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INVESTIGATION OF THE NATURE OF AMYLASE ENZYMES FROM INCUBATED, DE-EMBRYONATED *ZEA MAYS* KERNELS

by

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ABSTRACT

Amylase activity is present in three maize tissue systems. the developing kernel, the germinating kernel, and the incubated de-embryonated kernel. Reaction product patterns and physicochemical analysis of amylase activity indicate the presence of both α and β-amylases in the endosperm of developing and germinating kernels and in the incubated de-embryonated kernel.

A maximum of four starch degrading zones are evident upon polyacrylamide gel electrophoresis (PAGE) analysis of the activity from the three endosperm samples. Correlating with the sequential appearance of starch degrading zones in the activity from the endosperm of the germinating kernel and from the de-embryonated kernel, total amylase activity rises over time of incubation.

Amylase activity in the de-embryonated kernel develops as a function of incubation, with the embryo removed and without the addition of gibberellic acid (GA_3). Expression of amylase activity in the de-embryonated kernel is markedly dependent upon the pretreatment conditions of the kernel and upon the presence of the aleurone and pericarp layers.

The action of GA_3 on the incubated de-embryonated kernel is quantitative, as evinced by the enhanced level of amylase activity during early incubation and the earlier appearance of starch degrading zones on PAGE analysis. PAGE patterns, physicochemical analysis and reaction product patterns for GA_3 and non-GA_3 treated de-embryonated kernels yield similar results, negating a qualitative effect.

The nature of the amylase activity changes in the de-embryonated kernel during incubation. Only β-amylase activity is present during early incubation, in both soluble and latent forms. Alpha-amylase activity arises during incubation. Treatment with RNA and protein synthesis inhibitors implicates a de novo synthesis for this latter activity. A decrease in amylase activity is noted, and the more anodic starch degrading bands fail to appear upon incubation with actinomycin D and cycloheximide.

On ion-exchange chromatography, the amylase activity purified from de-embryonated kernels in mid-course incubation (day 5) is resolved into three major activities. Peaks I, II, and III elute at 0.17 M, 0.42 M, and 0.65 M, NaCl concentration, respectively. PAGE analysis verifies a separation of the amylase electrophoretic complement into these three separate activities. Reaction product patterns and physicochemical analysis of the separated activities point to a β-amylase in Peak I and α-amylase activities in Peaks II and III.

Ion-exchange chromatography of a purified amylase extract from de-embryonated kernels during early incubation (day 1) indicates the presence of one peak (Peak I), eluting at 0.13 M NaCl. The activity of this peak is characteristic of β-amylase. Similarity of physicochemical analysis, reaction product patterns, PAGE analysis and elution profile for Peak I from both early and mid-course preparations suggest that this is the same amylase, that the Peak I activities contain a β-amylase existing in early incubation in both soluble and latent forms, and not requiring RNA or protein synthesis for expression.

Data from this study thus supports the existence of two types of

amylase activity in the incubated de-embryonated kernel. Beta-amylase most likely exists preformed in the maize kernel with early expression during incubation in both soluble and latent forms. Alpha-amylase arises from the de-embryonated kernel during incubation, apparently via de novo synthesis and without the assistance of an embryo factor.

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Part I. Occurrence and Development of Amylase Activity in Maize Kernels.

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INTRODUCTION

The germination of the cereal grain is a complex process. A host of factors are involved in the sequence of events leading to seed germination. The breakdown of the storage carbohydrate in the endosperm of the germinating seed furnishes simple sugars to the growing embryo and is but one aspect of this complex process.

Enzymes involved in the degradation of starch vary widely in their substrate specificity and mode of action. Hydrolytic enzymes, such as the debranching enzyme, play but a small part in supplying soluble carbohydrate to the growing embryo (17). Formation of sugars via the phosphorylytic pathway adds little (55,65). By far, the bulk of the simple sugars is supplied by the complementary actions of α and β-amylases (1). The amylases have been identified in a number of cereal grains (13).

Investigation of the origin of amylases and elucidation of their in situ behaviour in cereal grains derives mostly from studies with barley (37, 82, 83, 93). It would appear that the expression of amylase activity in the germinating grain is dependent upon three factors. biochemical control mechanisms (which include new protein and RNA synthesis), hormonal control, and activation and secretion mechanisms.

Previous work gives indication that the maize system is in some ways similar to that of barley (17, 27, 28, 35). However, further work is necessary to fully understand the expression of amylase activity in the developing and germinating seed.

The present study is divided into two segments. The first of these

deals with the occurrence and development of amylase activity in the maize kernel. The latter deals with the isolation and characterization of part of this activity. The results of this study will be directed toward elucidating the origin and nature of amylases in the maize kernel.

LITERATURE REVIEW

1. Definition and Classification of the Amylases.

The amylases are hydrolytic enzymes. Hydrolysis of the starch substrate, a glucose polymer, is achieved by the cleavage of an α 1,4 bond with the transfer of a glucosyl fragment to water.

Alpha-amylase (α 1,4 glucan 4 glucanohydrolase, E.C.3.2.1.1.) is termed thus because its hydrolytic reaction products possess the α configuration (23, 32). Alpha-amylase is an endoamylase, i.e., it randomnly cleaves large linear substrates at internal bonds. In its attack the amylase has the ability to bypass α 1,6 linkages or branch points within the starch molecule. Thus, the enzyme's reaction products are of varying molecular weight and size (23).

Beta-amylase (α 1,4 glucan maltohydrolase, E.C.3.2.1.2.) is an exoamylase. It cleaves alternate linkages in a linear glucose polymer from the non-reducing end. However, the enzyme does not have the ability to bypass branch points within a starch molecule. Thus, its sole reaction product is the dissaccharide, maltose, of the β configuration (88).

Because starch, glycogen and related glucose polymers serve as universal sources of dietary carbohydrate, one would expect the starch degrading enzymes to be ubiquitous as well. Alpha-amylase, indeed, is found throughout the plant and animal kingdoms. In contrast, β-amylase is limited to the plant kingdom, where it is found in seeds of higher plants, in cereal grains and in the sweet potato (88).

Molecular weights for α and β-amylases from a variety of sources are in the range of about 50,000 (88). For the most part, α and β-amylases

exist in monomeric forms, although a tetramer β-amylase has been reported for the sweet potato (88). The physical properties of the amylases are typically those of globular proteins.

Differentiation between α and β-amylases takes advantage of inherent physicochemical differences. These properties constitute the methods generally used by workers in the field to identify what are regarded as classical α and β-amylases (86, 17, 25, 37, 97).

All plant and animal α-amylases studied thus far contain calcium, and its removal results in either reversible or irreversible inactivation, or in great loss of thermal stability (88). Cereal α-amylases bind calcium weakly and its removal is easily effected by dialysis against EDTA, a metal chelating agent. Thermal inactivation of cereal α-amylases is greatly retarded at temperatures above 50°C when high concentrations of calcium ion are present (88). It would appear that the calcium ion not only stabilizes the structure of the α-amylase molecule, but helps to maintain the enzymatically active conformation as well. In contrast, β-amylase does not appear to require calcium or any other metals for activation or stabilization (24).

Beta-amylase is a sulfhydryl containing enzyme. The β-amylase from wheat flour contains four sulfhydryl groups and one disulfide bond (90). Implication of β-amylase thiol groups in direct substrate catalysis or binding seems unlikely. Chemical modification studies of β-amylase activity indicate that the thiol sites may play a regulatory role <u>in vivo</u>, with the enzyme reversibly inactivated via disulfide interchanges (84). This observation is consistent with the diverse effects of a wide variety of sulfhydryl reagents of β-amylase activity (71, 82, 83, 91).

Beta-amylase is also sensitive to the presence of heavy metal ions (Cu^{++}, Hg^{++}, Ag^{++}, Pb^{++}). These heavy metals act as non-competitive inhibitors of the enzyme. They form mercaptides with the free sulfhydryl groups, bringing about changes in enzyme activity due to alteration of the enzyme conformation (52).

2. Alpha and Beta-Amylase Enzymes in the Developing Cereal Grain.

An early report indicates that amylase activity appears in the cereal grain immediately after anthesis. As grains of wheat, oats, rye and barley ripen, total activity gradually decreases (13).

Alpha-amylase is present in the immature grains of wheat (67, 7), barley (5, 15, 16, 50), and maize (22). Activity reaches a peak level in the immature grain (7, 15, 16). Thereafter, α-amylase activity decreases to a low level during ripening and does not appear again in appreciable quantities until germination (7, 67). Olered and Jonsson (1970) postulate that the decreasing moisture level of the grain upon maturation may play a role in the decreasing levels of α-amylase in wheat. They found that α-amylase was continuously inactivated as the grain matured. However, the process was reversed when evaporation of moisture from the grain was retarded. The α-amylase activity was thought to increase as a consequence of a higher amount of dissolved enzyme.

The expression of α-amylase activity in the developing grain may be under hormonal control (15). Duffus (1969) found that α-amylase in developing barley was mainly localized in the endosperm and aleurone layers. The formation of α-amylase was inhibited by application of the gibberellic acid synthesis inhibitor, chlorocholine chloride (CCC). Inhibition could be overcome by subsequent addition of gibberellic acid (GA_3). Gibberellins

are known to be synthesized in the scutellum of developing barley (72, 73), and to be inhibited by the addition of CCC (72, 99). Duffus (1969) suggests that α-amylase synthesis in the developing barley may be controlled by gibberellins, similar to the mechanism found in the germinating barley, where GA_3 induced de novo synthesis of α-amylase is known to occur (21, 37).

Beta-amylase is thought to be stored in the ungerminated cereal grain in an insoluble and inactive zymogen form (82, 83). Zymogen granules containing an inactive glucosidase enzyme have been identified in ungerminated peas (58). Evidence for the presence of both active and latent forms of β-amylase in the developing grain have been reported by a number of researchers (5, 16, 22, 50, 82, 83).

In the developing barley seed total β-amylase appears to remain constant, while the free, soluble enzyme gradually decreases and the bound, insoluble enzyme gradually increases with maturation (16, 82, 83). On maturation portions of the active β-amylase are most likely denatured, as with α-amylase, or changed into a latent form, due to rapid decrease in moisture levels of the grain in the latter stages of ripening (82). Early reports indicate β-amylase to be stored in the sub-aleurone layers of barley, wheat and rye (18). However, latent and soluble forms of β-amylase have been found in the barley endosperm as well (16).

The nature of zymogen granules and the mechanism of release of bound β-amylase have been studied in barley (82, 83) and in wheat (75, 76, 77, 78). The latent enzyme is believed to be chemically bound to cereal protein by disulfide bonds. Release of the enzyme is effected by treatment with sulfhydryl reagent (2-mercaptoethanol), which reduce disulfide linkages, or by proteolytic enzymes (papain), which split peptide bonds, releasing

the β-amylase from its zymogen form (76, 78, 82, 83). Additions of GA_3 increased the active β-amylase, probably via GA_3 enhanced synthesis of protease, which subsequently releases the bound β-amylase (33, 38, 77, 78, 82).

Alternatively, and consistent with the proposed regulatory role of β-amylase thiol groups, β-amylase activation could be achieved by the action of protein disulfide reductase, known to be present in barley (84). The disulfide reductase may catalyze the reaction of disulfides to sulfhydryls during germination, leading to β-amylase conformational changes which activate the enzyme.

The bulk of the research dealing with the occurrence of α and B-amylases in developing cereal grains has been with grains other than maize. However, evidence from electrophoretic studies (9, 22) indicate the presence of at least one β-amylase in the liquid endosperm of developing maize kernels. Dure (1960) reported β-amylase to be the only amylase present in the endosperm of the resting, mature seed, and concluded that the enzyme is formed in the endosperm during development of the grain.

Evidence for the release of a latent β-amylase in maize is supported by the work of Harvey and Oaks (1974a,b). These researchers found two types of protease in the maize endosperm. One protease is present in the endosperm prior to germination, responsible for the breakdown of the storage proteins (zein and glutelin), and regulated by the level of its amino acid reaction products. The other in GA_3 enhanced, formed in the aleurone layer upon germination, and responsible for the activation of bound β-amylase.

The presence of at least one α-amylase in the endosperm of developing maize kernels has been confirmed by several researchers (9, 10, 22).

Total amylase activity in the developing maize endosperm reaches its maximum 30 to 35 days after pollination, thereafter decreasing. Dry, harvested maize kernels were found to have very low levels of activity (22).

