increases as incubation time increases. The untreated endosperms show a delay in initial activity onset and slower activity development but exhibit full expression of activity as incubation time increases.

Although the alpha-amylase activity was delayed in development without exogenous gibberellic acid, it appears that the endogenous level of gibberellic acid in de-embryonated endosperms is sufficient for the full development of activity. Goldstein and Jennings (1975) speculated that some tissue in the endosperm possesses the ability to synthesize gibberellic acid. This synthesis would bring the endogenous level of gibberellic acid up to the critical level needed for maximal induction. This suggestion is supported by the work of Atzorn and Weiler (1983) who found using antibodies against gibberellic acid that the aleurone layer synthesizes some gibberellic acid. An alternative hypothesis proposed by Harvey and Oaks (1974) involves an endogenous inhibitor. This inhibitor leaches out, lowering the level of gibberellic acid necessary for alpha-amylase induction.

Two observations suggest that although exogenous gibberellic acid is not necessary for synthesis of alpha-amylase, it is involved in the mechanism of induction. One ob-

servation is that abscisic acid acts antagonistically to gibberellic acid, it inhibits the synthesis of alpha-amylase in maize endosperms. The inhibition can be overcome by the addition of gibberellic acid (Harvey and Oaks, 1974). Dwarf corn endosperms normally deficient in endogenous gibberellic acid show a good response to addition of gibberellic acid. Thus, the differences in results in the various studies can be readily explained by differences in the endogenous levels of gibberellic acid between cultivars of *Zea mays.*

The isozyme pattern of alpha-amylase was similar between the gibberellic acid treated and untreated endosperms. Comparisons with kernel endosperm activity patterns were also indicated remarkable similarities. These data indicate that the activity seen in the untreated and treated endosperms is due to the same isozymes and not to differential expression of one isozyme over another. Goldstein and Jennings (1975) also found the activities showed excellent similarities in reaction products and in physicochemical characteristics. In RNA inhibitor studies they showed that the alpha-amylase activity is due to *de novo* synthesis of the enzyme.

In other cereal grains , alpha-amylase activity is also the result of *de novo* synthesis. This activity in wheat and barley is divided into two alpha-amylase fractions or isozyme groups which can be separated on the basis of a number of characteristics including lag time of appearence and response to exogenous gibberellic acid (Jacobsen and Higgins, 1982). In barley and wheat, one group of isozymes, the low pI isozymes can be found in unstimulated aleurone layer cells and in gibberellic acid insensitive mutants (Baulcombe et al., 1983 and MacGregor, 1976). Synthesis of these isozymes can be enhanced by the addition of gibberellic acid into the medium. These observations correlate well with maize alpha-amylase. The pattern of development in germinating grains of this isozyme group and the maize group is strikingly similar. Perhaps the low pI isozyme group of wheat and barley and the maize isozymes share a common genetic basis.

In wheat and barley the genes for the low pI isozyme family have been isolated. Comparisons in the sequences between wheat and barley show greater similarities in the coding region and in the 5′ and 3′ noncoding regulatory regions between the two species than in comparisons to the high pI isozyme genes within the same species. This demonstrated a common protein structure as well as a common regulatory mechanism in the low pI isozyme group genes of the two species. But the question of how maize alpha-amylase fits into the scheme remains to be answered. Perhaps the answer lies in further characterization of the maize isozymes and their genetic basis.

Chapter Two

The Purification and Properties of Maize Alpha-Amylase

Introduction

A starch-degrading enzyme in barley was first found in 1833 by Pazyen and Persoz, who gave it the name diastase. Since that time the enzyme has been renamed amylase to conform to modern enzyme nomenclature. It is characterized by the hydrolysis of α-1,4 linked glucose polymers at most internal bonds. The random cleavage produces maltose, a mixture of oligosaccharides and glucose (Barman, 1969).

An interesting aspect of cereal α-amylase is its induction by the plant hormone gibberellic acid. This induction is well established in barley aleurone layers, serving as a model for hormonally-regulated gene expression (Chrispeels and Varner, 1967; Pagel, 1960; and Yomo, 1960). Gibberellic acid induction has been reported in rice, wild oats, and wheat (Miyata and Akazawa, 1982; Zwar and Hooley, 1986; and Gale and

Ainsworth, 1979). In maize, hormonal induction of α-amylase is a matter of controversy.

Isozymes or electrophoretic variants of α-amylase have been found in barley, wheat, sorghum and maize (Tanaka and Akazawa, 1970; Ockelen and Verbeek, 1969; Callis and Ho, 1983; Frydenburg and Nielson, 1960; Marchylo et al., 1980; Gale and Ainsworth, 1984; Botes et al., 1967; Chao and Scandalios, 1969; Goldstein and Jennings, 1975). Isozymes catalyze the same reaction but differ in amino acid composition and thus in their isoelectric points (pI). They may exist in different tissues or cellular organelles and may play a role in metabolic control (Goodwin and Mercer, 1982). Goldstein and Jennings (1978) found three isozymes of maize α-amylase with pI's of 4.3, 4.95 and 5.05. All three exhibit broad pH activity profiles (Goldstein and Jennings, 1978).

Researchers have sought to isolate and purify cereal α-amylases by a number of techniques including; differential solubility in organic solvents and salts, glycogen precipitation, column chromatography, and electrophoresis (fixed pH native and SDS, and isoelectric focusing). This study attempts to determine a means of purifying maize α-amylase and once purified to characterize it's physicochemical properties. It also includes isozyme separation and characterization studies.

Materials and Methods

Extraction of Alpha-Amylase

Lyophilized endosperms were ground to a fine powder in a Wiley Mill. The powder was dispersed using a 10 to 1 volume to weight ratio in a buffer containing 10 mM Tris-HCl, 1 mM $CaCl_2$ and 2 mM Phenylmethylsulfonyl fluoride (PMSF) pH 7.5 for the α-amylase extraction. The samples were gently agitated for a period of 14 to 18 hrs. at 4°C.

Extracts were then centrifuged at 10,000xg for 15 min. to remove the debris. The supernatant was removed and heated to 70°C for 16 min. to inactivate the β-amylase (Kneed, 1946). After heating, the extract was quickly cooled and centrifuged at 10,000xg for 15 min. This supernatant fraction was used as an α-amylase source in further studies.

Measurement of Alpha-Amylase Activity

The α-amylase activity was measured using a modification of the Varner and Mense (1972) spectrophotometric assay. The modifications involved scaling down the procedure to perform it in microtiter plates and measuring the absorbance at 600 nm. Use of the microtiter plates allowed replicates to be preformed at the same time and read almost simultaneously on an ELISA reader (a modified spectrophotometer, (Dynatech MR600). Enzyme activity was measured in units defined as: ΔA600 nm/min. x ml. To convert the readings to arbitrary units the following formula was applied:

$$\text{unit} = \frac{1000}{\text{vol. of enzyme sol.}(\mu l)} \times \frac{1}{\text{length of incubatn.}} \times (\text{initial A600nm - final A600nm})$$

*adapted from Varner and Mense (1972) by R. Hammerton (unpublished).

Protein Determination

The protein content of the samples was determined using a modification of the Bradford (1976) procedure. The procedure measures protein content by Coomassie Blue dye binding in free solution. The modification involved scaling down the amount of sample and reagent for use in microtiter plates. *Bacillus subtilis* α-amylase was used as a standard.

Fixed pH Electrophoresis

Native and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed according to the Ornstein and Davis (1969) and Laemmli (1970) procedures, respectively. Gels (7-14%, nongradient) were run using a vertical Protean Cell (BioRad) electrophoresis unit. Protein bands on the SDS-PAGE gels were visualized using a Commassie Blue stain dissolved in isopropanol in lieu of ethanol.

Native gels were stained for α-amylase activity using starch and iodine solutions. Upon completion of a run the gels were immersed in a 2 to 5% starch solution, depending upon the thickness and density of the gel for one-half to two hrs. The gel was rinsed and placed in an iodine solution (0.1% iodine and 1% potassium iodide) to visualize the clear α-amylase bands against the dark-blue background formed by the starch-iodine complexes.

Isoelectric Focusing

Isoelectric focusing was carried out on a flat bed apparatus (Pharmacia) using polyacrylamide gels. The pH ranges of the gradients used were either 3-10 or 3.5-6.5. Ampholytes were purchased from LKB.

Visualization of α-amylase bands was carried out by incubating the gel in 2% starch solutions one-half to one hr. The gels were then rinsed and placed in an iodine solution (0.1% iodine, and 1% potassium iodide). The pH gradient was determined using a surface electrode prior to staining for α-amylase activity.

Ion-Exchange Chromatography

The clear supernatant after heating to 70°C and spinning to remove the precipitated proteins was applied to a DEAE cellulose column which had been prepared according to Peterson and Sober (1962). The column was equilibrated with 10 mM Tris-HCl and 1 mM $CaCl_2$ pH 7.5, the same as the extraction buffer, and was eluted with a linear salt (NaCl) gradient from 0.1 M to 1.0 M. The eluate was collected in 10 ml fractions (flow rate of 2.5 ml/min) and the fractions with α-amylase activity pooled, dialyzed against 50 mM ammonium bicarbonate and 50 uM $CaCl_2$ and freeze-dried.

Immunological Studies

An acetone precipitate, obtained by 70% acetone precipitation of the heated α-amylase extract, which is enriched for α-amylase was electrophoresed on a 10% preparative SDS-PAGE gel and the band representing α-amylase was cut and dehydrated with alcohol. Before injection into a rabbit the band was rehydrated in 8 M urea, homogenized and mixed with Freund's adjuvant (alternating an injection in Freund's

complete and then incomplete). Booster injections were administered every two to three weeks. Bleedings were taken just prior to booster injections and antibody titer tested using an enzyme linked immunosorbant assay (ELISA) method (Engvall and Perlmann, 1972)

ELISA tests were conducted according to the procedure of Conroy and Esen (1984). Antigen prepared in a phosphate buffer was allowed to bind overnight to microtiter plates and was then reacted with protein A peroxidase and the peroxidase substrate (ABTS), respectively. The absorbance of the peroxidase reaction product was read using a Dynatech MR600 ELISA Reader.

Organic Solvent Precipitation

Precipitation studies were performed to determine at what percent organic solvent the α-amylase begins to precipitate from solution. Using freshly extracted and heated α-amylase samples, the same amount of extract was added to 1.5 ml polypropylene tubes. Ice cold acetone or ethanol (absolute) was added to the tubes to make the final concentration of organic solvent 10, 20, 30, 40, 50, 60, 70, and 80 percent in the different tubes. After gentle agitation the tubes were allowed to stand in the cold for one-half to an hr. and were then centrifuged for 15 min. (12,000 rpm) in the cold. The supernatant was discarded, the pellets were dried and resuspended in extraction buffer and then tested for α-amylase activity.

Precipitation of Alpha-Amylase by Ammonium Sulfate

Ammonium sulfate precipitation studies were undertaken to determine the concentration of salt required to precipitate α-amylase. Aliquots of equal volumes of an

α-amylase sample were placed in 1.5 ml polypropylene tubes and saturated ammonium sulfate was added. Ammonium sulfate was added up to the 20, 40, 60, and 80 percent final concentrations in different tubes. The tubes were gently agitated and then allowed to stand in the cold for one-half to an hr. The tubes were then centrifuged in a microfuge, the supernatant discarded and the pellet redissolved in the extraction buffer. The resuspended pellet was tested for α-amylase activity.

Glycogen Precipitation of Alpha-Amylase

Alpha-amylase was extracted from lyophilized maize endosperms as previously described. The extract was then brought up to 35% alcohol and was quickly centrifuged to remove precipitating proteins. The α-amylase was then precipitated from the supernatant by addition of 2% (w/v) oyster glycogen (Sigma) and incubation of the mixture for one hr. in the cold. The α-amylase-glycogen complexes were pelleted by centrifugation at 10,000 g for 10 min. and then resuspended in 2 mM $CaCl_2$.

pH Optima Determination in Gel Slices

The pH optima of α-amylase isozymes was tested in IEF gels to determine whether there were significant differences in the pH optima among the four major isozymes. Native IEF gels (3.5-6.5) were run as previously described using an extract enriched in α-amylase activity. After electrophoresis the gel was cut into slices and each slice was placed in a buffer solution of different pH. The universal buffer described by Antibus et al., (1986) was used to minimize changes due to differences in the salts used. The gel slices were equilibrated for 15 min. in the buffers and then the 2% starch solutions mixed in the appropriate pH buffers were added. These were incubated at 37° C for one-half

to three-fourths hr., rinsed and the iodine solution was added (0.1% I_2 and 1% KI) to visualize the bands.

Temperature Optima Determination in Gel Slices

The temperature optima of α-amylase was tested in IEF gels to determine if there were significant differences among the four major isozymes. The 2% starch solutions were aliquoted, placed at the desired temperatures and allowed to equilibrate for one hr.. A native IEF gel (3.5-6.5) using a fraction enriched for α-amylase activity was run and then cut into slices. The slices were placed in the different temperature solutions and equilibrated at those temperatures for 15 min. The buffer was then removed and starch added and the slices again incubated at the desired temperatures for one-half to an hr. The starch was then removed, gels rinsed and the iodine solution added to visualize the α-amylase bands.

pH Optima Determination in Free Solution

A universal buffer (Skujins et al., 1962) containing several kinds of salts was used to minimize differential salt stabilities of α-amylase. The universal buffer hydrogen ion concentration was changed to pH's in the range of 3 to 11. The α-amylase extract was then added to the buffers and the pH measured to ensure that no shift in pH had occurred due to the addition of the extract. The buffer and extract solutions were allowed to equilibrate for 15 min. and then the solutions were assayed for α-amylase activity. No buffering was used in the starch spectrophotometeric assay reagent to ensure that pH's remained constant over the assay period.