3. Cereal Grain Physiology.

The germination of the cereal grain involves two distinct processes. The synthesis of macromolecules in the embryo, and the degradation of storage materials in the endosperm (66). The development of the enzymatic machinery to convert polymeric reserves of the endosperm to supply the needs of the growing embryo presents one of the first challenges to the germinating seed. In the cereal grain the relevant enzymes are the amylases and the proteases (45).

The endosperm of the cereal grain is a relatively inert tissue, characterized by lack of cell division, lack of photosynthesis, and a low respiration rate (45). Microscopic examination of endosperm cells from maize reveals the presence of starch granules and protein bodies composed of zein, both of which are held together by a glutelin protein matrix (12).

Metabolic changes associated with germination first occur in the maize embryo, proceeding through the scutellum to the endosperm (36). It is inviting to assume that new activities in the storage endosperm are directly related to the new metabolic activities in the embryo.

Changes in the endosperm during germination could be mediated via the aleurone layer, which surrounds the endosperm. The aleurone layer from mature barley is composed of highly differentiated cells, three to four cell layers in thickness (42). The morphological specialization which characterizes these cells is the presence of aleurone grains, single membrane bound storage organelles of protein and phytin (42).

Ultrastructural studies by Jones (1969) lend support to the role of aleurone storage proteins as substrates for enzyme synthesis, while phytin could be a principal phosphorous reserve. The aleurone layer is capable of both respiration and amino acid incorporation (95).

4. Amylases in the Germinating Cereal Grain.

In 1960 Paleg and others, working with barley, discovered that GA_3 could substitute for the embryo in the initiation of events leading to substrate mobilization. Subsequently, Varner and associates (21, 38, 93) established the GA_3 directed de novo synthesis of α-amylase in barley endosperm and isolated barley aleurone layers.

The relationship between the embryo and other seed parts in barley has now been fairly well established. Gibberellins similar to GA_1 and GA_3 are produced in the embryonic axis and/or scutellum and transported to the aleurone layer (45, 72, 73). The gibberellins induce de novo synthesis of hydrolytic enzymes within the aleurone layer (21, 38). These enzymes are secreted into the endosperm where hydrolysis of the stored substrates takes place (45, 44, 46). The products of enzyme hydrolysis are absorbed by the scutellum and translocated to the growing embryo to maintain early seedling development (45).

At least four hydrolytic enzymes have been found to be controlled by GA_3 and synthesized de novo in barley. β 1,3 glucanase (a cell wall hydrolysing enzyme) (2), protease (38), ribonuclease (2, 11), and α-amylase (11, 21).

Inhibition of α-amylase production in the barley aleurone by the protein synthesis inhibitor, cycloheximide, is consistent with the observation that this synthesis is de novo (93). Likewise, de novo

synthesis of α-amylase has been implicated in other cereal grains. Additions of cycloheximide prevented α-amylase expression in the maize endosperm (27, 28, 34). Palmiano and Juliano (1972) found inhibition of expression of protease and α-amylase in rice during germination when cycloheximide was added.

In barley the production of α-amylase can also be inhibited by inhibitors of RNA synthesis, e.g. actinomycin D and 6 methyl purine (11, 95). Studies by Goodwin and Carr (1972) show that actinomycin D inhibition in barley is especially evident during the 'lag phase', before the activity of α-amylase is measurable and during the period when gene activation and/or reactions preparatory to enzyme synthesis are most likely taking place.

The GA_3 enhanced de novo synthesis of α-amylase according to this scheme would obviate the need for biochemical apparatus necessary for new protein synthesis. This is borne out by the observation that during the 'lag phase' of α-amylase production in GA_3 treated barley aleurone layers, there is an increase in ribosomes, an increase in the percentage of ribosomes associated as polyribosomes, and an increase in membrane formation, particularly in endoplasmic reticulum (ER), over that in the control tissue (19, 20). Evins and Varner (1972) speculate that these polysomes bound to ER are functional in hydrolase synthesis. GA_3 could function quantitatively to regulate changes in ribosome synthesis, or qualitatively to regulate changes in the amount of ER. Ultrastructural studies on GA3 treated barley aleurone layers confirm increases in the above cellular constituents, as well as showing degradation of aleurone grain protein, which could serve as substrate for new enzyme synthesis (47).

The exogenous GA_3 enhancement of α-amylase synthesis lends itself to other interpretations. In studies with the aleurone layers of a domestic oat variety, incubation with GA_3 showed only an enhanced rate of formation of α-amylase. The total amount of the enzyme formed was independent of the hormone (63). With wild oat, where a strong GA_3 requirement was apparent for initiation of α-amylase synthesis, a partial effect could be obtained by replacing GA_3 with an amino acid complement. As a consequence, Naylor (1969) suggested that the primary site of GA_3 activity might be in proteolysis, thereby making available amino acids for synthesis of a broad range of enzymes. The lack of requirement for GA_3 in the domestic oat variety could reflect a genetic situation, where the endogenous supply of gibberellins is greater or autonomous proteolysis occurs.

The maize kernel has been subjected to much less scrutiny than other grains, and germination studies have resulted in conflicting results.

Bernstein (1943) first suggested separate origins for the amylases. α-amylase arising as an embryo characteristic, and β-amylase formed in situ in the endosperm. Consistent with Berstein's genetic studies, Dure (1960) reported β-amylase to be present in the maize endosperm prior to germination, while α-amylase develops in the embryo during germination, is secreted into the endosperm, and accounts for the greater proportion of the endosperm amylase activity during germination.

In line with studies on barley, Ingle and Hageman (1965) suggest that GA_3 replaces an embryo factor produced during germination. These researchers found carbohydrate catabolism in the excised maize endosperm to be initiated and completely dependent upon exogenous GA_3. Thus the implication that gibberellins control the synthesis of amylase enzymes

which arise in the endosperm and/or aleurone layers of the maize kernel during germination.

More recent work suggests that the maize endosperm is capable of digesting its own starch reserves, independent of the embryo (27, 28, 33, 34). Working with cultivars of Seneca Chief, Goldstein and Jennings (1975) reported the presence of both α and β-amylase activities arising from incubated, de-embryonated kernels. Additions of GA_3 to the de-embryonated kernels resulted in a quantitative effect, as evidenced by a faster increase in amylase activity. The nature of the amylase activity and the total activity realized in both GA_3 and control samples was similar. Additions of actinomycin D and cycloheximide were capable of inhibiting amylase development during early incubation of the de-embryonated kernels, suggesting the involvement of protein synthesis in amylase expression.

Harvey and Oaks (1974a,b) also present evidence for the de novo synthesis of amylase in the endosperm of the maize kernel. They propose that gibberellins are instrumental in the development of α-amylase, where they may function to overcome the abscisic acid (ABA) inhibition to α-amylase development. Variation in reported results on the effect of GA_3 in the maize kernel could thus be due to differences in relative endogenous levels of gibberellins and ABA in the maize cultivar used for experimentation (34).

ABA has frequently been implicated as a dormancy imposing factor in the resting seed (98), and has been shown to inhibit α-amylase synthesis in the barley aleurone (11). The counteracting of the GA_3 response by ABA in a GA_3 treated barley aleurone may be a direct effect where ABA

enhances the conversion of GA_3 to inactive intermediates (61). Indirectly, ABA prevents the GA_3 enhanced increase in polysome content in the barley aleurone, a phenomenum which may be preparatory to the GA_3 controlled de novo synthesis of α-amylase (20).

5. Isozymes and Genetic Control.

In addition to the influences of environmental and hormonal factors, genetic controls play a role in the expression of amylase activity. Both α and B amylases have been found to exist in multiple molecular forms (25, 79, 80, 87, 90) in the cereal grain. These multiple forms may be separated by means of gel electrophoresis, indicating differences in size or net electrical charge of the enzymes (81). Different molecular forms of an enzyme which exhibit the same enzymatic specificity have been termed isozymes or isoenzymes (81).

Isozymes, although catalyzing the same reaction, may differ in catalytic properties, such as substrate affinity, inhibitor sensitivity, pH optima, thermal stability and specific activity, pointing to possible variations in their in vivo roles (81). Although the above criteria may be used in characterizing isozyme activities, true evidence as to the existence of isozymes within a tissue comes from demonstration of isozyme structural differences or by demonstration that the isozymes are synthesized under independent genetic control (81).

In this regard a number of researchers have shown multiple forms of amylases in cereal grains to be discreet entities, not arising from artifacts of preparation (89, 93). The level of acidic amino acids appears solely responsible for differences in net electrical charge of

the amylase isozymes, as evidenced by differences in amino acid composition and tryptic peptide maps (54). In addition, genetic evidence lends further support for the presence of real isozyme expression (9, 10).

Amylase isozymes have been reported in developing wheat (67), and developing barley (5). Electrophoretic studies have been employed to identify α and β-amylase variants in germinating barley (6, 25), germinating rice (87), germinating rye (97), and in wheat (49, 89, 90).

In maize several researchers using the technique of starch gel electrophoresis to identify individual activities, have examined the genetic control mechanisms underlying the presence of multiple starch degrading enzymes (9, 10, 22, 79, 80).

Finnigan (1969) found two zones of starch degrading activity in the endosperm of maize kernels harvested 30 days after pollination. The first zone was clear on starch gel electrophoresis, implying complete degradation of the substrate, and it was identified as an α-amylase. Three bands (A,B,C) in the second zone appeared rust colored due to incomplete hydrolysis of the substrate. That zone was identified as having β-amylase activity.

In all progeny and crosses, the single clear band with α-amylase activity remained and exhibited the same mobility. However, the β-amylase variants, A,B, and C, were found to be each controlled by a single allele, (Sd^A, Sd^B, Sd^C) with the heterozygote having two bands corresponding with its two alleles, but no hybrid banding (22).

Chao and Scandalios (1969) reported three zones of starch degrading activity from the liquid endosperm of 16 to 20 day old maize kernels. These were identified as α-amylase (zone 1), β-amylase (zone 2), and

α-glucan phosphorylase (zone 3). Results from genetic crosses show that zone 2 was controlled by two co-dominant alleles (Amy^{2A}, Amy^{2B}). A and B, the respective fast and slow moving electrophoretic variants were thought to be each one type of β-amylase monomer, with the heterozygote possessing both active variants.

Chao and Scandalios (1971) subsequently investigated the Amy 1 α-amylase. The two genetic variants (A and B) were found to be also controlled by two co-dominant alleles (Amy^{1A}, Amy^{1B}). The variants were identical in molecular weight, but differed in isoelectric pH and electrophoretic mobility. Aberrant F_2 segration of the Amy 1 variants supported a differential allelic expression for the Amy 1 locus which was found to be both tissue and age dependent. The maize endosperm is a triploid tissue, having two doses of maternal genes to one of paternal. Investigation of gene dosage effects in the endosperm of the germinating seedling indicated that for the Amy 1 amylase, gene dosage may be either additive or the result of preferential expression for one of the two alleles (10).

6. Purification, Fractionation, and Characterization of Amylases.

A. Purification. The amylases are typical protein and may be purified by conventional protein fractionation techniques (23). For preliminary work, concentrations of ammonium sulfate or cold acetone have been used to flocculate protein containing the active enzyme fraction from a number of cereal grain preparations (27, 51, 89). The precipitation accomplishes two purposes. The active fraction is separated from extraneous materials and concentrated for further purification.

Gel filtration chromatography is a purification technique primarily

based upon differences in size. The gel filtration medium consists of hydrated dextran, polyacrylamide or agarose gels. Gels of various fractionation ranges are used to effect separation of lower molecular weight substances from the higher molecular weight compact globular proteins, such as the amylases. Application of a gel with specific exclusion limit (molecular weight of the smallest molecule incapable of penetrating the pores of the gel matrix and thus passing through the column) allows not only separation of components but preliminary information as to the molecular weight of the enzymes in question (6, 51, 82).

B. Fractionation. The separation of multiple forms of amylases almose invariably takes advantage of differences in net electrical charge among the enzymes. Gel electrophoresis and ion-exchange chromatography are the techniques most commonly employed.

Electrophoresis is presently the most powerful analytical technique available to separate enzymes. The theory underlying electrophoresis is relatively simple. The application of direct current separates the enzymes according to the different and characteristic electrical charge of each enzyme. Mobility of an enzyme on a solid support in gel electrophoresis may be altered by the substitution of a single amino acid in the enzyme, with a charge different from the one it replaces (81).

Electrophoretic analysis has been widely used to study multiple forms of amylases in developing (5, 9, 22, 67) and germinating (25, 92) grains. In addition electrophoresis may serve as a means of verifying amylase homogeneity after purification procedures (89), and as an analytical tool to study the effects of physicochemical treatments (6, 37, 97) and hormone application (87, 93, 95).

Ion-exchange chromatography has been successfully employed for the resolution of multiple forms of amylases (59, 83, 89). Adsorption of the enzyme to ion-exchange celluloses involves the formation of multiple ionic bonds between charged groups on the enzyme and available groups of opposite charge on the adsorbent. Differential elution of the adsorbed enzymes is then effected by altering the charged enzyme state or by use of agents capable of competing with the adsorbed enzyme for charged sites on the adsorbent (35).

Amylase variants have been fractionated by ion-exchange chromatography from extracts of barley (59, 83) and wheat (89). Tipples and Trachuk (1965) demonstrated the presence of distinct β-amylase isozyme in wheat. These researchers analyzed bound β-amylase by extraction with NaCl solutions, eliminating possible artifacts arising from the extraction of bound β-amylases with proteases or thiol reagents.

C. Characterization. 1. Physicochemical Properties and Reaction Products. Differentiation between α and β-amylases relies greatly upon analysis of their physicochemical properties and their reaction products.

Differences in the physicochemical properties of classical α and β-amylases have been discussed earlier (1. Definition and Classification of α and β-Amylases). Selective denaturation of α-amylase can be achieved by dialysis against calcium chelating agents, such as ethylenediamine-tetra-acetic acid (EDTA), or subjection of the α-amylase to low pH (pH 3.5) (16, 17, 97). Denaturation of classical β-amylase, insensitive to the above treatments, may be effected by incubation in the presence of excess calcium ions at a high temperature (70°C) for a time period which will not inactivate classical α-amylase (12 minutes). Also, β-amylase denaturation

is effected by reaction with heavy metal ions, a treatment to which classical α-amylase is insensitive (16, 17, 97).

Reaction product analysis by paper chromatography (74) yields data not only on the type of reaction products from amylase hydrolysis, but on their relative amounts and order of appearance, when incubation periods with the substrate are timed. Reaction products patterns for β-amylase acting on a soluble starch substrate will yield only maltose as the initial reaction product. The presence of high molecular weight oliogo-saccharides occurs later, as the large polymer is cleaved (88). Alpha-amylase action characteristically shows the presence of a variety of oliogosaccharides earlier in the hydrolysis, an indication of more randomn attack on the glucose polymer (32). Glucose is present from α-amylase hydrolysis, but absent in β-amylase hydrolysis, as the latter enzyme does not cleave maltose or maltotriose (88).

2. Kinetic Analysis. The stability and catalytic efficiency of an amylase are pH dependent factors. Cereal α-amylases usually exhibit optimal activity between pH 5.0 and pH 6.0 (32). Below pH 5.0, irreversible inactivation occurs (32). Optimum pH values for crystalline malt α-amylase (pH 5.3) (60), and α-amylase from pea cotyledon (pH 5.3 to 5.9) (86) have been reported. Lee and Unrau (1969) found the pH optimum and pH stability range for wheat α-amylase to be pH 4.5 and pH 4.0 to 8.4, respectively. Rye α-amylase showed a pH optimum of pH 4.9 to 5.0, and a range of pH 4.0 to 8.1 (51).

The pH optima for β-amylases lie between pH 4.0 and pH 6.0, and the enzyme is relatively stable at pH 3.5 (32). Values for pH optima within this range. pH 4.6 to 5.2 for wheat β-amylase (90), pH 4.0 for rye

β-amylase (60), and pH 4.8 to 5.0 for sweet potato β-amylase (60), have been reported.

Rate constants for enzyme reactions universally exhibit temperature dependence. Temperature optima for amylases usually range between 50°C and 65°C (51). Above this range, the reaction rate decreases due to the denaturation of the amylase. Disruption of intramolecular hydrogen bonding may cause changes in secondary and tertiary enzyme structure (51).

Energy of activation (E_a) for amylase catalyzed hydrolysis may be evaluated from an Arrhenius plot of activity. The amylase activity in arbitrary units is plotted as a logarithm against the reciprocal of the absolute temperature. The E_a may then be calculated within a given temperature range from the slope of the resulting curve. The relationship is expressed by the following equation (57).

$$E_a = -2.303\ R(\text{slope})$$

Activation energies for amylases are usually about 14 Kcal/mole at 10°C, decreasing to a few Kcal/mole at 40 to 50°C (88). Activation energies at 10 to 20°C of 13.5 Kcal/mole for wheat α-amylase and 8.2 Kcal/mole for rye α-amylase have been reported (51).

Curvature of Arrhenius plots under widely varying conditions are usually interpreted as decreases in E_a with increasing temperatures. However, a decrease in E_a may also reflect an increase in the dissociation constant of the enzyme-substrate complex as well as greater denaturation rates at higher temperatures (32, 88).

As the rate of an enzyme catalyzed reaction ordinarily increases with increasing substrate concentration, barring cases where the

reaction product(s) acts as an enzyme inhibitor, a rate curve may be plotted. Michaelis-Menten constants, K_m and V_m, are determined from a Lineweaver-Burke plot.

The K_m varies with the substrate used (60). Raw starch granules in addition to having generally complicated and undefined structures, are very resistant to amylase action for kinetic work (60). Although perhaps not indicative of in situ substrate properties, the soluble starches, such as Lintner starch (which is acid-modified) have often been used in determination of kinetic parameters (60). The K_m values for amylases are in the range of 2.0×10^{-4} to 5.0×10^{-3} grams of soluble starch/ml (51, 32, 86).

MATERIALS AND METHODS

1. Plant Material

Seed of a commercial variety of field corn, Funk's G-17, was used for all studies.

2. Tissue Preparation

a. Developing Kernels. Ears were harvested 24 days after pollination. Kernels were removed from several ears and intact embryos were separated from the soft kernels with a scalpel. Endosperm and embryo samples were kept at 0°C prior to enzyme extraction.

b. Mature Kernels. The mature seeds, obtained commercially, were de-embryonated by hand, using a file and scalpel. Differences in color and texture were used as the criteria for the complete removal of the softer embryo from the hard endosperm. For some studies the pericarp and aleurone layers of the intact kernels were mechanically removed by rotating the kernels in a sandpaper-lined canister over a 10 hour period. The kernels were then de-embryonated and any adhering outer layers were manually filed away.

The de-embryonated kernels with or without pericarp and aleurone layers were stored at 4 to 6°C either in a humidity chamber (75% R.H.), or in a desiccator (less than 10% R.H.) for at least two months prior to experimentation. Intact kernels were stored at the low temperature in the high humidity environment.

3. Tissue Incubation

a. De-embryonated and Germinating Kernels. Either intact or

de-embryonated kernels were surface sterilized with 1% (w/v) calcium hypochlorite solution for 15 min. with constant agitation. This was followed by rinsing with 10 changes of sterile, de-ionized water. The kernels were transferred under sterile conditions to 9 cm diamter petri dishes, 10 kernels/plate. The incubation medium consisted of 10 ml of sterile water containing 50 ug Penicillin G and 3.0 ug of Streptomycin sulfate. The kernels were incubated at 30°C in the dark.

4. Preparation of the Enzyme Extract

a. Developing Kernels. Fifteen ml of 0.05 M Tris-HCl buffer, pH 7.6, were added to samples of 20 endosperms. The sample was homogenized for 1.5 min. (0.5 min low speed, 1.0 min. high speed) in a Virtis 45 homogenizer. The resultant homogenate was centrifuged at 20,000 g for 15 min., and the supernatant collected. The pellet was resuspended in 4 ml of the Tris-HCl buffer and centrifuged as above. The combined supernatants were decanted through 4 layers of cheesecloth, made up to a 20 ml volume with the Tris-HCl buffer, and served as the crude endosperm extract.

Fine grade sand was added to samples of 20 embryos, which were ground with mortar and pestle. Ten ml of 0.05 M Tris-HCl buffer, pH 7.6, were added to each sample. The homogenate was centrifuged as above. The pellet was resuspended with 2 ml of the Tris-HCl buffer and again centrifuged as above. The combined supernatants were decanted through 4 layers of cheesecloth, made up to a 15 ml volume, and served as the crude embryo extract.

b. De-embryonated Kernels. After incubation for the specified period of time, 20 kernel samples with residual incubation medium plus 2 ml of Tris-HCl buffer, pH 7.6, were homogenized for two min. (0.5 min low speed,

0.5 min. medium speed, 1.0 min. high speed) using a Virtis 45 homogenizer. The homogenate was centrifuged at 20,000 g for 15 min.,and the supernatant collected. The pellet was resuspended with 4 ml of the Tris-HCl buffer, and centrifuged as above. The combined supernatants were decanted through 4 layers of cheesecloth, made up to a 25 ml volume with Tris-HCl buffer, and served as the crude endosperm extract.

For determination of bound enzyme in incubated, de-embryonated kernels, the pellet obtained from the above preparation was resuspended with 4 ml of the Tris-HCl buffer, centrifuged as above and the supernatant decanted through 4 layers of cheesecloth. This procedure was repeated three times, after which no amylase activity could be detected in the resulting, collected supernatants. The washed pellet was then resuspended in 20 ml of 0.05 M citrate buffer, pH 5.4, containing 0.1% papain and 0.1% cysteine. The suspension was incubated at 30°C with frequent stirring. At specified time periods, the suspension was centrifuged for 15 min. at 20,000g. The supernatant was collected and decanted through 4 layers of cheesecloth, and served as the source of soluble enzyme released from a bound form in the pellet.

c. Germinating Kernels. After a specified incubation period, the embryo including root and shoot was separated from the endosperm with a scalpel. The endosperm fraction from 20 kernels with 2 ml of Tris-HCl buffer and the residual incubation medium was homogenized, centrifuged, and resuspended as described for incubated, de-embryonated kernels (4b). The collected supernatants were decanted through 4 layers of cheesecloth, made up to 25 ml volume with 0.05 M Tris-HCl buffer, pH 7.6, and served as the crude endosperm extract.

Twenty ml of the Tris-HCl buffer were added to the germinating embryo fraction, including root and shoot, separated from the 20 kernels. The embryos were homogenized for 1.5 min (0.5 min low speed, 1.0 min. high speed) in a Virtis 45 homogenizer. The homogenate was centrifuged at 20,000 g for 15 min., and the supernatant collected. The pellet was resuspended in 3 ml of the Tris-HCl buffer and centrifuged as above. The collected supernatants were decanted through four layers of cheesecloth, made up to a 25 ml volume with the Tris-HCl buffer and served as the crude embryo extract.

5. Determination of Enzyme Activity

Total amylase activity for all crude and purified enzyme extracts was determined by the method of Bernfeld (1955). The assay measured reducing sugars released from the amylase catalyzed hydrolysis of a soluble starch substrate.

The standard reaction mixture consisted of 1 ml of 2% Merck soluble starch (prepared according to Lintner), 0.1 to 0.5 ml of the enzyme extract (depending upon activity), and 0.05 M acetate buffer, pH 5.0, adjusted to give a final volume of 5 ml for the reaction mixture.

The soluble starch and buffer were equilibrated at 30°C. The assay, initiated by addition of the enzyme, was carried out at 30°C in a constant temperature water bath. At incubation times ranging from 5 to 20 min., 1 ml aliquots of the reaction mixture were withdrawn and added to 3 ml of dinitroslicylic acid reagent (DNSA). Reducing power was determined spectrophotometrically (575 nm), according to Sumner and Somers (1949). Standard curves using maltose as the reducing sugar, were prepared and the enzyme activity was expressed as mg maltose/unit time.

6. Determination of Protein

Total protein for crude and purified enzyme extracts was determined by the method of Lowry et al. (1951), using the Folin-Ciocaltau reagent. A standard curve was prepared using bovine serum albumin.

7. Determination of Enzyme Complement

Crude and purified enzyme extracts were subjected to polyacrylamide gel electrophoresis (PAGE) as a method for determining the presence and number of starch degrading enzyme in the extracts. A modified method of Davis (1964) was used.