Temperature Optima Determination in Free Solution

A water or ethylene glycol bath was used to equilibrate the α-amylase sample and the starch substrate at the desired temperatures. Samples and substrate were allowed to equilibrate for 15 min. before assaying for α-amylase. During the assay the solutions were held at the equilibration temperatures to ensure correct readings.

Western Blotting

Analytical SDS-PAGE gels (10%) were electroblotted onto nitrocellulose (Fisher) using a BioRad Model 250 unit. These blots were either reacted with antibodies raised to maize α-amylase or stained with india ink according to Hancock and Tsang (1983) to visualize protein bands.

Ouchterlony Double Immunodiffusion Assay

Ouchterlony testing was used to try to determine antigenic differences among the four major isozymes. The Ouchterlony procedure was carried out on 1% agarose plates containing 0.1% sodium azide to prevent contamination. Antigen and antibody were routinely diluted with a phosphate buffer (PBS). When denatured antigen was used, antigen was mixed in 6 M urea or 0.1% SDS.

Immunoelectrophoresis

Immunoelectrophoresis of α-amylase was used to determine antigenic differences among the four major isozymes. Immunoelectrophoresis was carried out according to

manufacturers specifications on a Helena Laboratories apparatus. Clean glass slides were coated with 1% agarose and were used for the electrophoresis.

Absorption of Antibodies to Alpha-Amylase

In order to obtain antibodies monospecific for α-amylase, absorption of polyspecific antibodies was carried out. A preparative (1.5 mm thick) 12% SDS gel was run and electroblotted onto nitrocellulose. The edges of the nitrocellulose were cut and stained with india ink to visualize the amylase band. These edges were lined up with the rest of the blot and used as a guide to cut the amylase band. After cutting the nitrocellulose, this piece was used to absorb α-amylase antibodies according to the Esen (unpublished) procedure.

Reactivity of Maize Alpha-Amylase Antibodies to Purification Samples

Antibodies specific for maize α-amylase were used to follow the reactivity of the maize α-amylase purification samples. These tests were carried out in microtiter plates. Samples used to coat the plate were the crude extract, the supernatant of the heated extract, the precipitate of the heated extract and the 70% acetone precipitated extract. The samples were dissolved in either 6M urea or a tris buffer (TBS) and were used to coat the microtiter plate overnight. After coating, the plate was flicked and treated with either TBS or 6M urea for another 4 hrs. The ELISA test was then completed as specified by Conroy and Esen (1984). Results were read on an ELISA reader (Dynatech MR600) at 450 nm.

Results

Purification of Maize Alpha-Amylase by Differential Solubility

It was demonstrated in earlier studies that α-amylase precipitates in organic solvents and ammonium salts. In the literature, values were given at which cereal α-amylase should precipitate out of solution. This study was to determine at what level maize α-amylase precipitated out of solution.

Freshly extracted maize α-amylase was precipitated using organic solvents such as ethanol and acetone or salts such as ammonium sulfate. After precipitation the α-amylase was resuspended in extraction buffer and tested for activity. The results of the ethanol, the acetone and the ammonium sulfate precipitation are shown in Figures 6 - 8.

The ammonium sulfate precipitation (figure 8) showed some precipitation of the maize α-amylase at the 20% level but most of the precipitation took place at the 40% level. There was no increase in precipitation after the 40% level.

Alpha-amylase began precipitating from solution at the 30% level with acetone (figure 7) and at about the 40% level in ethanol (figure 6). Precipitation increased as the level of organic solvent increased until the 50% level in acetone and the 60% level in ethanol. Both organic solvents precipitated α-amylase without irreversibly denaturing it.

Purification of Maize Aipha-Amylase by Glycogen Precipitation,

Column Chromatography, and Gel Eelectrophoresis

Previous studies purifying cereal α-amylases have used techniques such as glycogen precipitation, column chromatography, and gel electrophoresis to obtain pure α-amylase. The present study tested these techniques to determine their usefulness in purifying maize α-amylase. The results of the methods tested are given in Figure 9.

Glycogen precipitation has been used extensively in wheat and barley α-amylase purification (Baulcombe, 1983; Rodaway, 1979). It involves extraction of the α-amylase, a 40% ethanol cut and addition of glycogen to the solution. Alpha-amylase recognizes glycogen as a substrate and binds to it. The α-amylase - glycogen complex then precipitates out of solution. This technique yielded the highest purity for maize α-amylase of all the methods tested (Figure 9).

Column chromatrography used a DEAE-cellulose column as an anion exchanger. This method was previously used by Goldstein and Jennings (1978) to purify maize α-amylase. A freshly extracted and heated sample of maize extract was applied to the DEAE-cellulose column and the column was washed with a low salt buffer. Then the material bound to the column was eluted using a sodium chloride gradient of 0.01 M to 1 M. The fractions were tested for α-amylase activity and protein content.

The results indicated no α-amylase activity in the column wash. Alpha-amylase activity eluted off the column in a broad peak. The fractions where a high activity was found were pooled, dialyzed and freeze-dried. This method proved the least suitable for purifying maize α-amylase of all the methods tested (Figure 9) due to the low activity found in these samples.

Although glycogen precipitation yielded the best purity, a great deal of protein and activity were lost in the manipulations. Routinely, for studies involving total activity, purified α-amylase was not necessary; thus the crude, heated extract was used.

SDS polyacrylamide gel electrophoresis was used to follow purification of maize α-amylase. Shown in figure 10 are the protein banding patterns of the extracts purified by the above mentioned techniques. As purification proceeds there was an enrichment of the α-amylase band and the loss of other bands. The reactivity of α-amylase purification samples using antibodies specific for maize α-amylase is shown in figure 11. Samples were dissolved in either 6M urea for denatured α-amylase or a tris buffer (TBS) for native α-amylase. Notice that the the antibodies are much more reactive to 'denatured' α-amylase than to the 'native' form. The heat precipitate showed a much higher reaction with the antibodies than the supernatant.

Isoelectric Focusing of Maize Alpha-Amylase

Previous researchers have found electrophoretic variants or isozymes of cereal α-amylases upon isoelectric focusing (IEF). Native IEF gels were run in the cold and stained for α-amylase activity using starch and iodine. Four major electrophoretic variants of maize α-amylase were found (figure 12). The major isozymes showed very acidic isoelectric points (pI's) which range from 3.8 to 4.3. One or two minor α-amylase bands were seen inconsistently on the gels with higher pI's. These bands were not found on every gel and frequently when one sample was used did not extend across the gel as did the major bands.

pH Optima for Maize Alpha-Amylase

A study was undertaken to determine whether the four major isozymes of maize α-amylase exhibit pH optima and profile differences as is found in the barley isozymes (Jacobsen and Higgins, 1982). Maize α-amylase was separated into its isozymes by

isoelectric focusing. The IEF gel was then cut into slices and slices equilibrated in buffers of various hydrogen ion concentrations. Each gel slice was incubated in a different buffer with pH's ranging from 3 to 10. After equilibration the gel pieces were incubated with a starch solution made of the same pH as the equilibration buffer. Alpha-amylase bands were detected by staining with an I-KI solution.

The behavior of the isozymes under different pH conditions is shown in figure 13. All four pI forms exhibited similar pH dependent activity profiles. In the gel slices they were active from pH 3 up to pH 10 showing the greatest activity around pH 4 to pH 5. Activity tapered off as the pH changed in either direction from pH 4 to pH 5.

The gel slice experiment was designed to demonstrate differences among the isozymes, but its limitation is that it is unable to show an exact pH optima, only a range. Free solution studies were performed to determine the exact pH optimum of maize α-amylase. The pH dependent activity profile of maize α-amylase is shown in figure 14. In free solution, α-amylase had little activity at pH 3.5; but the activity level quickly rose between pH 4 and pH 5 and peaked at pH 5. The activity level quickly dropped off after pH 5. There was very little activity at pH 8 and above.

Temperature Optima of Alpha-Amylase

A study using IEF gel slices was performed to determine possible differences in the temperature profile and optima of the four major isozymes. The IEF gel was run to separate the α-amylase into isozymes, once separation was complete the gels were equilibrated in different temperature buffers. After equilibration the gel slices were incubated with a starch solution, equilibrated to the same temperature as the buffer used, and the activity was visualized by staining with an iodine solution.

The effect of temperature on the activity and stability of α-amylase was examined over the range of 0°C to 50°C. The four major components of α-amylase showed a similar broad range of activity (Figure 15). They were active at all of the temperatures tested. The peak of activity was between 30°C and 40°C with activity levels decreasing on either side of the optima.

Since the gel slices studies only show differences between the isozymes and cannot show an exact optima, free solution studies were done to determine the exact temperature optima of maize α-amylase. The results demonstrate that the temperature optimun of maize α-amylase in free solution (figure 16) was between 30°C and 40°C The temperature optima correlates well with the optima found in the gel slice studies. Alpha-amylase was active over a broad temperature range with the activity being greatest at 37°C and tapering off as the temperature changed in either direction.

Antibodies Specific for Alpha-Amylase

A fraction enriched for maize α-amylase was electrophoresed on a preparative 10% SDS gel. The α-amylase band was cut from the gel, dehydrated, mixed with adjuvant and injected into a rabbit. There was an increase in antibody titer as the number of injections increased (Figure 17) based on the ELISA results.

To determine the specificity of the antibodies produced, western blot analysis was performed. Western blots of 10% SDS gels were reacted with pre-immune and immune sera. There was no reaction in the pre-immune sera but after 9 injections the pattern contained a number of bands (Figure 18). The bands directly above and below the α-amylase band also reacted with the antibodies. Western blot analysis also showed that barley α-amylase reacted with the antibodies against maize α-amylase.

Antigenicity of Isozymes

In barley, α-amylase isozymes can be divided into two groups on the basis of a number of characteristics. One of these characteristics was antigenicity, that is barley α-amylase isozymes groups being antigenically distinct. With this in mind, tests were initiated to demonstrate possible antigenic differences between the four major isozymes in maize.

Ouchterlony double immuno-diffusion assays using 1% agar plates and antisera prepared against maize α-amylase were initiated. These tests proved to be unsuccessful; no visible precipitation lines formed. Staining of the Ouchterlony plate with Coomassie Blue for protein after incubation also yielded no detectable precipitin lines. To rule out reasons for no precipitation lines forming, immunoelectrophoresis (IEP) was initiated. This method also yielded negative results.

A possible reason for negative results was that the antibody was much more reactive with the denatured rather than the native form of the enzyme. This would limit the sites recognized in the native protein by the antibodies and thus cause no lattice work of precipitation to be formed. To determine if the antibody against α-amylase was for the denatured form, ELISA tests using native and denatured enzyme to coat the microtiter plate were initiated. Figure 19 demonstrates that the antibodies are more active with the denatured form than the native form. In fact, the antibodies are about 4 times as reactive toward the denatured form than the native form.

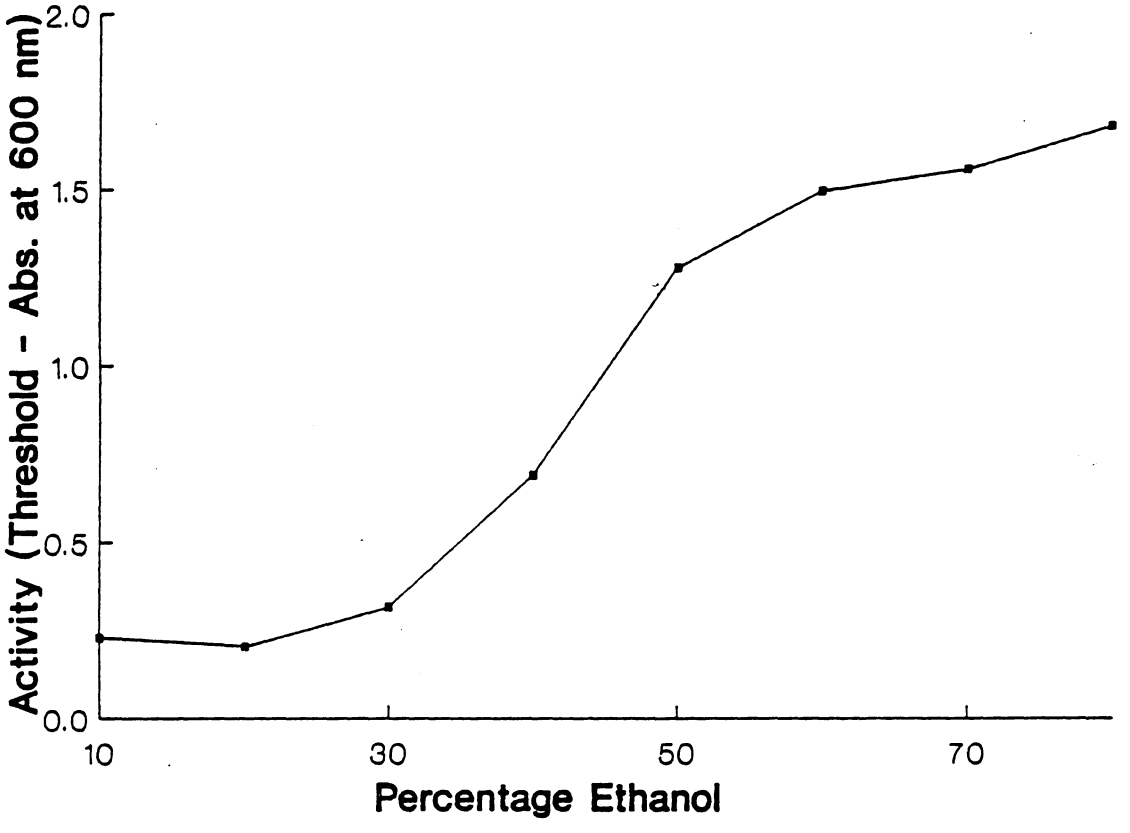

Figure 6. Ethanol Precipitation of Maize Alpha-Amylase: Alpha-amylase begins precipitating out of solution when ethanol reaches the 40% level. Precipitation of amylase activity was essentially complete at ethanol concentrations of 60% or greater.