Twenty ml of a small pore gel preparation containing 8.25% gel solution and 2.5 ml of a 4.0% iodometric starch solution were placed in 12 cylindrical glass tubes (5 mm x 0.5 mm ID). Large pore gel (0.4 mm in depth) was placed on top of the solidified small pore gel. The enzyme extracts (0.1 to 0.3 ml) were placed on top of the gels, and the gels were run vertically at 3 mAmp/tube at 4 to 6°C for approximately one hour. A bromophenol blue tracking dye was used to determine when electrophoresis was completed.

The gels were extruded from the tubes and incubated for 40 min. at room temperature with a 4% iodometric starch solution. At the end of incubation, the gels were washed and stained with an iodine solution (12 g KI and 1.2 g I_2/L). Areas of starch degrading activity appeared as clear zones against a dark blue background.

8. Enzyme Purification Procedures

a. Bulk Enzyme Extraction. Bulk enzyme extracts from 200 de-embryonated kernels were prepared after one and five days of incubation. Day 1 kernels

with residual incubation medium were homogenized with 30 ml of 0.05 M Tris-HCl buffer, pH 7.6 and with 50 ml of the Tris-HCl buffer for the day 5 preparation. Homogenization times in the Virtis 45 homogenizer for day 1 kernels was 0.5 min low speed, 1.0 min. medium speed, and 1.5 min. high speed. Homogenization time for the day 5 kernels was 0.5 min. low speed, 0.5 min. medium speed, and 1.0 min. high speed. The homogenates were centrifuged for 15 min. at 20,000 g and the supernatants collected. The pellets from both preparations were resuspended in 40 ml of the Tris-HCl buffer and centrifuged as above. The combined supernatants for both day 1 and 5 preparations were decanted through 4 layers of cheesecloth. The amount of buffer added to the kernels for homogenization and the time of homogenization were dictated by the amount of residual incubation medium and the softness of the kernels.

Precipitation of the soluble enzyme from the supernatant was effected by the addition of ammonium sulfate to give 75% saturation (w/v). The ammonium sulfate was gradually added with constant stirring, and the mixture was allowed to stand for 15 min. at 4 to 6 °C at allow flocculation of the protein. The pellet containing the precipitated protein was collected after centrifuging at 35,000 g for 15 minutes.

b. Gel Filtration Chromatography. Elimination of low molecular weight compounds from the pellet containing amylase activity was effectd by the use of gel filtration chromatography. Sephadex G-50 (Pharmacia) was hydrated with 0.05 M Tris-HCl buffer, pH 7.6, packed into a 2.5 x 20 cm column, and equilibrated with the buffer. A nylon mesh disk was placed over the top of the sephadex to eliminate mixing or uneveness of the gel surface. The pellet containing amylase activity was resuspended

with 2.5 ml of the Tris-HCl buffer and placed onto the column. The sample was eluted from the column with the Tris-HCl buffer by gravity flow at the rate of 5 ml/3 minutes. Active fractions obtained from the column were bulked and precipitated with ammonium acetate as described above (8a).

9. Enzyme Fractionation Procedures

a. Ion-Exchange Chromatography. Ion-exchange chromatography was employed as a method of separating the partially purified amylase complement into activities based on charge difference. The differential elution of activities from the column was effected by use of a linear salt gradient. Diethylaminoethyl cellulose (DEAE-cellulose) was used as an anion exchanger and was prepared according to the method of Peterson and Sober (1962). The matrix material was equilibrated with 0.05 M Tris-HCl buffer, pH 8.0, packed into a 2.5 x 20 cm column, and fitted with a nylon mesh disk.

The pellet obtained from the precipitation of activity in the bulked Sephadex G-50 fractions was taken up in 2.5 ml of the Tris-HCl buffer, pH 8.0, and applied to the column. Elution of the active fractions was effected with a 200 ml linear gradient of 0.01 to 1.0 M NaCl in 0.05 M Tris-HCl buffer, pH 8.0. Five ml fractions were collected at the rate of seven minutes per fraction.

Peak fractions containing amylase activity were subjected to characterization of activity or rechromatographed. The latter involved bulking active fractions from respective peaks, precipitating with ammonium sulfate (8a), and resuspending the pellet in 2.5 ml of the Tris-HCl buffer, pH 8.0. The sample was again applied to a DEAE-cellulose

column and eluted over the appropriate salt concentration range.

10. Procedures for Enzyme Characterization

a. Reaction Product Analysis. The reaction products of amylase hydrolysis of a soluble starch substrate were determined by paper chromatography according to the method of Robyt and French (1963). Pellets containing amylase activity from ammonium sulfate precipitation (8a) were taken up in 2.5 ml of 0.05 M Tris-HCl buffer, pH 8.0, and served as the source of enzyme for reaction product analysis. An enzyme aliquot (0.1 ml) was incubated with the standard reaction mixture (5a) for 10m 20 and 30 min. intervals, at which time aliquots were withdrawn and placed into a boiling water bath to inactivate the enzyme.

One hundred ul of the aliquots were spotted on Whatman No. 1 Chromatography paper strips. The chromatograms were developed with a mixture of water-95% ethanol-nitromethane (21.44.35) for 14 hours by descending flow. Reaction products were visualized by first dipping the papers in an $AgNO_3$ solution (0.1 ml saturated $AgNO_3$/20 ml acetone), drying and dipping in a second solution containing 0.1 ml of 10 N NaOH/100 ml of 95% ethanol. Glucose and maltose were run as standards for comparison.

b. Polyacrylamide Gel Electrophoresis. PAGE analysis as described previously (7) was applied to amylase activities at all stages of purification to verify homogeneity of activities during the purification procedures.

c. Physicochemical Characterization. Physicochemical treatments were used alone or in conjunction with PAGE analysis as a criterion for differentiation between α and β-amylases. The following treatments were performed on aliquots of crude and purified enzyme extracts prior to

standard amylase assay (5):

1. Calcium ions (Ca^{+2}) Enzyme eliquot incubated with a final concentration of 10^{-3} M $CaCl_2$ for one hour at 0 to 4°C.

2. Heat (H) Enzyme aliquot incubated for 12 min. at 70°C in a constant temperature water bath.

3. Calcium ions plus Heat (Ca^{+2} + H) Procedure 1 followed by procedure 2.

4. Mercury ions (Hg^{+2}) Enzyme aliquot incubated with a final concentration of 10^{-5} M $HgCl_2$ for 15 min. at 0 to 4°C.

5. Dithiothreitol reagent (DTT) Enzyme aliquot incubated with a final concentration of 1 mM DTT for 15 min. at 0 to 4°C.

6. Mercury ions plus Dithiothreitol reagent (Hg^{+2} + DTT) Procedure 4 followed by procedure 5.

7. Dithiothreitol reagent plus Mercury ions (DTT + Hg^{+2}) Procedure 5 followed by procedure 4.

d. pH Optima Determination. Effect of pH on amylase activities from purified extracts was determined over the range of pH 2.5 to pH 8.0. Buffers of 0.025 M concentration were prepared according to the method of Gomori (1955). They are as follows. citrate-phosphate (pH 2.6 to 3.5), acetate (pH 3.6 to 5.6), citrate-phosphate (pH 5.7 to 7.0), Tris-HCl (pH 7.0 to 8.0). Amylase activity was determined by standard assy (5), using 1 ml of soluble starch, 3.5 ml of the respective buffer, and 0.5 ml of the enzyme. The pH values of the reaction mixture were taken with a single probe electrode at the termination of the 20 min. assay.

e. Determination of Temperature Optima and Energy of Activation. The temperature optima for the purified amylase activity was determined for a specified set of conditions. Standard assays (5) were run in a constant temperature water bath set at 5°C increments from 15°C to 55°C. The reaction mixture consisted of 0.5 ml of the enzyme, 1 ml of 2% soluble

starch and 3.5 ml of 0.05 M actate buffer, pH 5.0.

Energy of Activation (E_a) for amylase activity over a specified temperature range was determined from data obtained from the temperature optima study. An Arrhenious graph of the reciprocal of the absolute temperature versus the log of the reaction rate was plotted. The E_a was calculated from the slope of the curve.

f. Determination of Michaelis-Menten Constants. A K_m determination was made by assaying the activity of the purified enzyme over a range of substrate concentrations. Reaction mixtures were prepared consisting of 2.0 to 40 mg of soluble starch/ml in 0.05 M acetate buffer, pH 5.0. The purified enzyme was added to the reaction mixture at 30°C and routine assays (5) were carried out for 20 minutes. The K_m was determined from a Lineweaver-Burke plot of amylase activity (1/v vs 1/(s)).

11. Verification of Experimental Data

For verification and unless otherwise noted, all experiments were repeated at least twice. The majority of the studies were repeated three times.

RESULTS

Part I. Occurrence and Development of Amylase Activity in Maize Kernels.

A. Amylase Activity in Incubated, De-embryonated Maize Kernels.

1. Environmental effects. The time course development of amylase activity in incubated, de-embryonated maize kernels is shown in Fig. 1. Maize kernels kept under conditions of high relative humidity (75%) for two months prior to experimentation exhibited no detectable activity at zero time but did show some amylase activity after 24 hours of incubation. A sharp rise in activity follows, which begins to plateau after 7 days of incubation. Concomitant with the increase in activity was an increase in the number of starch degrading zones visualized using polyacrylamide gel electrophoresis (PAGE) (Fig. 2, zymogram 1).

Maize kernels which have been kept in a desiccator at a R.H. of less than 10% for two months prior to experimentation, exhibited a low level of total amylase activity, extractable throughout the time course, when compared with the activity from the kernels in a high humidity environment (Fig. 1). A similar initial increase in activity occurred in both cases but after 9 days of incubation the kernels stored under high humidity had three times the activity as the low humidity stored kernels. An investigation of the electrophoretic banding pattern from the crude extracts of these "dry" kernels showed a maximum of two bands, the second one occurring at about 5 days of incubation (Fig. 2, zymogram 2).

Table 1 indicates the moisture loss of whole and de-embryonated kernels previously subjected to humid or dry conditions. Surprisingly, the percentage of moisture loss is virtually the same for "dry" kernels,

FIGURE 1

Time course development of total amylase activity in incubated, de-embryonated maize kernels stored for two months under humid (75% R.H.) or dry (less than 10% R.H.) conditions.

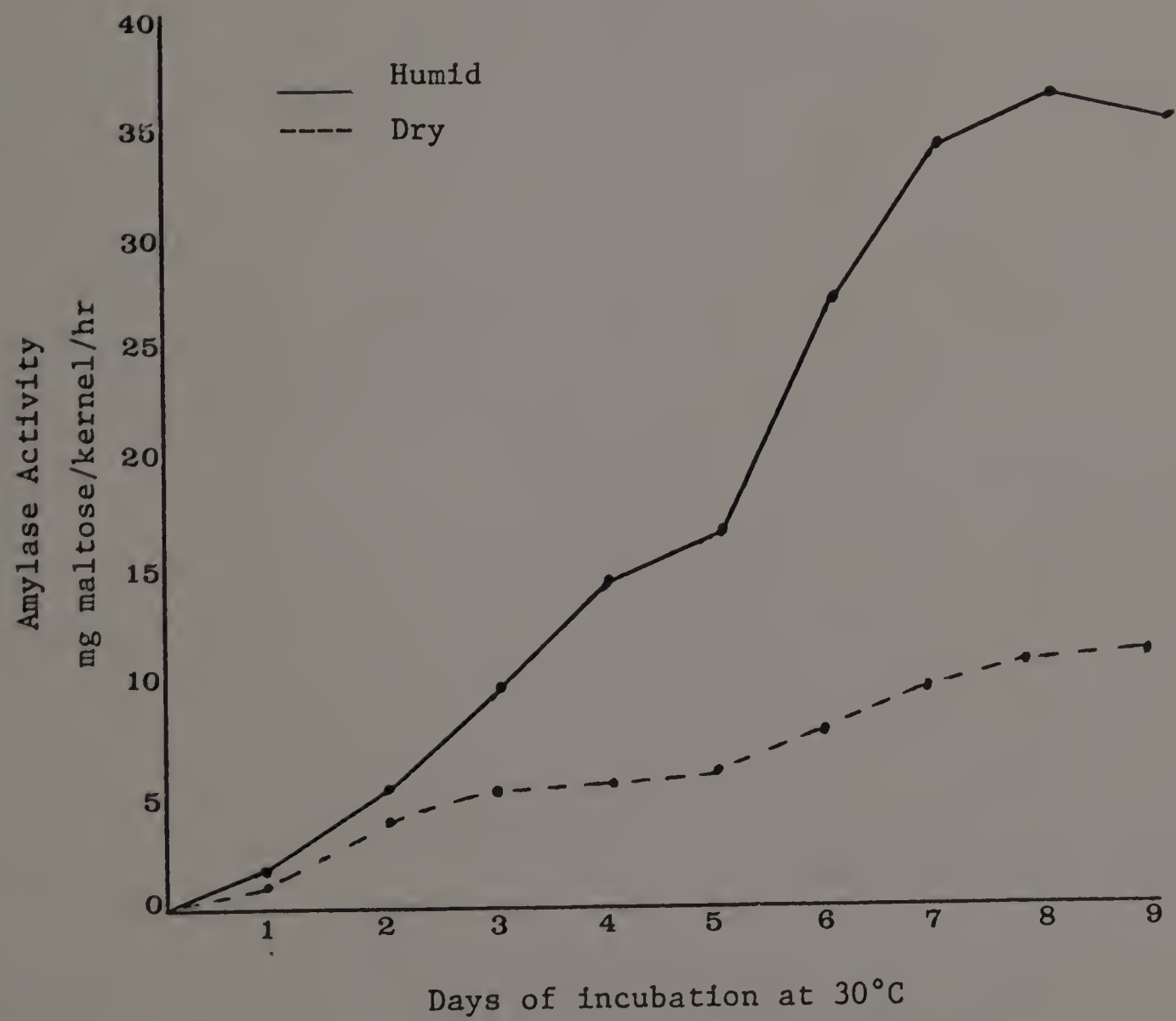


FIGURE 2

Polyacrylamide gel electrophoresis zymograms of starch degrading bands from the time course development of amylase activity in incubated, de-embryonated maize kernels stored under humid (75% R.H.) or dry (less than 10% R.H.) conditions for two months.