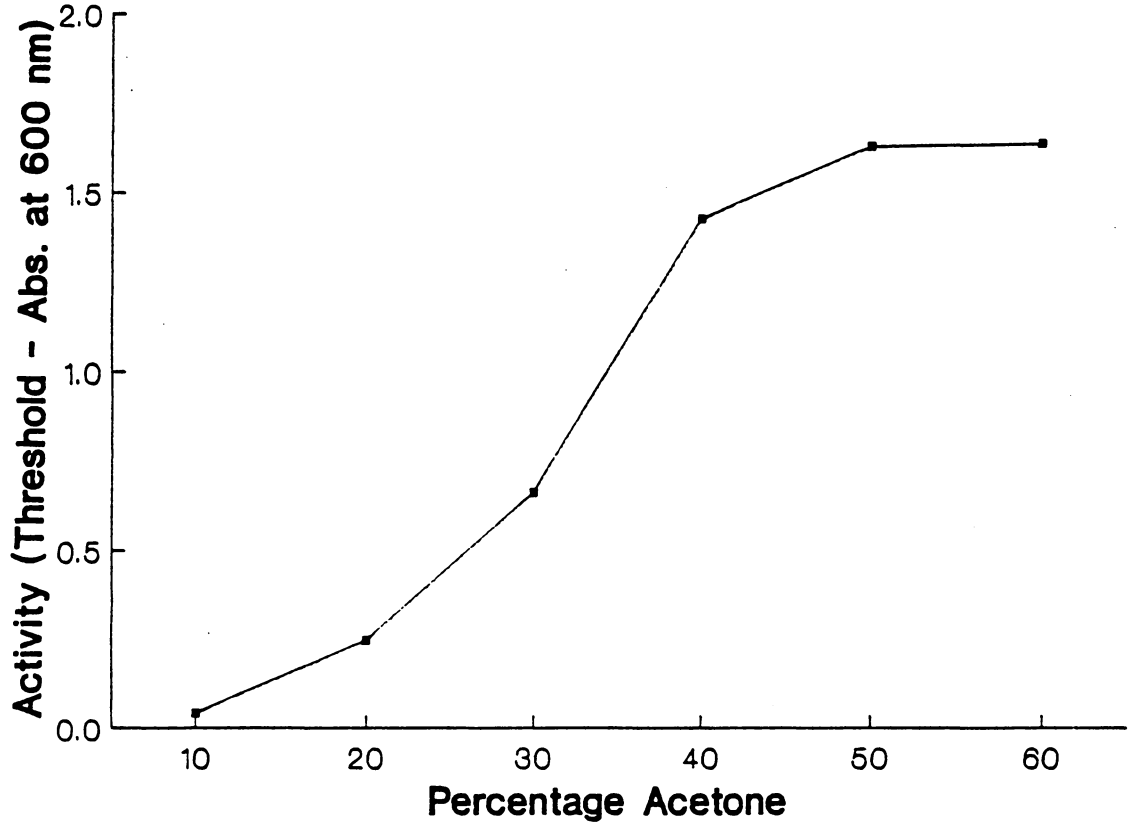

Figure 7. Acetone Precipitation of Maize Alpha-Amylase: Acetone begins precipitating maize α-amylase at the 30% level. Precipitation increases as the concentration of acetone increases until about 50% acetone. Precipitation was essentially complete at acetone concentrations of 50% or greater.

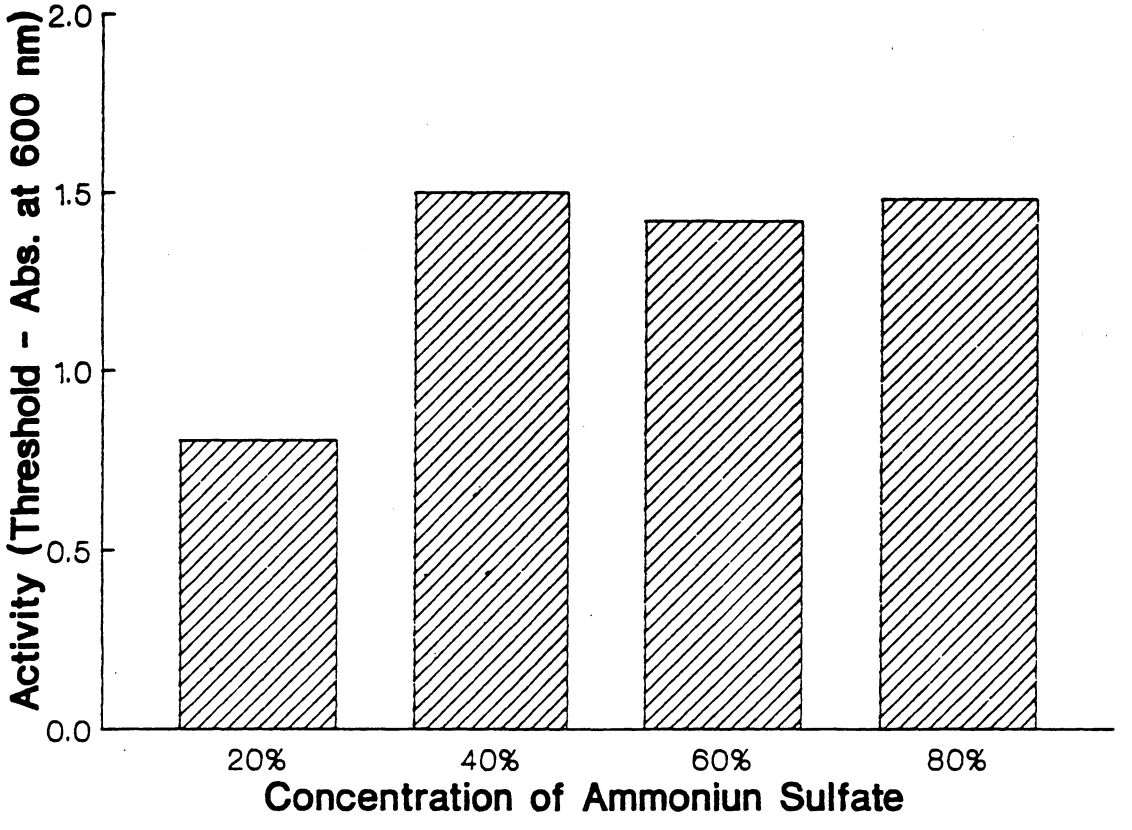

Figure 8. Ammonium Sulfate Precipitation of Maize Alpha-Amylase: Ammonium sulfate was used to precipitate α-amylase. Precipitation was first noted at the 20% level and was essentially complete by the 40% level or above.

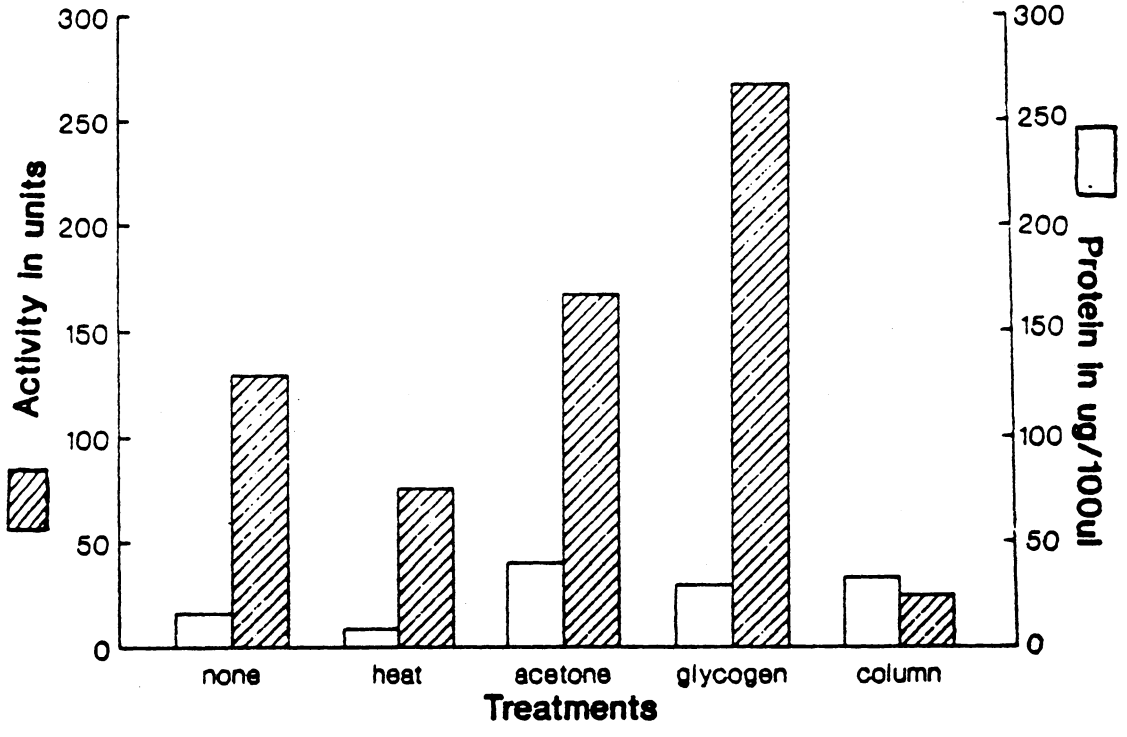

Figure 9. Purification of Maize Alpha-Amylase: Various techniques of purifying α-amylase were tested. 'none' indicates the crude α-amylase sample; 'heat' is the sample after heating to 70°C; '70%' is the extract after 70% precipitation with acetone; 'glycogen' is the glycogen precipitated amylase sample and 'column' is the pooled DEAE-cellulose column fractions. Alpha-amylase activity is shown in (▨) and the amount of protein is shown as (□). The glycogen precipitation yielded the highest specific activity of α-amylase while the DEAE column yielded the lowest.

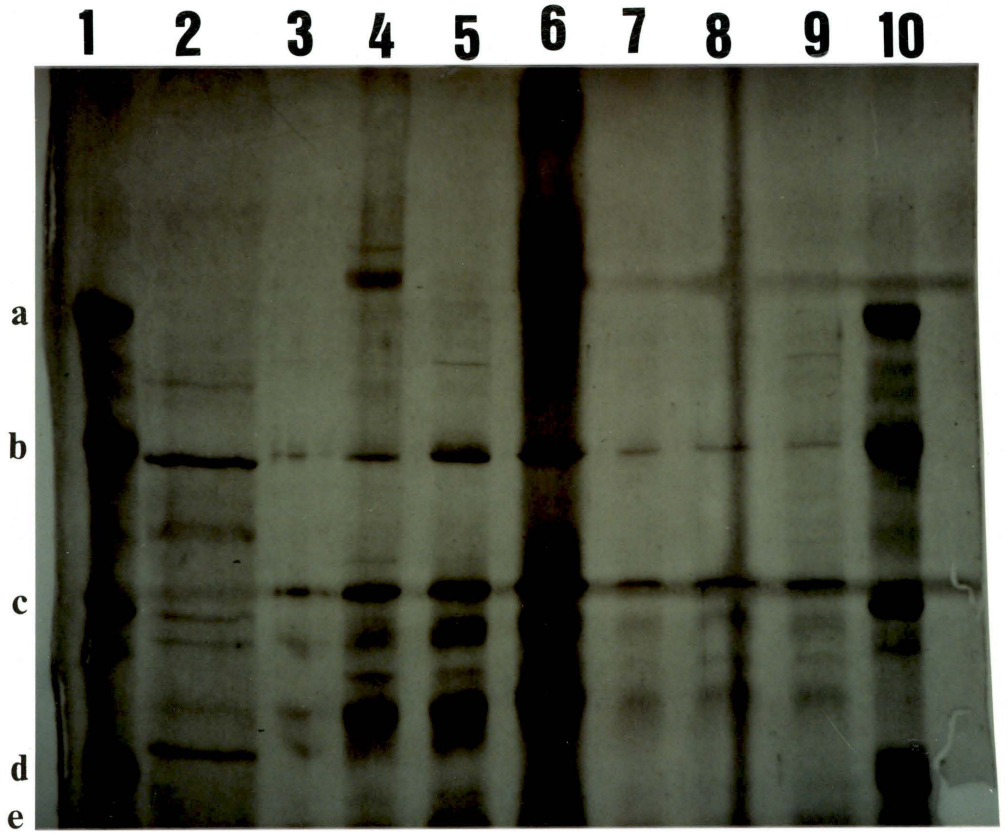

Figure 10. SDS-PAGE Gel Showing Purification of Maize Alpha-Amylase: Lane 1 (1) contains molecular weight standards consisting of (a) BSA (66,000 daltons), (b) Egg Albumin (45,000 d), (c) Carbonic Anhydrase (29,000 d), (d) Soybean Trypsin Inhibitor (20,000 d), (e) Lysozyme (14,000 d). 2, glycogen-precipitated barley extract. The arrow indicates the α-amylase band. 3, the supernatant after glycogen precipitation of a maize extract. 4, a glycogen precipitated maize extract. The arrow indicates the α-amylase band. 5, a resuspended sample of a 70% acetone precipitation. 6, a 70% acetone precipitate which has not been resuspended. 7, the maize extract after heating and a 30% acetone cut. 8, the extract after heating the sample. 9, the crude extract. 10, the molecular weight standards again. Note that the alpha-amylase band is enriched for during the purification and that other bands above below the α-amylase band are reduced.

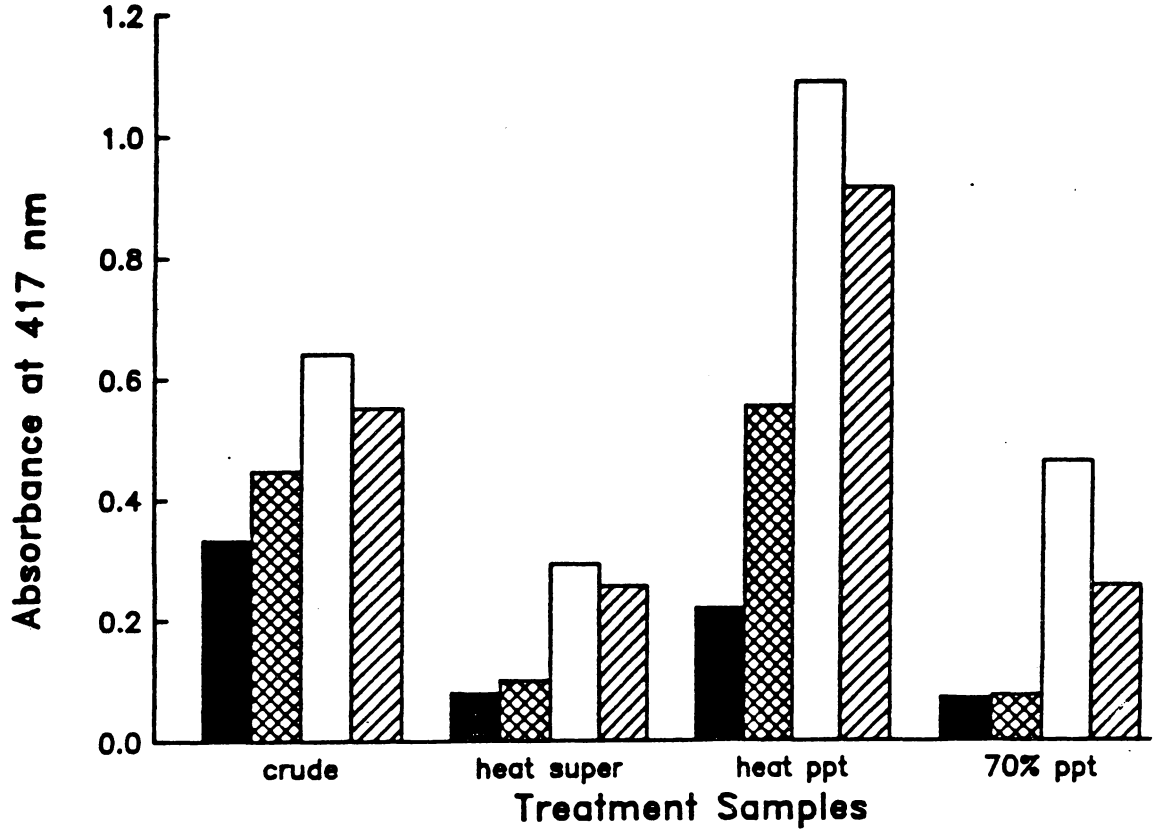

Figure 11. Reactivity of Purified Samples with Maize Alpha-Amylase Antisera: Antibodies specific for maize alpha-amylase were used to follow the the reactivity after subjecting the alpha-amylase preparations to various treatments. These tests were carried out in microtiter plates. The various treatment samples were dissolved in either urea or a tris buffer (TBS) and used to coat the microtiter plate. After overnight coating, the plate was rinsed and treated with either TBS or urea for another 4 hours. ( ) means the plate was coated with 6 M urea sample then reincubated in 6 M for another 4 hours. ( ) means that the plate was coated with a 6 M urea sample then reincubated in TBS. ( ) means that the sample was dissolved in TBS and then reincubated in 6 M urea. ( ) indicates that the sample was dissolved in TBS and then reincubated in TBS. The treatment samples used to coat the plate are: 'crude' = the crude extract, 'heat super' = the supernatant of the heated extract, 'heat ppt' = the precipitate of the heated extract, '70%' = the 70% acetone precipitated extract. Notice that the antibodies are more reactive with the samples dissolved in 6 M urea and the reactivity of the TBS samples is increased with additional incubation in urea after the initial coating.

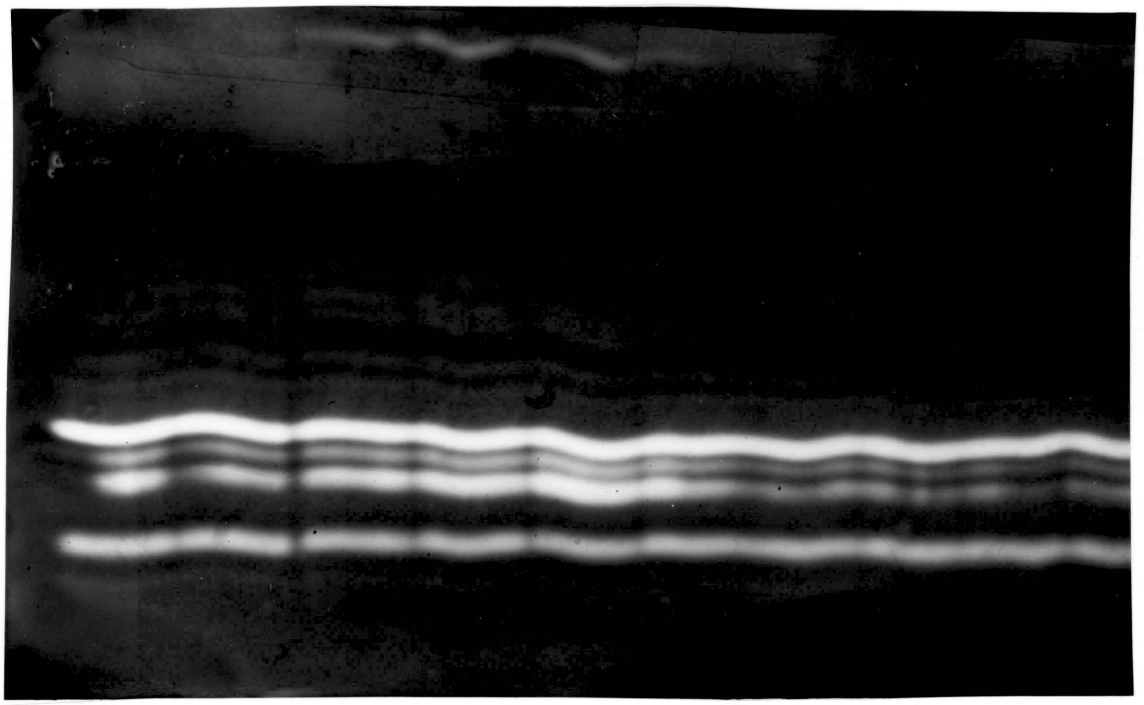

Figure 12. Maize Alpha-Amylase Isozymes: There are four major isozymes of maize α-amylase. The pI's for these isozymes are very acidic and range from 3.8 to 4.3.

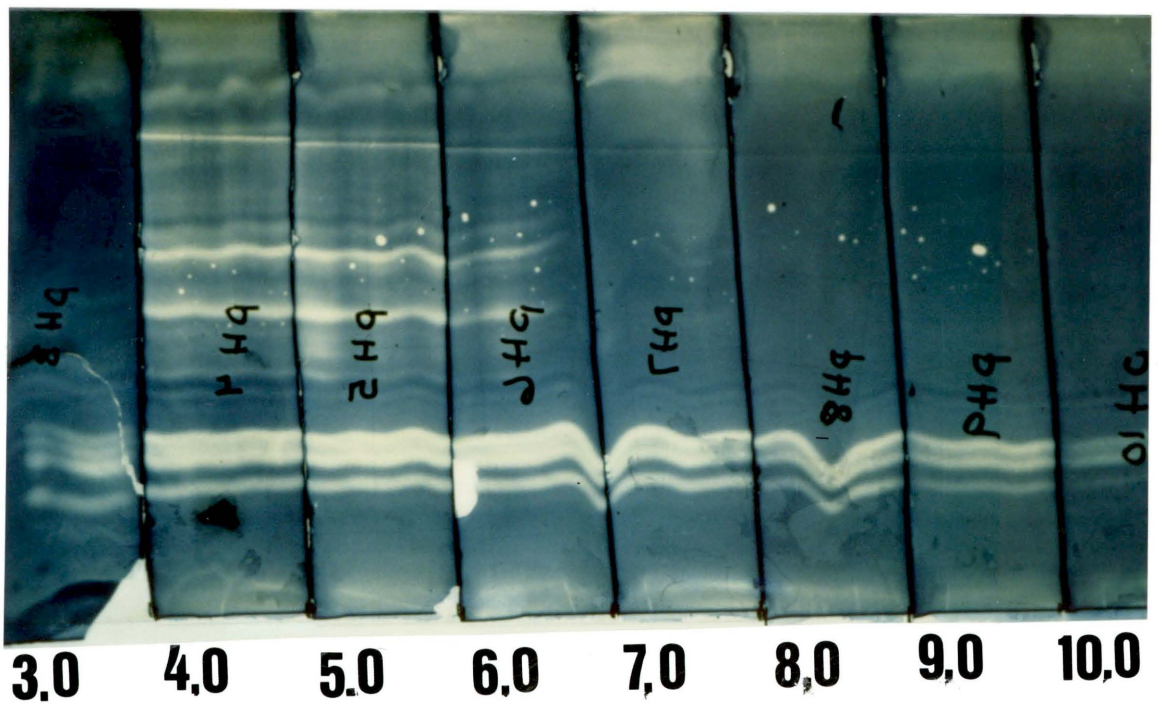

Figure 13. pH Dependent Activity Profile of Maize Alpha-Amylase Isozymes: The numbers at the bottom of the gel indicate the pH at which the gel was incubated and the activity assayed. All four isozymes exhibit similar pH dependent activity profiles. Peak activity appears to be in the range of pH 4.0 to pH 6.0.

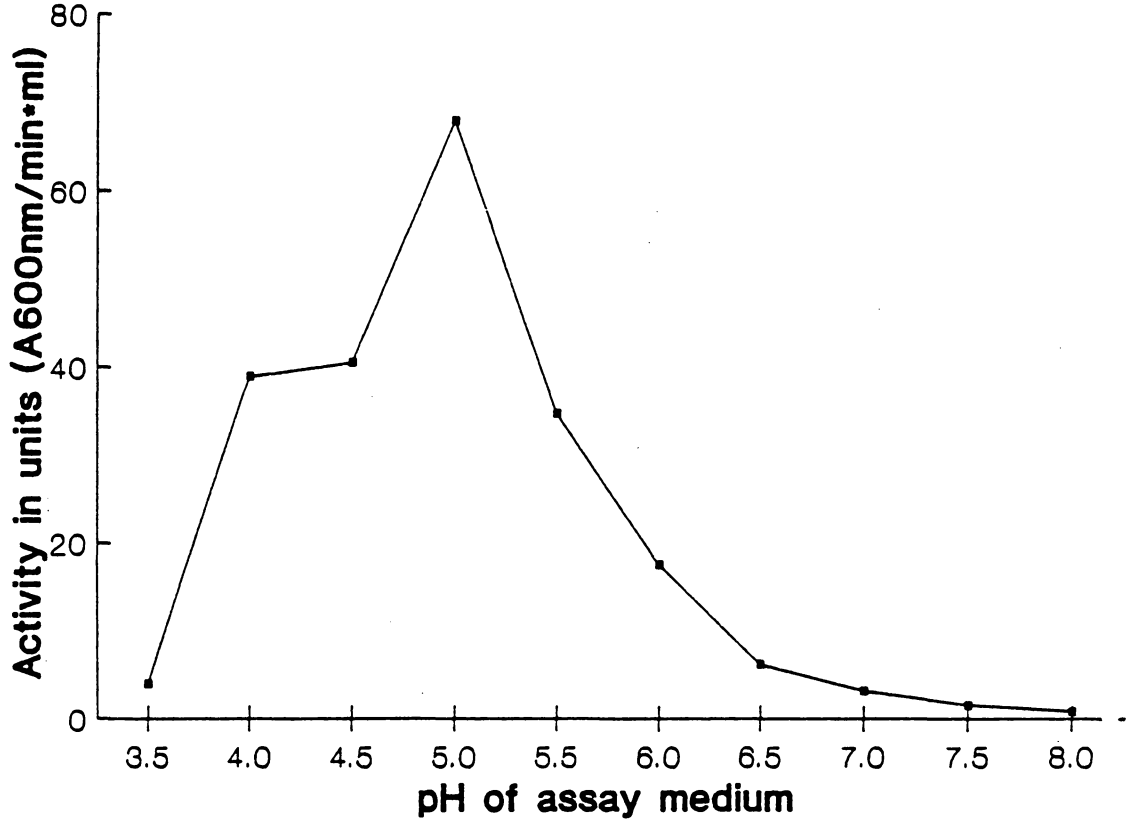

Figure 14. pH Profile of Maize Alpha-Amylase in Free Solution: The α-amylase has little activity at pH 3.5 and above pH 8. Between these extremes the activity level quickly rises between pH 4 and pH 5 and peaks at pH 5. After pH 5 the activity level quickly drops off.