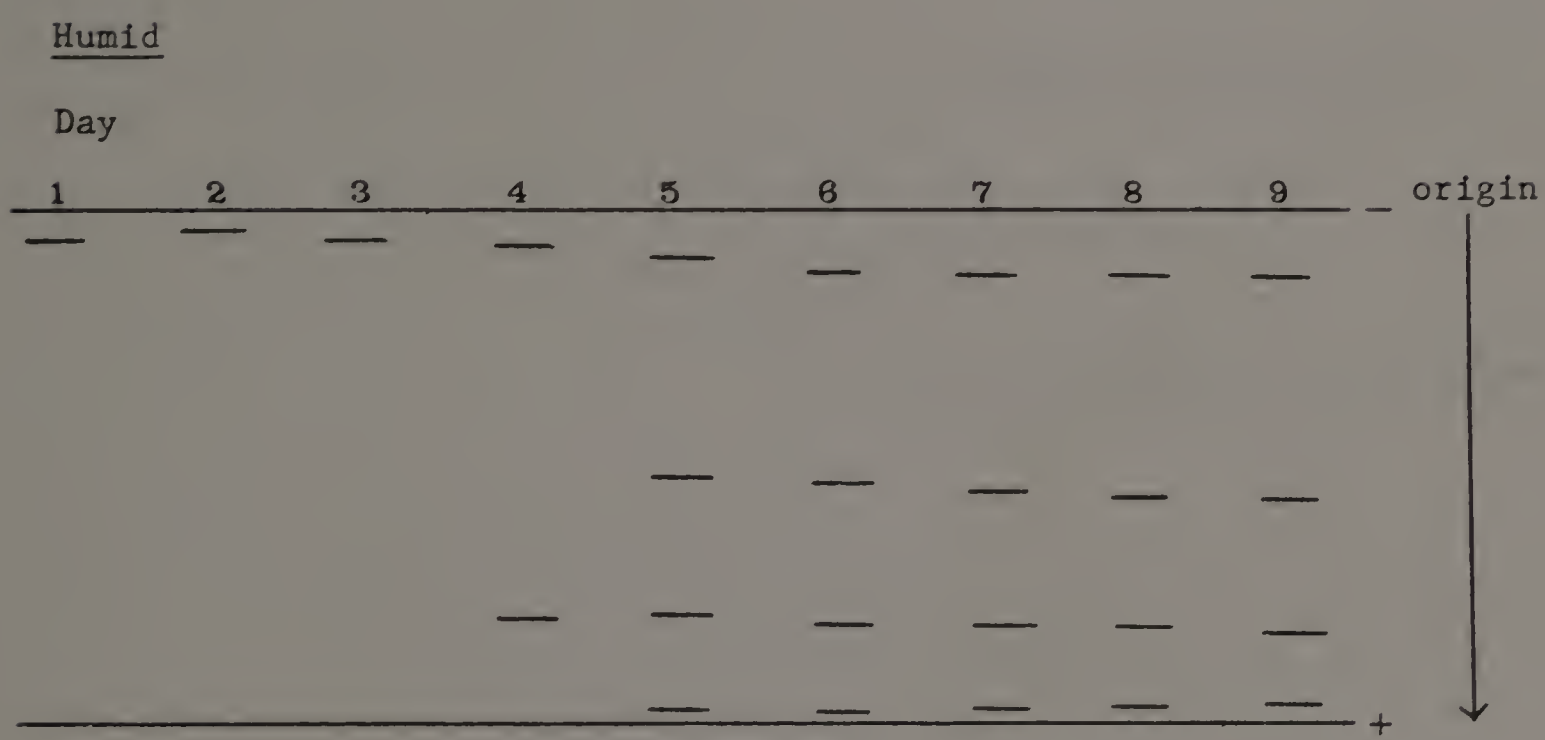


TABLE 1

Moisture content of developing kernels, mature kernels, and mature, de-embryonated kernels

	Sample				
Average weight per kernel (grams)	de-embryonated dry kernels	whole dry kernels	de-embryonated humid kernels	whole humid kernels	developing kernels 24 days
initial	0.3104	0.3660	0.3258	0.3761	0.2632
final	0.2960	0.3490	0.2860	0.3312	0.1059
Average moisture loss (per cent)	4.52	4.64	12.2	11.9	59.8

Initial weights were determined from 20 kernel samples. Moisture determination was made by oven drying the samples at 70°C for 26 days, after which no further weight loss was apparent.

"Dry" kernels were subjected to a two month period in a dessicator (less than 10% R.H.)

"Humid" kernels were subjected to a two month period in a humidity chamber (75% R.H.)

whether they are intact or minus the embryo with the endosperm tissue exposed. The same observation holds true for "humid" kernels. As would be expected, however, moisture loss is almost three times as great from the kernels exposed to humid conditions as it is for the "dry" kernels, suggesting that the free moisture in the kernels is a function of the environmental conditions.

An indication that all free moisture has been removed from the kernels is supported by the observation that the average dried weights of the intact kernels are similar, regardless of pretreatment in either humid or dry environment. The same observation holds true for de-embryonated kernels from both environments.

Developing maize kernels examined 24 days after pollination, weigh less than mature kernels from either humid or dry environments (Table 1). However, almost 60% of the weight of the developing kernel is moisture, versus the 11.9% moisture in the mature "humid" kernel. This is an indication that a reduction in free moisture occurs as the maize kernel matures.

2. Physical effects. Several reports have indicated that only β-amylase is present in the ungerminated cereal grain and that it is located in the aleurone or sub-aleurone layers (17, 18). To further investigate the origin of the complement of amylase enzymes arising from the de-embryonated kernel upon incubation, the pericarp and aleurone layers were removed from the kernels. Samples of kernels kept under conditions of low and high relative humidity for two months as previously described, were subjected to mechanical abrasion to remove the pericarp and aleurone layers and then were manually de-embryonated. On incubation a complete absence of amylase activity in the crude extracts from both samples was

noted. In addition PAGE analysis failed to detect any starch degrading zones for either sample (results not presented).

As the major amylase activity was obtained from de-embryonated kernels with the outer layers intact, and kept under conditions of high relative humidity at 0°C, all subsequent work was based upon this prior treatment of the maize kernels.

B. Amylase Activity in Germinating Kernels

Total amylase activity was determined in the endosperm and excised embryo from germinating kernels separated at time of sampling. Activity was present in both the endosperm fraction and the excised embryo, which included the developing root and shoot (Fig. 3).

Initially amylase activity in the endosperm from germinating kernels was higher than that found in the endosperm from previously de-embryonated kernels (Fig. 3). Activity in the former began to plateau after 5 days of incubation, while maximum activity in the latter was reached after 7 days of incubation. Approximately the same total activity was reached in both cases. Total activity from the excised embryo from germinating kernels was lower than that from the endosperm fractions, although an increase in activity was noted over the time course of incubation.

PAGE analysis of the crude extracts from the three samples (Fig. 4) shows a complement of starch degrading bands appearing earlier in the endosperm from the germinating kernels than from the de-embryonated kernels. This earlier appearance of starch degrading bands correlates well with the higher amylase activity detected in the extracts (Fig. 3). Comparison of day 6 zymograms for the two endosperm fractions shows some similarity in banding patterns (Fig. 4). The slowest moving, least anodic starch

FIGURE 3

Time course development of total amylase activity in de-embryonated maize kernels and in the embryo and endosperm from germinating maize kernel.

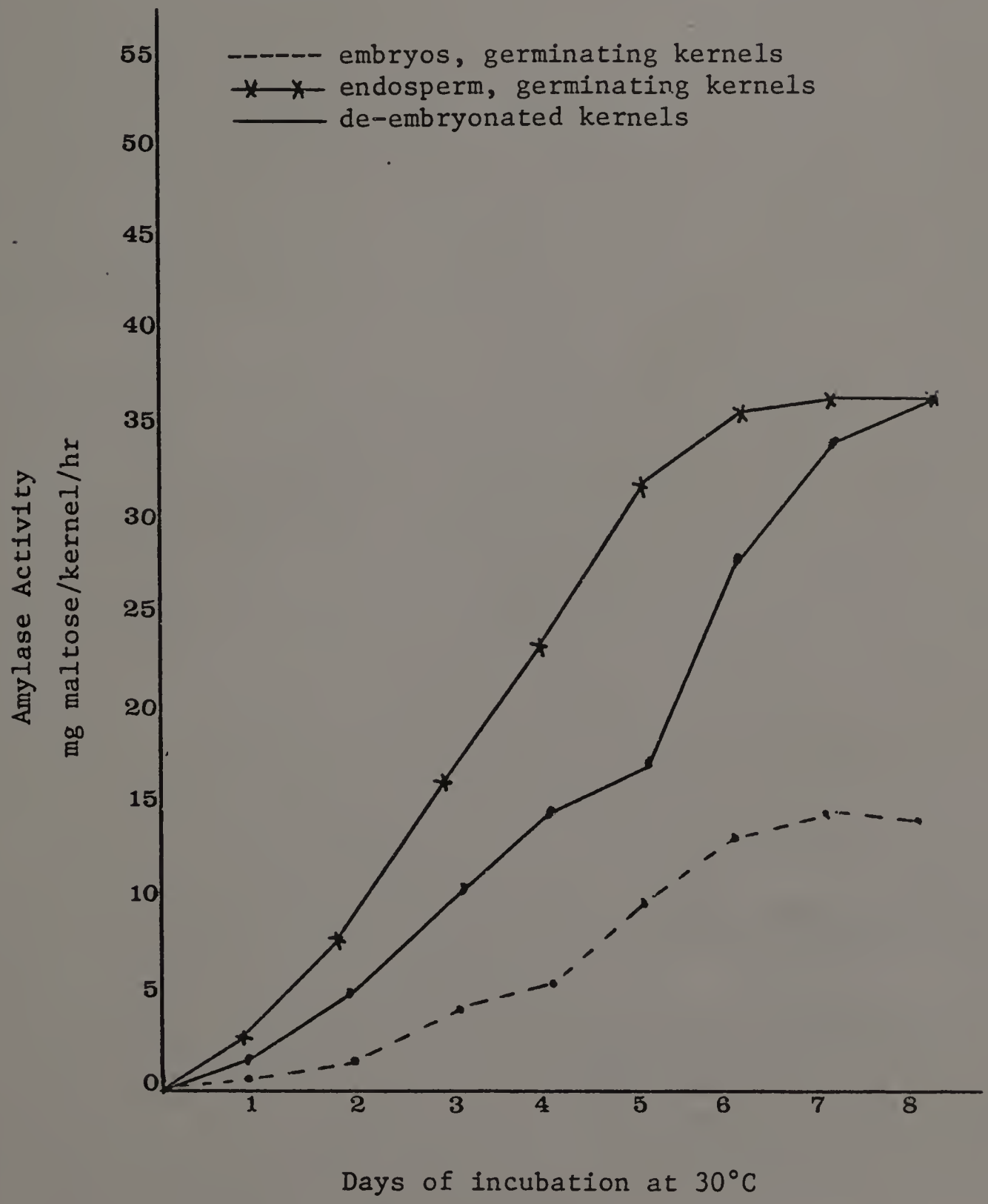
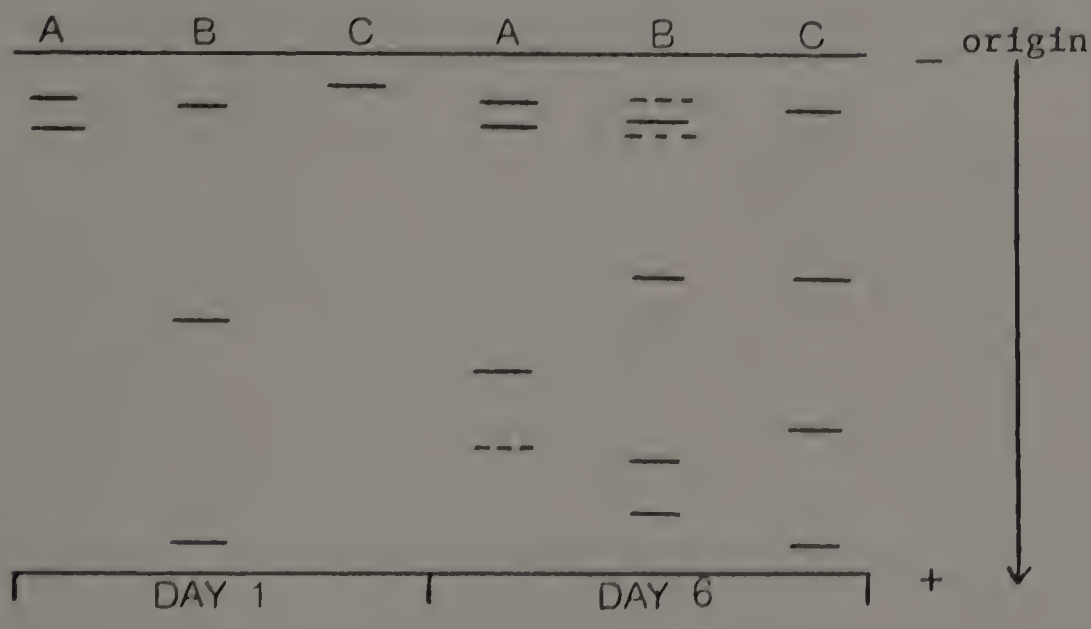