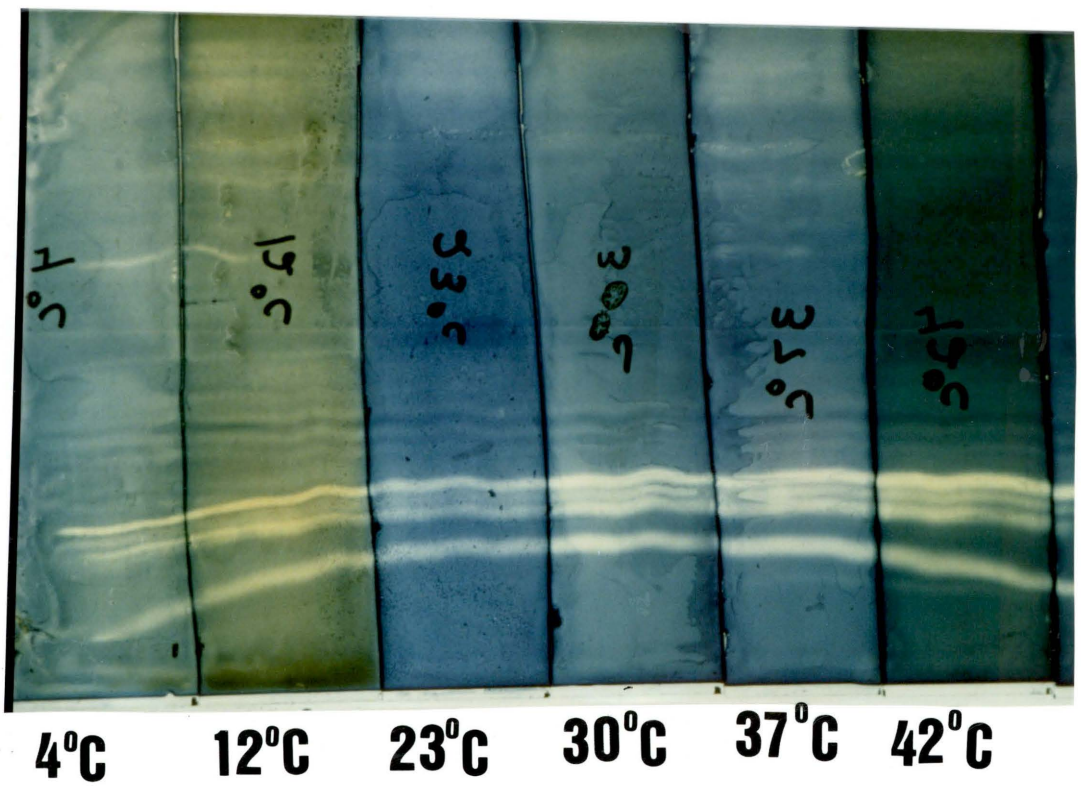

Figure 15. Temperature Dependent Profile of Maize Alpha-Amylase Isozymes: The numbers at the bottom of the gel indicate the incubation and assay temperatures that the gel slices were subjected. All four major isozymes of maize α-amylase exhibit a broad range of activity peaking at about 37°C.

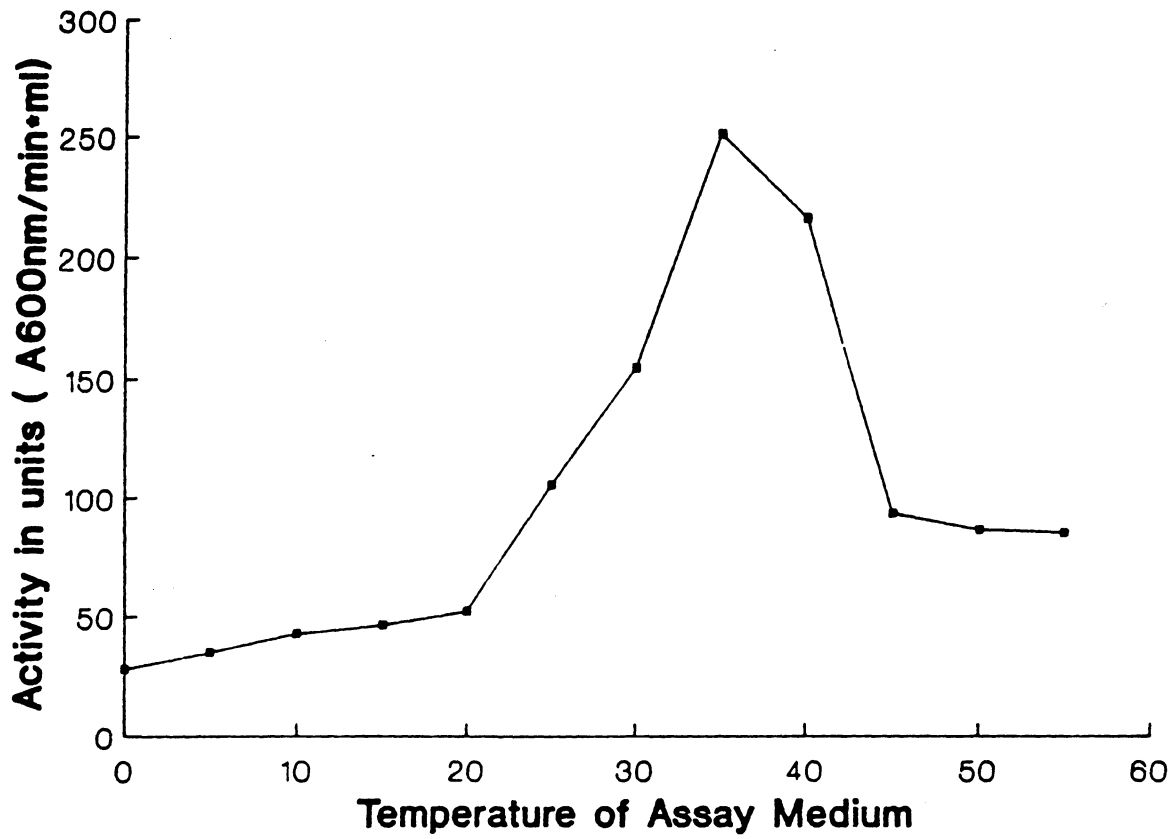

Figure 16. Temperature Profile of Maize α-Amylase Activity in Solution: Alpha-Amylase is active over a broad temperature range. The activity was greatest at 35°C to 37°C and tapered off as the temperature was changed in either direction.

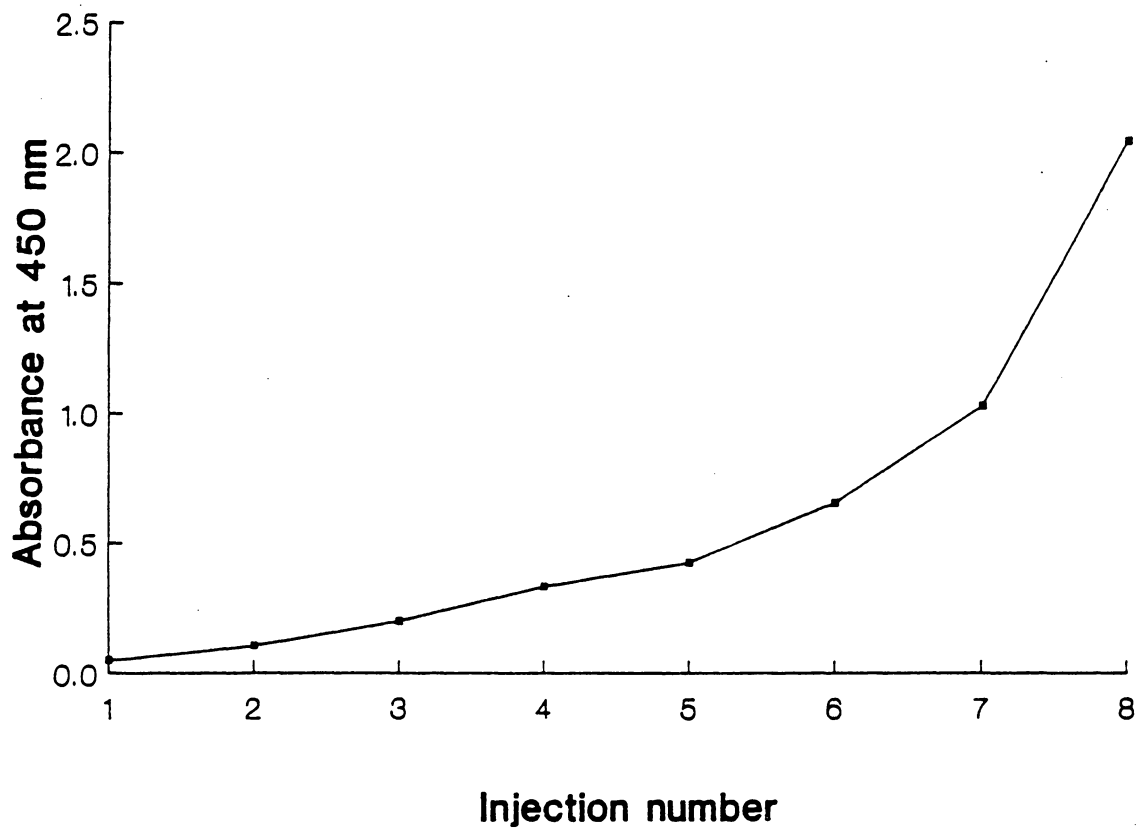

Figure 17. Antibody Titer of Maize Alpha-Amylase Antisera: The figure demonstrates the increase in antibody titer of antisera as the number of immunizations increases. 9 immunizations were given at two to three week intervals with bleedings taken just prior to immunization. A fixed dilution of antibody (1/250) was used to demonstrate the rise in titer. The highest titer was reached after the 9th immunization.

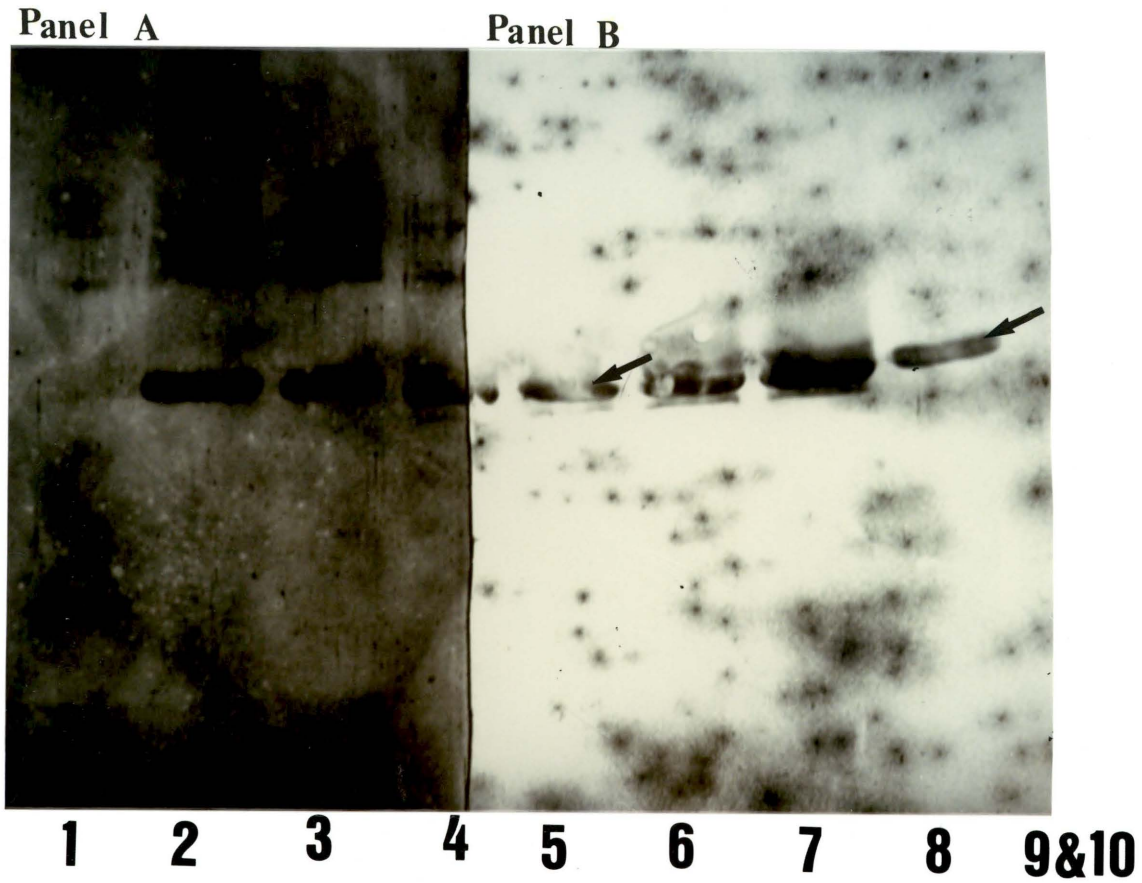

Figure 18. Western Blot of Fractions Enriched for Maize Alpha-Amylase: Panel A, the protein profile of the blot which had been stained with india ink. Panel B, the blot after reaction with maize α-amylase antibodies. The lanes are as follows: Panel A: 1, a molecular weight standard. 2, a barley α-amylase glycogen precipitate. 3, a DEAE-cellulose column eluate of a maize extract. Panel B: 4, and 5, a 70% acetone precipitated sample of a maize extract. 6 and 7, a DEAE-cellulose column eluate of a maize extract. 8, a barley α-amylase glycogen precipitate. 9, and 10, two different molecular weight standards. The arrow indicates the alpha-amylase band. The bands directly above and below the α-amylase band cross-react with the α-amylase. Note that barley α-amylase cross-reacts with the maize α-amylase antisera.

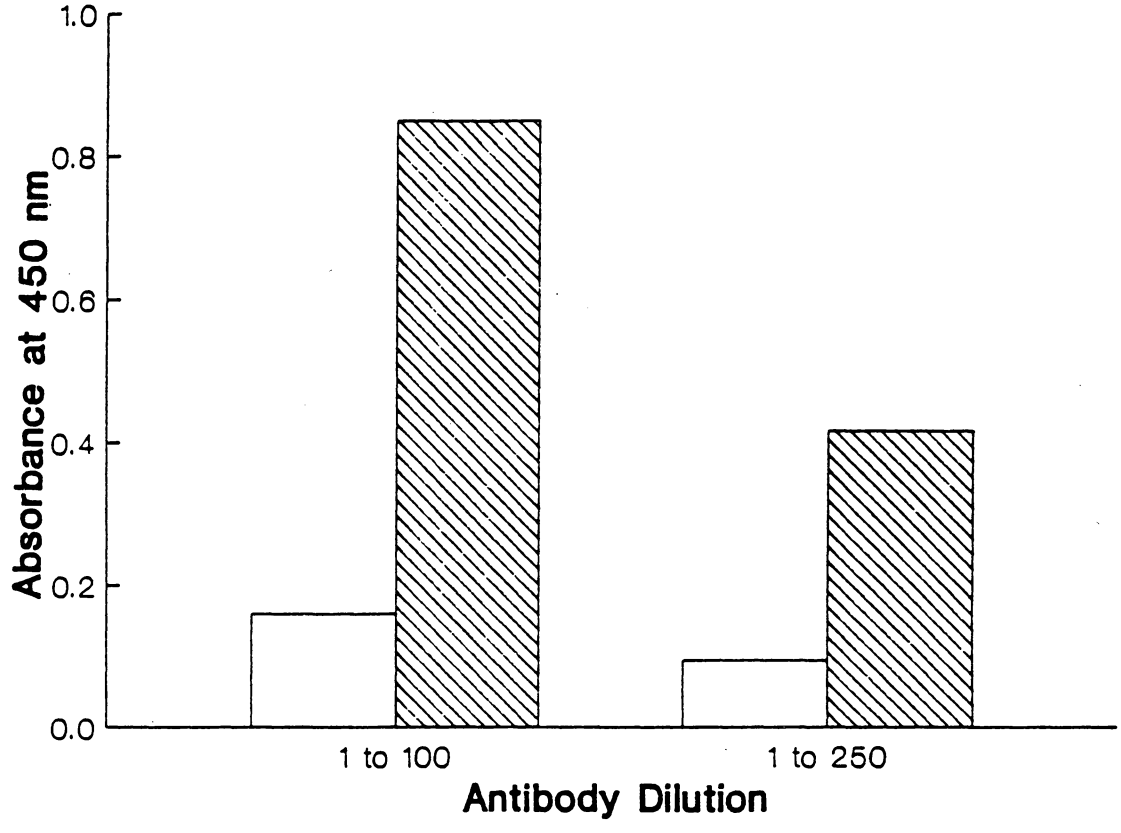

Figure 19. Maize Alpha-Amylase Antibody Reactivity with Native and Denatured Antigen: (▭) indicates that the native form of α-amylase (dissolved in a phosphate buffer (PBS)) was used to coat the ELISA plate. (▨) indicates that the denatured form of α-amylase (dissolved in 6 M urea) was used to coat the ELISA plate. Note that the antibodies are about 4 times as reactive with the denatured form than with the native form.

Discussion

Several methods of purification were used to isolate maize α-amylase. These methods included organic solvent precipitation, ammonium sulfate precipitation, glycogen precipitation, ion-exchange chromatography, and electrophoresis. Of the methods the glycogen precipitation gave the highest specific activity. Since this method was originally designed to specifically purify only α-amylase, the fact that it was the most successful was not surprising. This method is also successfully used in wheat and barley α-amylase purification by other researchers.

Although purification was best with the glycogen precipitation the material so purified still included several Coomassie Blue stainable bands on an SDS gel. These bands especially included two with mobilities slightly lower and higher than the α-amylase band. Separation of the α-amylase band from these bands was not possible with the techniques of differential solubility and chromatography. Previous maize α-amylase work did not include SDS gel electrophoresis.

The anion exchange column also failed to resolve the closely migrating bands from the amylase but did remove other contaminating bands. This shows that these bands

not only are close in molecular weight but also show similar characteristics on an anion exchange column.

In this study the column procedure did not yield as high a specific activity as the glycogen precipitation or the 70% acetone precipitation. The activity was very low in the freeze-dried column sample. The low activity may partially be due to the dialysis steps and freeze-drying steps which followed the column elution. Goldstein and Jennings (1975) were able to isolate three isozymes of α-amylase and one of beta-amylase using a DEAE cellulose anion exchange column. I was unable to cleanly separate the isozymes using the same type of column; the isozymes eluted in a broad peak of activity. Although the buffering systems were identical, Goldstein and Jennings buffered the system at pH 8.0 while I buffered at a pH of 7.5. The isozymes isolated by Goldstein and Jennings (1978) had different pI's than those found in this study. This may also account for the differences in DEAE cellulose column elution.

The acetone precipitated α-amylase had high specific activity but also a higher protein yield thus showing a lower specific activity than the glycogen precipitate. Although the acetone precipitate had a lower specific activity less protein was lost in the methods and so this fraction was used in conjunction with electrophoresis to isolate the denatured α-amylase protein.

Glycogen precipitated samples of barley show a prominent α-amylase band co-migrating with the maize band. To convince ourselves that the band which co-migrated with the barley α-amylase band was indeed maize α-amylase several tests were done. Antibodies against barley α-amylase were tested with this band and proved positive. Amylase activity bands, cut from IEF gels were electroeluted and run on an SDS gel showed a band co-migrating with barley α-amylase. Thirdly, antibodies obtained against maize α-amylase tested positively to the barley α-amylase band on Western blots. These findings suggest that cereal α-amylases have very similar molecular weights and are

antigenically cross-reactive. This finding correlates well with previous work with cereal α-amylases.

Isoelectric focusing of maize α-amylase revealed four major electrophoretic variants or isozymes. Native fixed pH gel electrophoresis first showed the heterogeneity and isoelectric focusing further separated the isozymes. The pI's of the isozymes range from 3.8 to 4.3. Isozymes of maize have previously been described by Scandalios (1969) and Goldstein and Jennings (1975). Goldstein and Jennings found three isozymes of maize α-amylase with pI's ranging from 4.3 to 5.05. In a closely related species, sorghum, four isozymes are found but their isoelectric points are not known (Boyes, et al, 1967). Differences in the number of isozymes and the isoelectric points can be attributed to either differences in the methods used or possibly to varietal differences in the species *Zea mays*. Scandalios (1969) found varietal differences in the isozymes in his study.

Physicochemical studies were performed to determine if the isozymes had different properties. The isozymes of barley and wheat were separated into two groups partially on the basis of physicochemical properties. In maize, the isozymes exhibit remarkably similar physicochemical properties. The pH and temperature testing done in gel slices showed no differences among the isozymes. The pH gels showed that α-amylase has a broad pH activity profile peaking at about 5.0. The free solution assay for pH optima showed the same results. This pH pattern of activity and optima agrees well with the findings of Goldstein and Jennings (1975). A related species, sorghum also has a pH optima of 5.0 (Botes et al., 1969). The barley isozyme groups exhibit different pH activity characteristics. The low pI isozyme group has a bell-shaped pH optima curve which peaks at pH 5.0 while the high pI group has a much broader curve and the activity at pH 5 is nearly the same as at pH 6.9. (Tanaka and Akazawa, 1970). The gel studies and the free solution assay showed a broad temperature activity range which

peaked in the area of 37°C to 40°C. In wheat the temperature optima is much higher peaking at 55°C (MacGregor, 1978).

Tissue specificity studies were done to determine possible tissue localization differences in the isozymes. The gels showed that the isozyme pattern in the root, shoot, embryo/scutellum and endosperm of 7 day germinated seedlings were identical. The difference was not in isozyme pattern but in levels of activity. Differences in the intensity levels of the isozymes was noted in the different tissues. It appears that the greatest activity is in the embryo/scutellum extract. This correlates well with Dure's finding that the chief tissue in the mobilization of starch from the endosperm in germinating maize kernels is the scutellum. These results point to similar gene expression and regulation in all the tissues tested. Perhaps isozyme regulation of maize α-amylase is at the intracellular or organelle level rather than the tissue level.

It is interesting to note the similarities between the low pI isozyme group of wheat and barley and the maize isozymes. In all three cereals the isozymes have acidic isoelectric points, similar pH optima and low requirement for calcium. Also, they are all found in immature kernels and in germinating kernels (Olered and Jonsson, 1970; Daussant and Mayer, 1979; Chao and Scandalios, 1969). This is in contrast to the wheat and barley high pI isozyme group which is found only in germinating kernels. Thus, the low pI group of wheat and barley and the maize isozymes exhibit similar patterns of expression in germinating seeds.

Another interesting similarity between maize α-amylase isozymes and the low pI group of wheat and barley is that an exogenous source of gibberellic acid is not necessary for expression of these isozymes. The low pI isozyme group is found in gibberellic acid insensitive mutants in wheat (Baulcombe et al., 1983) and in barley the mRNA for this group is found in relatively high abundance in unstimulated aleurone layer cells (Rogers, 1985). MacGregor (1976) suggests that the formation of the low pI isozymes

in barley doesn't depend upon the embryo or an exogenous source of gibberellic acid. Although these isozymes are found in the absence of exogenous gibberellic acid, enhancement of the isozymes with the addition of gibberellic acid is found in wheat, barley and maize (Baulcombe et al., 1983; Rogers, 1985; Goldstein and Jennings, 1975).

Baulcombe suggests two distinct regulatory mechanisms for expression of the low pI isozyme group only one of which is sensitive to gibberellic acid. Perhaps a more conservative hypothesis would be one regulatory mechanism insensitive to gibberellic acid but with 'enhancer' mechanisms sensitive to gibberellic acid. This would account for expression of the low pI genes in the absence of exogenous gibberellic acid and then enhancement of synthesis with addition of exogenous gibberellic acid.

When the two isozyme groups of α-amylase in barley and wheat are compared with those in maize a surprising pattern emerges. The maize isozymes are similar to only one of the isozyme groups of barley and wheat, the low pI group. Although not all the results are conclusive, the high pI isozyme group appears absent in the maize inbred K55. There are several explanations for this observation. The absence of the high pI group may be due to the procedures used to isolate and to purify the protein. At the genetic level, the absence may be accounted for by inactivation of the gene or genes encoding the high pI group. Another possibility at the genetic level is that the genes for the high pI group may be absent entirely. If this is the case, it leads us to questions about the evolution of the two α-amylase gene families. Perhaps one multigene family, represented by the maize isozymes, and the low pI group is ancestral to the second multigene family represented by the high pI isozyme group of wheat and barley.