FIGURE 4

PAGE zymograms of starch degrading bands from extracts of de-embryonated kernels and extracts of the embryo and endosperm of germinating kernels after one and six days of incubation.



A- Extract from embryos of germinating kernels

B- Extract from endosperm of germinating kernels

C- Extract from de-embryonated kernels

degrading zone in the germinating endosperm sample consists of a cluster of bands, while that from the de-embryonated sample is one clear band. The increase in the number of bands from the embryo extracts correlates well with its increase in activity during incubation.

The reaction product patterns produced by activity from the purified endosperm extracts indicates the presence of α-amylase activity, at least in part (Fig. 5). Similar reaction patterns for endosperm extracts from germinating and de-embryonated kernels suggest the presence of similar amylase activities (Fig. 5).

C. Amylase Activity in Developing Maize Kernels

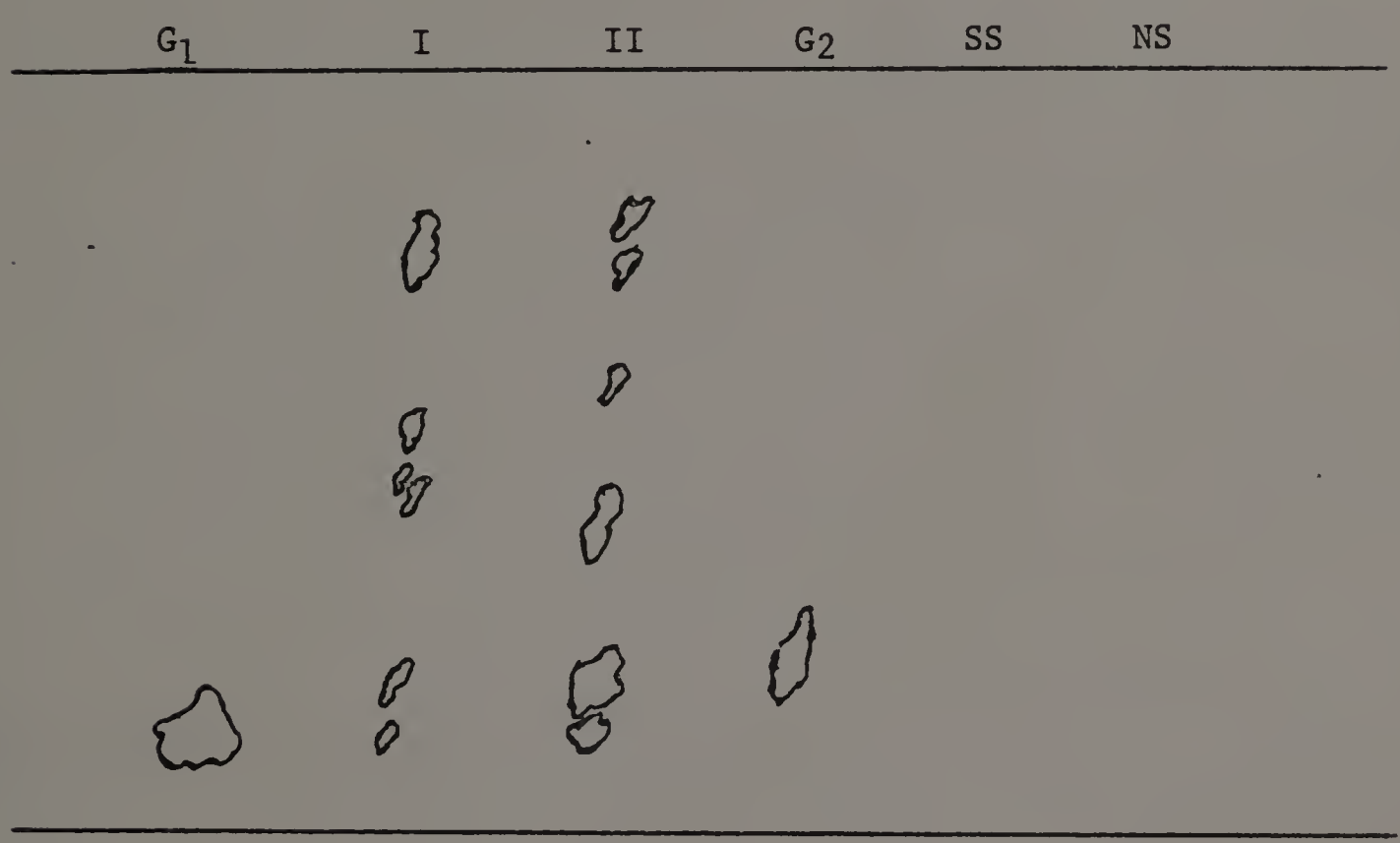
To assess the amylase activity in developing kernels of the Funk's G-17 variety, ears were harvested 24 days after pollination and kernels removed.

Total amylase activity in the endosperm and embryo of developing kernels was higher than that found for the respective activities from the germinating kernels on the first day of incubation (Table 2). PAGE analysis (Fig. 6) indicates a complex banding pattern for both the endosperm and embryo from the developing kernels. There is some similarity in PAGE patterns from the endosperm of day 6 germinating kernels (Fig. 4) and the endosperm of day 24 developing kernels (Fig. 6). The cluster of slow moving bands is again present in the endosperm of the developing kernel.

Reaction of the crude extracts prepared from the endosperm and embryo of the developing kernels to various physicochemical treatments suggests the presence of α and β-amylase activities in both of the tissues (Table 3). Beta-amylase activity is indicated by the protective effects

FIGURE 5

Zymograms of paper chromatograms of reaction product patterns of amylase activity from de-embryonated maize kernels and from the endosperm of germinating maize kernels, after 6 days of incubation.



I- De-embryonated kernels, 6 days of incubation

II- Germinating maize endosperm, 6 days of incubation

G_1- Glucose standard

G_2- Maltose standard

SS- Soluble starch substrate

NS- No substrate

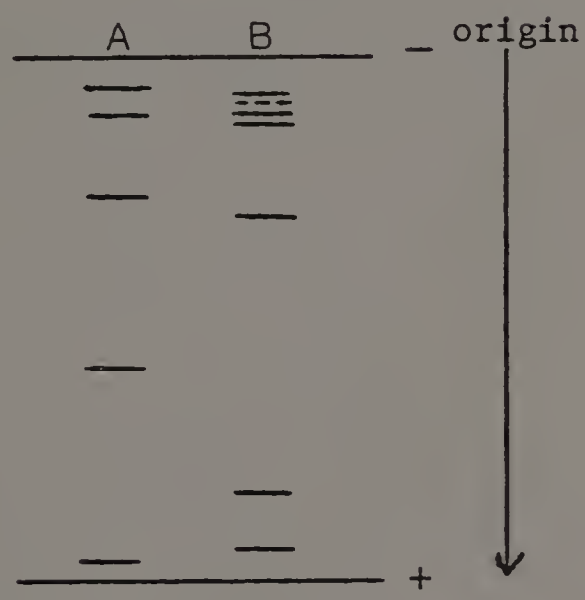
TABLE 2

Amylase activity in developing, germinating and incubated, de-embryonated maize kernels.

Sample	Amylase Activity mg maltose/tissue/hr
developing kernels, 24 days after pollination	
embryo	1.71
endosperm	4.02
germinating kernels, after one day of incubation at 30°C	
embryo (including root and shoot)	1.00
endosperm	2.29
de-embryonated kernels, after one day of incubation at 30°C	1.26

FIGURE 6

PAGE zymograms of starch degrading bands from extracts of the embryo and endosperm of developing maize kernels 24 days after pollination.



A- Extract from embryos of developing kernels

B- Extract from endosperms of developing kernels

TABLE 3

Physicochemical analysis of amylase activity in the embryo and endosperm of developing maize kernels, 24 days after pollination.

Treatment	Amylase Activity as % of Control	
	Endosperm	Embryo
Control (no treatment)	100.	100.
Ca^{+2} (10^{-3} M, 1 hr, 0°C)	78.3	86.5
Heat (12 min, 70°C)	13.5	14.9
Ca^{+2} + Heat	46.7	53.2
Hg^{+2} (10^{-5} M, 15 min, 0°C)	41.3	80.0
DTT (1mM, 15 min, 0°C)	103.	100.
Hg^{+2} + DTT	50.3	75.9
DTT + Hg^{+2}	77.2	94.5

of dithiothreitol (DTT) against inactivation of some of the activity by mercury ions. However, a typical reaction of classical α-amylase is also seen in both embryo and endosperm extracts. Heat treatment (70°C for 12 min.) greatly reduces activity, but the amylase activity is partially protected upon addition of excess calcium ions prior to heat treatment.

Amylase activity in the developing grain is thought to be in part a function of the grain moisture level, and hence the amount of soluble enzyme in the grain. Moisture level decreases as the grain matures (67). In this regard, moisture content for the developing kernel was found to be 59.8% versus the 11.9% for the fully mature kernel kept in a humid environment (Table 1). As well, activity from the de-embryonated kernel after one day of incubation was found to be only about one third of that from the endosperm of the developing kernel (Table 2).

D. Incubation Studies with De-embryonated Kernels

1. Gibberellic acid (GA_3) effects. The hormone, gibberellic acid, has been implicated in the process of cereal grain germination since Paleg (1960) and others first discovered that GA_3 could substitute for the embryo in the initiation of events leading to substrate mobilization. In barley, Varner and co-workers (21, 37, 93) subsequently established the GA_3 directed de novo synthesis of α-amylase and protease. Protease, in turn, may play a role in the expression of latent β-amylase (78).

As a similar GA_3 requirement for substrate mobilization has been reported for maize (36), it was thought of interest to determine the effect of GA_3 additions on the incubated, de-embryonated kernel.