There is indirect evidence to support this hypothesis and the evidence comes from genetic analysis of the two gene families in wheat and barley. In wheat and barley the low pI isozyme genes are more similar to each other in the regulatory, noncoding 5'and 3' regions and in the sequence than to the other gene family. This implies that there is

conservation between species of the two groups and that there are differences in the regulatory mechanisms. Also if the low pI group genes are older than the high pI genes then they should demonstrate more genetic heterogeneity owing to a longer time period for changes to accumulate. This is the case in barley, two mRNA's are found for the group A (low pI group) while only 1 mRNA is found for group B (high pI group) (Rogers, 1985).

Although the protein analysis reveals some tantalizing results, the complete picture lies in the genetic analysis of the maize α-amylase gene or gene family. The maize α-amylase gene(s) needs to be isolated and characterized before the questions about the evolution of the gene families can be clearly understood.

Conclusions

These data indicate that after incubation, de-embryonated maize kernels (inbred K55) show an increase in α-amylase activity whether or not exogenous gibberellic acid is present in the medium. The effect of gibberellic acid is quantitative rather than qualitative. The endosperms treated with gibberellic acid showed higher levels of α-amylase activity earlier than the control endosperms. The greatest difference in activity between the gibberellic acid treated and control endosperms was after 5 days of incubation where the gibberellic acid-treated endosperms showed 2 to 3 fold greater amylase activities. The α-amylase activity development pattern exactly parallels the activity development pattern found in whole kernels.

Concomitant with development of α-amylase activity, several starch-degrading bands appeared on polyacrylamide gels stained with an activity stain. Three bands are consistently seen with relative mobility rates of .56, .58, and .60. The quantitative effect of gibberellic acid treatment is also seen in intensity differences in the banding pattern. In the gibberellic acid-treated endosperm pattern, the 3 bands were all more intense at days 3 to 8 than the control endosperm bands. After that time the bands in the control and gibberellic acid-treated endosperms were of approximately equal intensity.

Gibberellic acid-treated endosperm, control endosperm and whole kernel endosperm banding patterns were similar at all incubation times. There was no change in the isozyme pattern over time indicating similar regulation of the isozymes.

Isozymes can exist in different tissues or organelles and so may have different regulatory mechanisms. Tissue-specificity studies revealed that there were no qualitative isozyme pattern differences in 7 day old maize tissues but there were quantitative differences in the patterns.

Several methods of enzyme purification were tested to determine which methods would be the most suitable in purifying maize α-amylase. Activity and protein analysis revealed that glycogen precipitation purified the α-amylase best. Differential solubility testing of maize α-amylase showed that it precipitated in organic solvents between 40% and 60% in ethanol and between 30% and 50% in acetone. Ammonium sulfate precipitates maize α-amylase at the 40% level.

SDS polyacrylamide gel electrophoresis showed the molecular weight of maize α-amylase to be approximately the same as barley α-amylase (42,500 daltons). This molecular weight correlates well with the molecular weights found in other cereal grains. In wheat the molecular weight of α-amylase is 42,000 d, rice is 44,000 d, and sorghum it is 44,000 d (Higgins et al., 1976; Okita et al., 1979; Miyata et al., 1981; Botes et al., 1967). An earlier estimate of the molecular weight of maize α-amylase was made by Scandalios et al. (1978), who estimated the molecular weight to be about 44,000 d.

Four electrophoretic variants or isozymes of maize α-amylase have been resolved using isoelectric focusing and the isoelectric points range from 3.8 to 4.3. pH and temperature optima studies of the isozymes carried out in gel slices showed similar profiles for all the isozymes. The maize α-amylase isozymes cannot be divided into two groups on the basis of the physical properties studied as can the barley and wheat isozymes.

Results of immunological studies aimed at determining antigenic differences between the isozymes were inconclusive.

In conclusion, comparisons between the barley and wheat isozymes and the maize α-amylase isozymes show some interesting differences. On the basis of the physical and chemical properties studied, maize α-amylase isozymes cannot be divided into two distinct groups as can the wheat and barley isozymes. This study raises questions about the evolution of the two cereal α-amylase multigene families.

Further studies in the area must include:

1. Analysis of the heterogeneity of maize α-amylase messenger RNA

2. Isolation and characterization of the maize α-amylase gene or genes

3. Analysis of homologies between the α-amylase genes from maize and other cereals at the nucleic acid level.

Complementary studies at the protein level should include:

1. Isolation and further characterization of the maize isozymes

2. Bioassay for the plant hormone gibberellic acid in the endosperm or immunological studies using an antibody directed against gibberellic acid to monitor the presence, quantity and movement of the hormone in the de-embryonated endosperm.

3. Testing for the role of the scutellum in the production of maize α-amylase during germination.

References

Antibus, R. K., C. J. Kroehler, and A. E. Linkins. 1986. The effect of external pH, temperature and substrate concentration on acid phosphatase activity of ectomycorrhizal fungi. Can. J. Bot. 64:2383- 2387.

Atzorn, R. and E. W. Weiler. 1983. The role of endogenous gibberellins in the formation of α-amylase by aleurone layers of germinating barley caryopses. Planta 159:289-299.

Barman, T. E. 1969. *Enzyme Handbook* Vol II. Springer-Verlag. New York, New York.

Baulcombe, D. C., C. Lazarus and R. Martienssen. 1984. Gibberellins and gene control in cereal aleurone cells. J. Embryol. Exp. Morphol. Suppl. 83:119-135.

Baulcombe, D. C. 1983. Wheat α-amylase genes: Cloning of a developmentally regulated gene family. In "Genetic engineering" Vol V, J. Setlow and A. Hollaender, eds., Plenum, New York.

Baulcombe, D. C. and D. Buffard. 1983. Gibberellic acid-regulated expression of α-amylase and six other genes in wheat aleurone layers. Planta 157:493-501.

Bilaerback, D. E. 1974. Amylases from aleurone layers and starchy endosperm of barley seeds. Plant Physiol. 53:480-484.

Bog-Hansen, T. C. and J. Daussant. 1974. Immunochemical Quantitation of Isozymes in barley malt. Analytical Biochemistry 61:522-527.

Botes, D. P., F. J. Joubert and L. Novellie. 1967. Kaffir corn malting and brewing studies XVII-Purification and properties of sorghum malt α-amylase. J. Sci. Food Agri. 18:409-412.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochem. 72:248.

Briggs, D. E. 1964. Origin and distribution of α-amylase in malt. J. Inst. Brew. 70: 14-24.

Briggs, D. E. 1972. Enzyme formation, cellular breakdown and the distribution of gibberellins in the endosperm of barley. Planta 108: 351-355.

Brown, A. H. D. and J. V. Jacobsen. 1982. Genetic basis and natural variation of α-amylase isozymes in barley. Genet. Res. 40:315-324.

Brown, H.T. and G. H. Morris. 1890. Researches on the germination of some of the *graminae*. J. Chem. Soc. 57:488-528.

Bush, D. S., M. J. Cornejo, C. N. Huang and R. L. Jones. 1986. Ca2+-Stimulated secretion of α-amylase during development in barley aleurone protoplasts. Plant Physiol. 82:566-574.

Caldwell, M. L. and Doebbeling, S. E. 1935. A study of the concentration and properties of two amylases of barley malt. J. Biol. Chem. 110:739-747.

Callis, J and T. H. D. Ho. 1983. Multiple molecular forms of the gibberellins-induced alpha-amylase from the aleurone layers of barley seeds. Arch. of Biochem. and Biophy. 224(1): 224-234.

Chandler, P. M., J. A. Zwar, J. V. Jacobsen, T. J. V. Higgins, A. S. Inglis. 1984. The effects of gibberellic acid and abscisic acid on α-amylase mRNA levels in barley aleurone layers; studies using an α-amylase cDNA clone. Plant Mol. Biol. 3:407-418.

Chao, S. E. and J. G. Scandalios. 1969. Identification and genetic control of starch-degrading enzymes in maize endosperm. Biochem Genet. 3: 537-547.

Chrispeels, M. J. and J. Evarner. 1966. Inhibition of gibberellic acid induced formation of α-amylase by abscissin II. Nature 212:1066-1067.

Chrispeels, M. J. and J. E. Varner. 1967. Gibberellic acid-enhanced synthesis and release of alpha-amylase and ribonuclease by isolated aleurone layers. Plant Physiol. 42: 398-406.

Chrispeels, M. J. and J. E. Varner. 1967. Hormonal control of enzyme synthesis: On the mode of action of gibberellic acid and abscisin in aleurone layers of barley. Plant. Physiol. 42:1008- 1016.

Conroy, J. M. and A. Esen. 1984. An enzyme-linked immunosorbant assay for zein and other proteins using unconventional solvents for antigen absorption. Anal. Biochem. 137:182-187.

Daussant, J. and P. Corvazier. 1970. Biosynthesis and modification of α- and β-amylases in germinating wheat seeds. FEBS. Letters. 7:191-194.

Davis, B. J. 1964. Disc Electrophoresis. II Methods and applications to human serum proteins. Ann N. Y. Acad. Sci 121:404.

Deikman, J. and R. L. Jones. 1985. Control at α-amylase mRNA accumulation by gibberellic acid and calcium in barley aleurone layers. Plant Physiol. 78:192-198.

Deikman, J. and R. L. Jones. 1986. Regulation of the accumulation of mRNA for α-amylase isoenzymes in barley aleurone. Plant Physiol. 80:672-675.

Doehlert, D. C. and S. H. Duke. 1983. Specific determination of alpha- amylase activity in crude plant extracts containing beta-amylase. Plant Physiol. 71: 229-234.

Dure, L. 1960. Site of origin and extent of activity of amylases in maize germination. Plant Physiol. 35:925-933.

Engvall, E. and P. Perlmann. 1972. Enzyme-linked immmunosorbent asssay (ELISA) quantitation of specific antibodies by enzyme labeled anti -immunoglobin in antigen coated tubes. J. Immmunol. 109:129-135.

Evins, W. H. 1971. Enhancement of polyribosome formation and induction of tryptophan-rich proteins by gibberellic acid. Biochemistry 10:4295- 4303.

Evins, W. H. and J. E. Varner. 1971. Hormonal-Control of polyribosome formation in barley aleurone layers. Plant Physiol. 49:348-352.

Evins, W. H. and J. E. Varner. 1972. Hormonally-Controlled synthesis of endoplasmic cells. Proc. Natl. Acad. Sci. U.S.A. 68:1631-1633.

Filner, P. and J. E. Varner. 1967. A test for *de novo* synthesis of enzymes by density labeling with $H2O^{18}$ of barley alpha-amylase induced by gibberellic acid. Proc. Nat. Acad. Sci. USA 58: 1520-1526.

Finnegan, D. S. 1969. Genetically controlled electrophoretic variants of a starch-degrading enzyme in *Zea mays*. Aust. J. Biol. Sci. 22:1055-1059.

Fischer, E. H. and E. A. Stein. 1960. Alpha-amylases in P. Boyer, H. Lardy and K. Myrback, eds., *The Enzymes*, Vol. 4, Ed. 2. Academic Press, New York pp. 313-343.

Frydenberg, O. and G. Nielsen. 1966. Amylase isozymes in germinating barley seeds. Hereditas 54: 123-139.

Gale, M. D., C. N. Law, A. J. Chojecki and R. A. Rempton. 1983. Genetic control of α-amylase production in wheat. Theor. Appl. Genet. 64:309-316.

Gale, M. D. and C. C. Ainsworth. 1984. The relationship between alpha- amylase species found in developing and germinating wheat grain. Biochem Genet. 22:1031-1036

Gale, M. P. and C. C. Ainsworth. 1984. The relationship between α-amylase species found in developing and germinating wheat grain. Biochem. Genet. 22:11-12.

Geddes, W. F. 1946. The amylases of wheat and their significance in milling & baking technology. Adv. Enzymol. 6:415-468.

Goldstein, L. D. and P. H. Jennings. 1975. The occurrence and development of amylase enzymes in incubated, de-embryonated maize kernels. Plant Physiol. 55: 893-898.

Goldstein, L. D. and P. H. Jennings. 1978. Amylase enzymes isolated from incubated de-embryonated maize kernels. New Phytol. 81: 233-242. Goodman, M. M. and C. W. Stuber. 1981. Genetic identification of lines and crosses using isoenzyme electrophoresis. 35th Annual Corn and Sorghum Research Conference.

Goodwin, T. W. and E. I. Mercer. 1983. *Introduction to Plant Biochemistry* Second edition, Pergamon Press, New York, New York.

Goodman, M. M. and C. W. Stuber. 1983. Maize: *in* "Isozymes in Plant Genetics and Breeding". Part B, eds. S. D. Tanksley and T. J. Orton.

Grabar, P. and C. A. Williams, Jr. 1953. Methodes permettant l'etude conjugee de proprietes electrphoretiques at immunochimiques d'une melange de proteines:application au serun sanguin. Biophys. Acta. 10:193-194.

Gregerson, E. and L. Taiz. 1985. Interpretation of hormone removal experiments in barley aleurone layers. J. Plant Physiol. 120:199- 205.

Haberlant, G. 1890. Die kleberschicht des g ras-Endosperms als diastase ausscheidendes drusengewebe. Ber. Deut. Bot. Ges. 8:40- 48.

Hancock, D. and V. Tsang. 1983. India ink staining of proteins on nitrocellulose paper. Anal. Biochem. 133: 157-162.