Figure 7 shows the development of total amylase activity in the de-embryonated kernels incubated with concentration of GA_3 (0.5 uM, 1.0 uM, 2.0 uM). As with the control, GA_3 treated kernels show an initial low level of activity which rises over time of incubation. However, between 1 and 5 days of incubation, extracts from GA_3-treated de-embryonated kernels exhibited a higher level of amylase activity than the control. The activities of all samples are similar after 7 days of incubation.

PAGE analysis (Fig. 8) of 1 uM GA_3-treated and control samples indicate the presence of one slow moving band on day 1 of the time course for both samples. However, the appearance of more anodic starch degrading bands in the GA_3-treated sample precedes that of the control, correlating with the earlier increase in activity of the GA_3-treated sample. The complete enzyme complement is present at day 4 for the 1 uM GA_3-treated sample and at day 5 for the control.

The order of appearance of starch degrading bands and the total of 4 bands in both the GA_3 and control samples may be one indication of a quantitative, rather than qualitative effect for the action of GA_3 in this system. This quantitative GA_3 effect has been observed, as well, in another study with incubated, de-embryonated maize kernels (27).

Concentration of GA_3 applied to the maize endosperm is reported to have a limiting value, after which higher concentrations of GA_3 do not bring about a corresponding greater increase in amylase activity (36). Data from this study support this observation. Maximum effect with GA_3 was achieved at 1 uM concentration, as higher concentration did not bring about an appreciably greater enhancement of amylase activity (Fig. 7). The quantitative nature of GA_3 on amylase activity in the incubated,

FIGURE 7

Effect of concentrations of GA_3 on the time course development of total amylase activity from incubated, de-embryonated maize kernels.

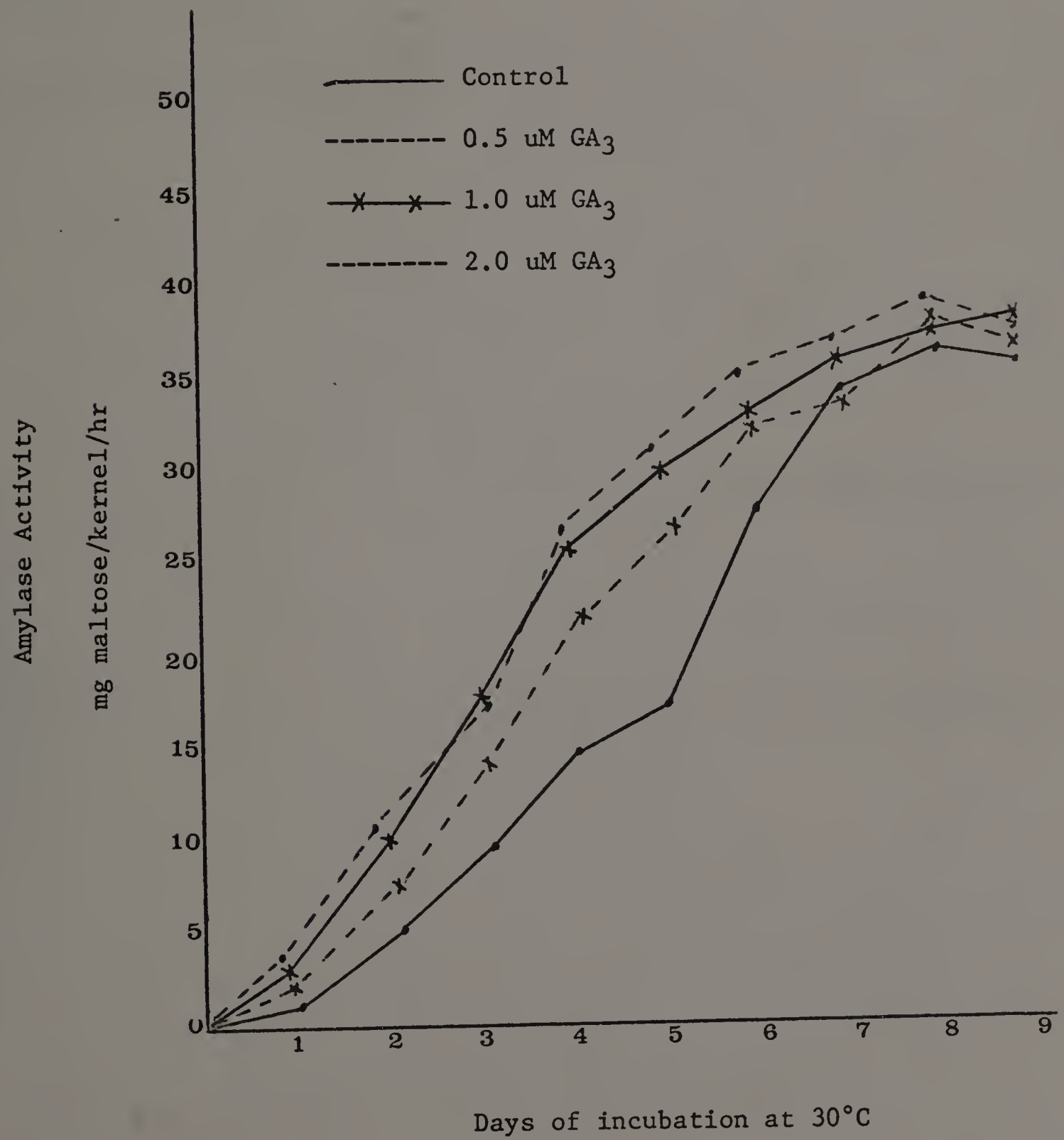
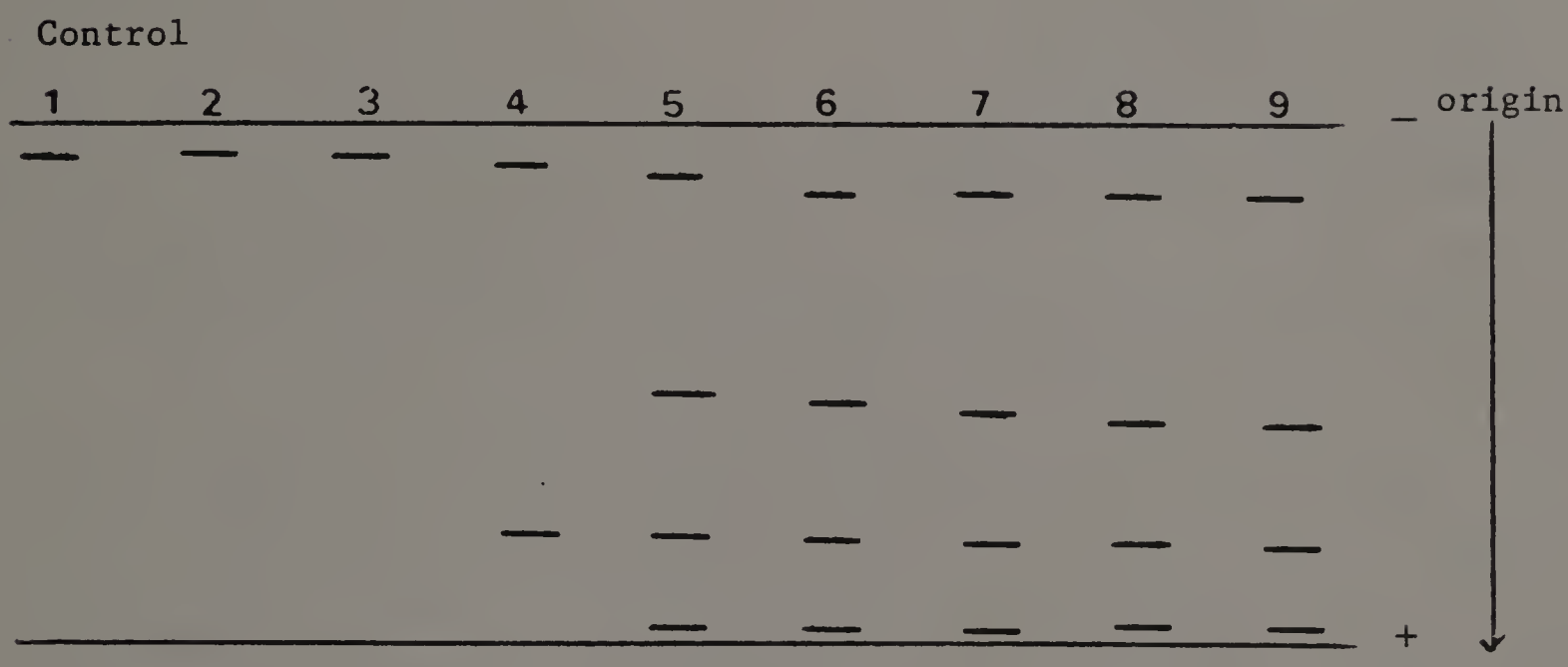


FIGURE 8

PAGE zymograms of starch degrading bands from the time course development of amylase activity in GA_3 (1uM) and non-GA_3 treated de-embryonated kernels.



de-embryonated kernel is further demonstrated by the lower GA_3 concentration (0.5 uM) which shows less enhancement of amylase activity than the higher concentration

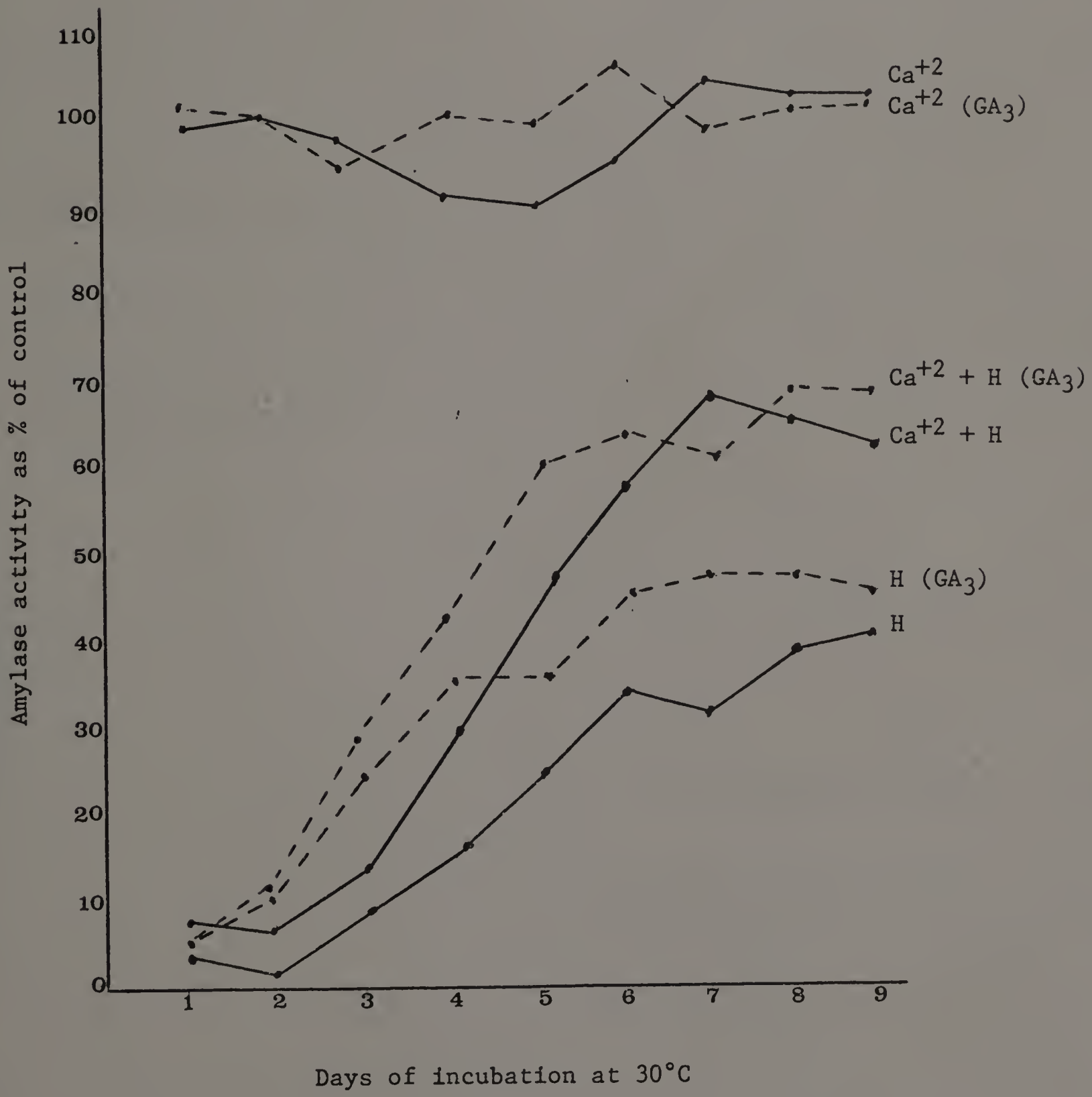
For 1 and 2 uM GA_3 additions, activity is increased two fold during early incubation. As full expression of amylase activity with GA_3 was obtained at 1 uM concentration, this level was used in all further experimentation.