Harvey, B. M. R and A. Oaks. 1974. The hydrolysis of endosperm protein in *Zea mays.* Plant Physiol. 53:453-457.

Higgins, T. J. V., J. V. Jacobsen, and J. A. Zwar. 1982. Gibberellic acid and abscisic acid modulate protein synthesis and mRNA levels in barley aleurone layers. Plant Mol. Biol. 1(3):191-215.

Higgins, T. J. V., J. A. Zwar, and J. V. Jacobsen. 1976. Gibberellic acid enhances the level of translatable mRNA for alpha-amylase in barley aleurone layers. Nature 260: 166-169.

Hopkins, R. H. 1946. The action of the amyloses. Advances in enzymology 6:389-412.

Ho, D. T. and J. E. Varner. 1974. Hormonal control of messenger ribonucleic acid metabolism in barley aleurone layers. Proc. Nat. Acad. Sci. USA 71: 4783-4786.

Ihara, H., T. Sasaki, A. Tsuboi, H. Yamagata, N. Tsukagoshi and S. Udaka. 1985. Complete nucleotide sequence of a thermophilic α- amylase gene. Homology between prokaryotic and eukaryotic α- amylases at the active site. J. Biochem. 98: 95-103.

Ingle, J. and R. H. Hageman. 1965. Metabolic changes associated with the germination of corn. III Effects of gibberellic acid on endosperm metabolism. Plant Physiol. 40: 672-675.

Jacobsen, J. V, J. G. Scandalios and J. E. Varner. 1970. Multiple forms of amylase induced by gibberellic acid in isolated barley aleurone layers. Plant Physiol. 45:367-371.

Jacobsen, J. V. and R. B. Knox. 1973. Cytochemical localization and antigenicity of alpha-amylase in barley aleurone tissue. Planta 112: 213-224.

Jacobsen, J. V. and J. A. Zwar. 1974. Gibberellic acid causes the increased synthesis of RNA which contains poly(A) in barley aleurone tissue. Proc. Nat. Acad. Sci. USA 71: 3290-3293.

Jacobsen, J. V. and T. J. V. Higgins. 1982. Characterization of the alpha-amylases synthesized by aleurone layers of Himalaya barley in response to gibberellic acid. Plant Physiol. 70: 1647-1653.

Jones, R. L. 1969a. The fine structure of barley aleurone cells. Planta 86:359-375.

Jones, R. L. 1969b. Gibberellic acid and the fine structure of barley aleurone layer cells. I. Changes during the lag-phase of α- amylase synthesis. Planta 87:119-133.

Jones, R. L. 1969c. Gibberellic acid and the fine structure of barley aleurone cells. II. Changes during the synthesis and secretion of α-amylase. Planta 88:73-86.

Jones, R. L. and J. E. Varner. 1967. The bioassay of gibberellins. Planta (Berl) 72: 155-161.

Jones, R. L. and J. M. Price. 1970. Gibberellic acid and the fine structure of the barley aleurone cells. III. Vacuolation of the aleurone cells during the phase of ribonuclease release. Planta 94:191-202.

Jones, R. L. and J. V. Jacobsen. 1983. Calcium regulation of the secretion of α-amylose isoenzymes and other proteins from barley aleurone layers. Planta 158:1-9.

Jones R. L. and J. Carbonell. 1984. Regulation of the synthesis of barley aleurone α-amylase by gibberellic acid and calcium ions. Plant Physiol. 76:213-218.

Kneed, E., R. M. Sandstedt and C. M. Hollenbeck. 1943. The differential stability of malt amylases. Separation of alpha and beta components. Cereal Chem. 20:399-423.

Kraus, J. P. and L. E. Rosenberg. 1982. Purification of low-abundance messenger RNAs from the rat liver by polysome immunoabsorption. Proc. Nat. Acad. Sci. USA 79: 4015-4019.

Laemmli, U. R. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 227:680-685.

Lehninger, A. L. 1975. *Biochemistry* Worth Publishers, New York, New York.

Linter, C. J. 1986. J. Prakt. Chem. 34:378.

Locy, R. and H. Kende. 1978. The made of secretion of α-amylase in barley aleurone layers. Planta 143:89-999.

Locy, R. and H. Kende. 1978. The mode of secretion of α-amylase in barley aleurone layers. Planta 143:89-99.

Loyter, A. and M. Schramm. 1962. The glycogen-amylase complex as a means of obtaining highly purified α-amylases. Biochim. Biophys. Acta. 65:200-206.

MacGregor, A. E., F. H. MacDougall, C. Mayer and J. Daussant. 1984. Changes in levels of alpha-amylase components in barley tissues during germination and early seed growth. Plant Physiol. 75: 203-206.

MacGregor, A. W., F. H. MacDougall, C. Mayer and J. Daussant. 1984. Changes in levels of α-amylase components in barley tissues during germination and early seedling growth. Plant Physiol. 75: 203-206.

MacGregor, A. W. 1976. A note on the formation of α-amylase in de-embryonated barley kernels. Cereal Chem. 53:792-796.

MacGregor, A. W. 1977. Isolation, purification and electrophoretic properties of an α-amylase from malted barley. J. Inst. Brew. 83:100-103.

MacGregor, A. W. 1976. A note on the formation of α-amylase in de-embryonated barley kernels. Cereal Chem. 53: 792-796.

MacGregor, A. W. and J. Daussant. 1981. Isoelectrofocusing and immunochemical analysis of germinating barley α-amylases after freeze-drying and kilning. J. Inst. Brew. 87:155-157.

Marchylo, B. A., L. J. Lacroix and J. E. Kruger, 1980. Alpha-amylase isoenzymes in canadian wheat cultivars during kernel growth and maturation. Can. J. Plant Sci. 60: 433-443.

Marker, M. 1879. Standpunkt unserer kenntnisse der diastatischer vorgange. Landw. Vers.-Sta. 23:69-70.

Miyata, S., and T. Akazawa. 1982. Alpha-amylase biosynthesis: Evidence for temporal sequence of NH2-terminal peptide cleavage and protein glycosylation. Proc. Nat. Acad. Sci. USA 79: 6566-6568.

Miyata, S., K. Okamoto, A. Watanabe, and T. Akazawa. 1981. Enzymic mechanism of starch breakdown in germinating rice seeds . 10. *In vivo* and *in vitro* synthesis of α- amylase. Plant Physiol. 68:1314-1318.

Miyata, S. and T. Akazawa. 1981. Enzymatic mechanism of starch breakdown in germinating rice seeds. 12. Biosynthesis of α- amylase in relation to glycosylation mechanism. Plant Physiol. 20:147-153.

Moll, B. A. and R. L. Jones. 1982. α-amylase secretion by single barley aleurone layers. Plant Physiol. 70:1148-1155.

Moser, T. J. 1980. Partial purification and characterization of the mRNA for alpha-amylase from barley aleurone layers. Plant Physiol. 65: 834-837.

Muthurishnan, S., G. R. Chandra, and E. S, Maxwell. 1979. Hormone- induced increase in levels of functional mRNA and alpha-amylase mRNA in barley aleurones. Pro. Nat. Acad. Sci. USA 76: 6161-6185.

Nishikawa, K. and M. Nobuhara. 1971. Genetic study of α-amylase isozymes in wheat. I. Localization of genes and variation in tetra- and hexaploid wheat. Jpn. J. Genet. 46:345-358.

Nitsan, J. and A. Lang. 1966. DNA synthesis in the elongating nondividing cells of the lentil epicotyl and its promotion by gibberellin. Plant Physiol. 41:965-970.

Ohisson, E. 1930. The two components of malt diastase especially with reference to the mutarotation of the products formed in the hydrolysis of starch. Physiol. Chem. 189:17-63.

Okamoto, K. and T. Okazawa. 1979. Enzymatic mechanism of starch breakdown in germinating rice seeds. 8. Immunohistochemical localization of β-amylase. Plant Physiol. 64:337-340.

Okamoto, R., H. Kitano and T. Akazawa. 1980. Biosynthesis and excretion of hydrolases in germinating cereal seeds. Plant Cell Physiol. 21:201-204.

Okamoto, R. and T. Akazawa. 1979. Enzymatic mechanism of starch breakdown in germinating rice seeds. α-amylase formation in the epithelium. Plant Physiol. 63:336-340.

Okita, T. W., R. DeClaeya and L. Rappaport. 1979. Synthesis of a possible precursor of alpha-amylase in wheat aleurone cells. Plant Physiol. 63: 195-200.

Ornstein, L. 1964. Ann. N.Y. Acad. Sci. 121:321-?. Osborne, T. B. 1895. On the chemical nature of diastase. J. Am. Chem. Sac. 17:587-603.

Ouchterlony, O.1949. Antigen antibody reactions in gels. Acta. Path. Microbiol. Scand. 26:507-515.

Pagel, L. 1960. Physiological effects of gibberllic acid. I . On carbohydrate metabolism and amylase activity of barley endosperm. Plant Physiol. 35:293-299.

Payen, A. and J. Persoz. 1833. Me miore sur la diastase, les principaux produits de ses reactions, et leurs applications aux arts industriels. Ann. Chim. Phy. (Ser.2) 53:73-92.

Peterson, E. A. and A. H. Sober. 1962. Column Chromatography of proteins: Substituted celluloses. in: *Methods of Enzymology.* Vol V. Ed. by S. P. Colowick and N. O. Kaplan. pp 3-27. Academic Press. New York, New York.

Radley, M. 1967. Site of production of gibberellin-like substances in germinating barley embryos. Planta 75:164-171.

Robyt, J. and D. French. 1963. Action pattern and specificity of an amylase from *B. Subtilis.* Arch. of Biochem. and Biophy. 100: 451-467.

Rodaway, S. J. 1978. Composition of α-amylase secreted by aleurone layers of grains of Himalaya barley. Phytochemistry 17:385-389.

Rogers, J. C. 1985. Two barley α-amylase gene families are regulated differently in aleurone cells. J. Biol. Chem. 260:3731- 3738.

Rogers, J. C. and C. Milliman. 1983. Isolation and sequence analysis of a barley α-amylase cDNA clone. J. Biol. Chem. 258:8169-8174.

Rogers, J. C. and C. Milliman. 1984. Coordinate increase in major transcripts from the high pI α-amylase multigene family in barley aleurone cells stimulated with gibberellic acid. J. Biol. Chem. 258:8169-8174.

Roswell, E. V. and J. L. Goad. 1962. The constituent of wheat binding latent α-amylase of wheat : its mode of attachment to glutenin and its release. Biochem. J. 84: 73-74.

Scandalios J. G., S. E. Chao, and J. C. Melville. 1978. Biochemical characterization of the major amylase form coded by the Amy-1 gene in maize. J. of Heredity 69:149-154.

Scandalios, J. G. 1966. Amylase isozyme polymorphism in maize. Planta 69:244.

Scandalios, J. G. 1969. Genetic control at multiple molecular forms of enzymes in plants. A review. Biochem Genet. 3:37-79.

Skujins, J. J., L. Broal and A. D.McLaren. 1960. Characterization of phosphatase in a terrestrial soil sterilized with an electron beam. Enzymologia 25: 125-133.

Tanaka, Y. and T. Akazawa. 1970. Alpha-amylase isozymes in gibberellic acid treated barley half-seeds. Plant Physiol. 46: 586-591.

Tanaka, Y., T. Ito and T.Akazawa. 1970. Enzymatic mechanism of starch breakdown in germinating rice seeds: α-amylase isozymes. Plant. Physiol. 46:650-654.

Tangl, E. 1886. Studien ubor das endospearm einiger gramineen wiener akad. Wissensch. 92:72-109.

Thomas, T. H., P. F. Wareing and R. M. Robinson. 1965. Action of the sycamore 'Dormin' as a gibberellin antagonist. Nature 205: 1270-1277.

Tkachuk, R. and J. E. Kruger. 1969. Wheat α-amylase I. Isolation. Cereal Chem. 46:219-226.

Tkachuk, R. and J. E. Kruger. 1974b. Wheat α-amylase II. Physical characterization. Cereal Chem. 51:508-529.

Tronier, B. and R. T. Ory. 1970. Association of β-amylase with proteins bodies in barley. Cereal Chem. 43:464-471.

Varner, J. E. 1964. Gibberellic acid controlled synthesis of α- amylase in barley endosperm. Plant Physiol. 39:413-415.

Varner, J. E. and G. R. Chandra. 1964. Hormonal control of enzyme synthesis in barley endosperm. Proc. Natl. Acad. Sci. U.s.a. 52:100-106.

Varner, J. E. and R. M. Mense. 1972. Characteristics of the process of enzyme release from secretory plant cells. Plant Physiol. 49:187-189.

Van Onckelen, H. A. and R. Verbeek. 1969. La formation des isozymes de l'alpha-amylase durant la germination de l'orge. Planta (Berl.) 88: 255-260.

Yomo, H. 1960. Studies on the α-amylase activating substances. IV. On the amylase activating action of gibberellin. Hakko kyokaishi, 18:600-602.

Zwar, J. A. and R. Hooley. 1960. Hormonal regulation of α-amylase gene transcription in wild oat (Auena Fatua L.) alerone protoplasts. Plant Physiol. 80:459-463.

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