2. Physicochemical effects. The number of starch degrading bands on PAGE analysis and the diversity of reaction products obtained by paper chromatography (Fig. 8, Fig. 5) suggested that both α and β-amylases were present in the incubated, de-embryonated maize kernel. It was of interest, then, to determine the proportion of activity due to α and or β-amylases during the time course and what effect the addition of GA_3 would have on this activity.

Figure 9 illustrates the time course development of activities from kernels incubated with or without 1 uM GA_3 and the effects of three physicochemical treatments used in the determination of α-amylase. Additions of calcium ions, alone, did not enhance the activity in either GA_3 or control samples, indicating that if α-amylase is present in this system and requires calcium, the endogenous concentration is adequate. Loss of activity on heat treatment is indicative of the heat sensitivity of both α and β-amylases. However, addition of calcium ions prior to heat treatment shows an increase in thermal stability of a portion of the amylase activity occurring later in the time course. This is an indication of the presence of α-amylase. Again, on heat treatment with or without calcium ions, the activities in GA_3 and control samples respond in a similar

FIGURE 9

Physicochemical analysis of the time course development of total amylase activity in GA_3 (1uM) and non-GA_3 treated de-embryonated maize kernels. Effects of Ca^{+2} (10^{-3} M), Heat (70°C, 12 min.), and Ca^{+2} plus Heat.



fashion.

PAGE patterns (Fig. 10) of the control and GA_3 samples after 8 days of incubation, show the full complement of starch degrading bands for extracts with no treatment and calcium-treated extracts. Heat treatment eliminates two bands from each sample, but the lower one is stabilized by the addition of calcium ions before heat treatment, an indication of α-amylase activity. For these treatments GA_3 and control samples respond in a similar fashion on PAGE analysis.

Figure 11 indicates the time course development of activities from de-embryonated kernels incubated with or without 1 uM GA_3 and subjected to three physicochemical treatments used in the determination of β-amylase. Beta-amylase is a sulfhydryl enzyme, which may be inhibited by heavy metal ions, such as Hg^{+2}. Thiol reagents such as dithiothreitol may be used to protect sulfhydryl enzymes against such inactivation. Addition of DTT increases the activity of both GA_3 and control samples early in the time course. Addition of Hg^{+2} dramatically decreases the activity in both samples, especially early in the time course. Addition of DTT after treatment with mercury ions protects against the effects of Hg^{+2} inhibition for both samples early in the time course.

PAGE analysis (Fig. 10) shows the full complement of bands for extracts with no treatment and DTT treatment in both samples. Treatment with Hg^{+2} eliminates the upper band and some of the lower ones. The least anodic band is the only one present in the early stages of incubation, and it again appears in samples treated with DTT subsequent to Hg^{+2}. However, one of the lower bands does not reappear on the double treatment. Both GA_3 and control samples respond similarly to the treatments, on PAGE analysis.

FIGURE 10

PAGE zymograms of starch degrading bands from physico-chemically treated amylase activity in GA_3 (1 uM) and non-GA_3 treated de-embryonated kernels after 8 days of incubation. Effects of Ca^{+2} (10^{-3}M) Heat (70°C, 12 min.), Ca^{+2} plus Heat, Hg^{+2} (10^{-5}M), DTT (1mM), and Hg^{+2} plus DTT.

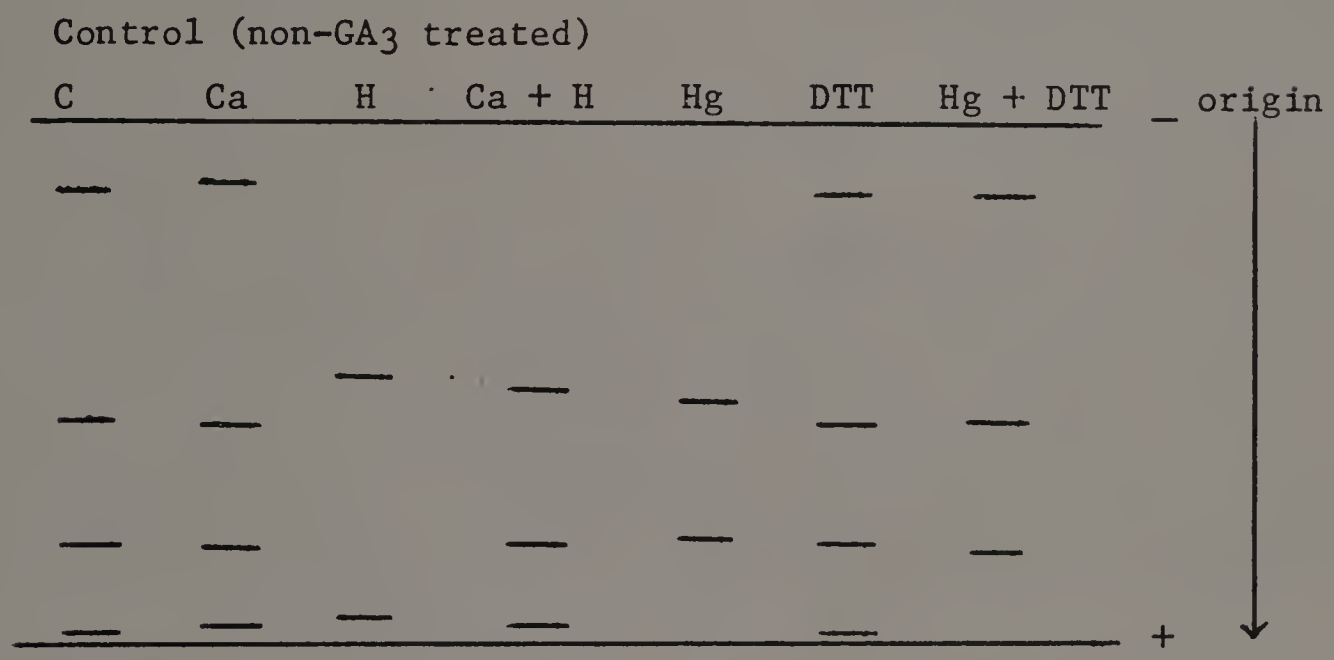
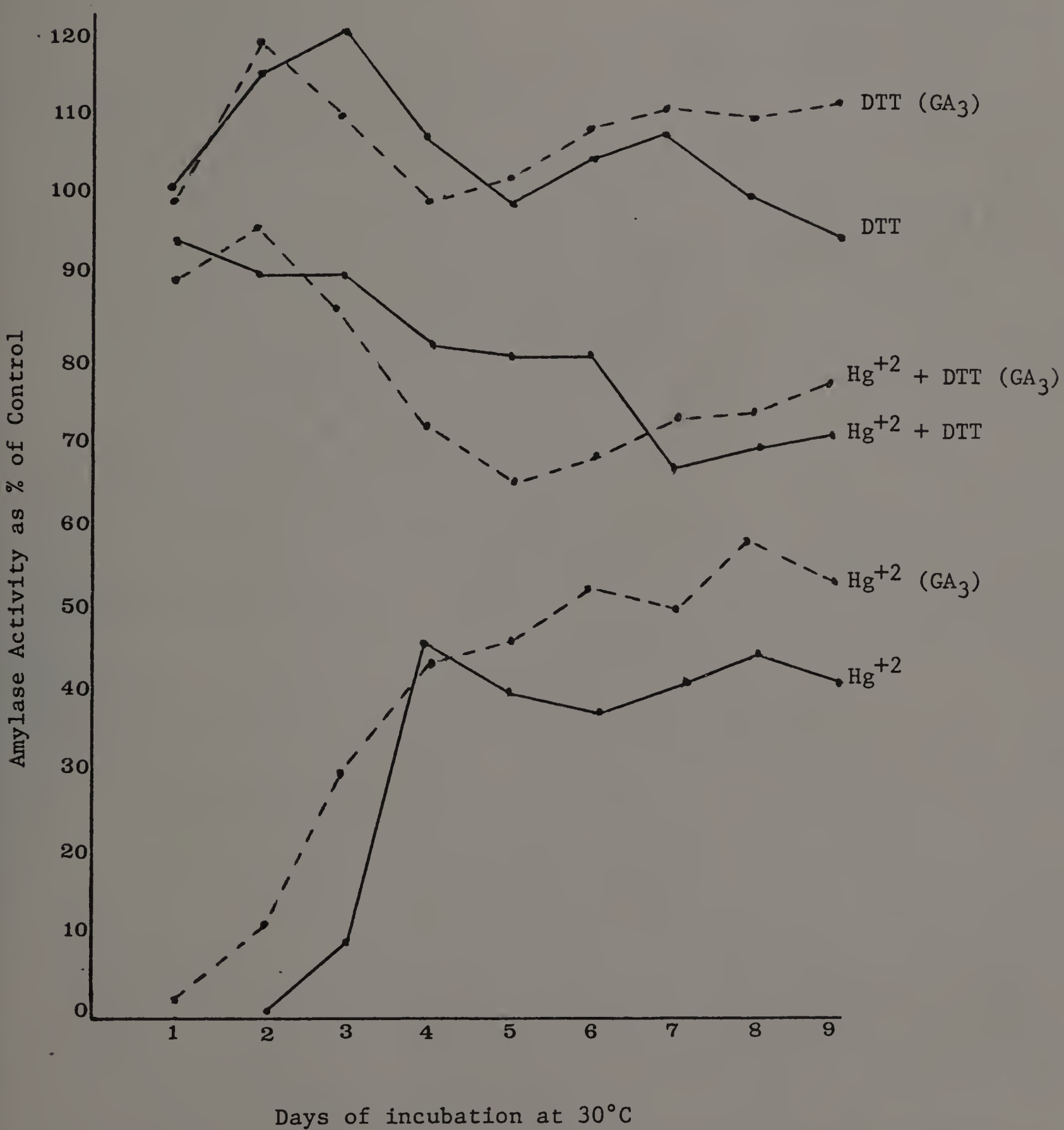


FIGURE 11

Physicochemical analysis of the time course development of amylase activity in GA_3 (1 uM) and non-GA_3 treated de-embryonated maize kernels. Effects of Hg^{+2} (10-5 M), DTT (1 mM), and Hg^{+2} plus DTT.



Physicochemical data thus point to the occurrence of β-amylase activity early in the incubation and to the presence of both α and β-amylase activities later in the time course. Physiocochemical treatments, reaction product patterns and PAGE analysis all indicated that the de-embryonated kernel develops both α and β-amylase activities during incubation and that the action of GA_3 on this system is quantitative, bringing about an earlier expression of activity.

3. Inhibitor effects. Activity from the incubated, de-embryonated maize kernel may arise from latent enzyme present in the resting seed, or from the synthesis of new enzyme. To determine the extent to which the development of amylase activity is dependent upon RNA and protein synthesis, the respective inhibitors of these processes, actinomycin D and cycloheximide were added to the incubation medium.

The inhibitors were added at the start of incubation or to controls at days 2, 4 and 6 of incubation (Fig. 12, Fig. 13). Addition of actinomycin D or cycloheximide at the start of incubation suppresses the development of amylase activity previously observed in incubated, de-embryonated kernels. Addition of the inhibitors at later times during incubation results in a dramatic decrease in activity compared with the control.

PAGE patterns (Fig. 14) indicated that the inhibitors prevented the appearance of the full complement of starch degrading bands, correlating with the observed decrease in activity.

It would appear that both the full development of amylase activity and the appearance of the full complement of starch degrading bands are mutually related processes, both dependent upon RNA and protein synthesis.

FIGURE 12

Effect of Actinomycin D (20 ug/ml) on the time course development of amylase activity in de-embryonated maize kernels.

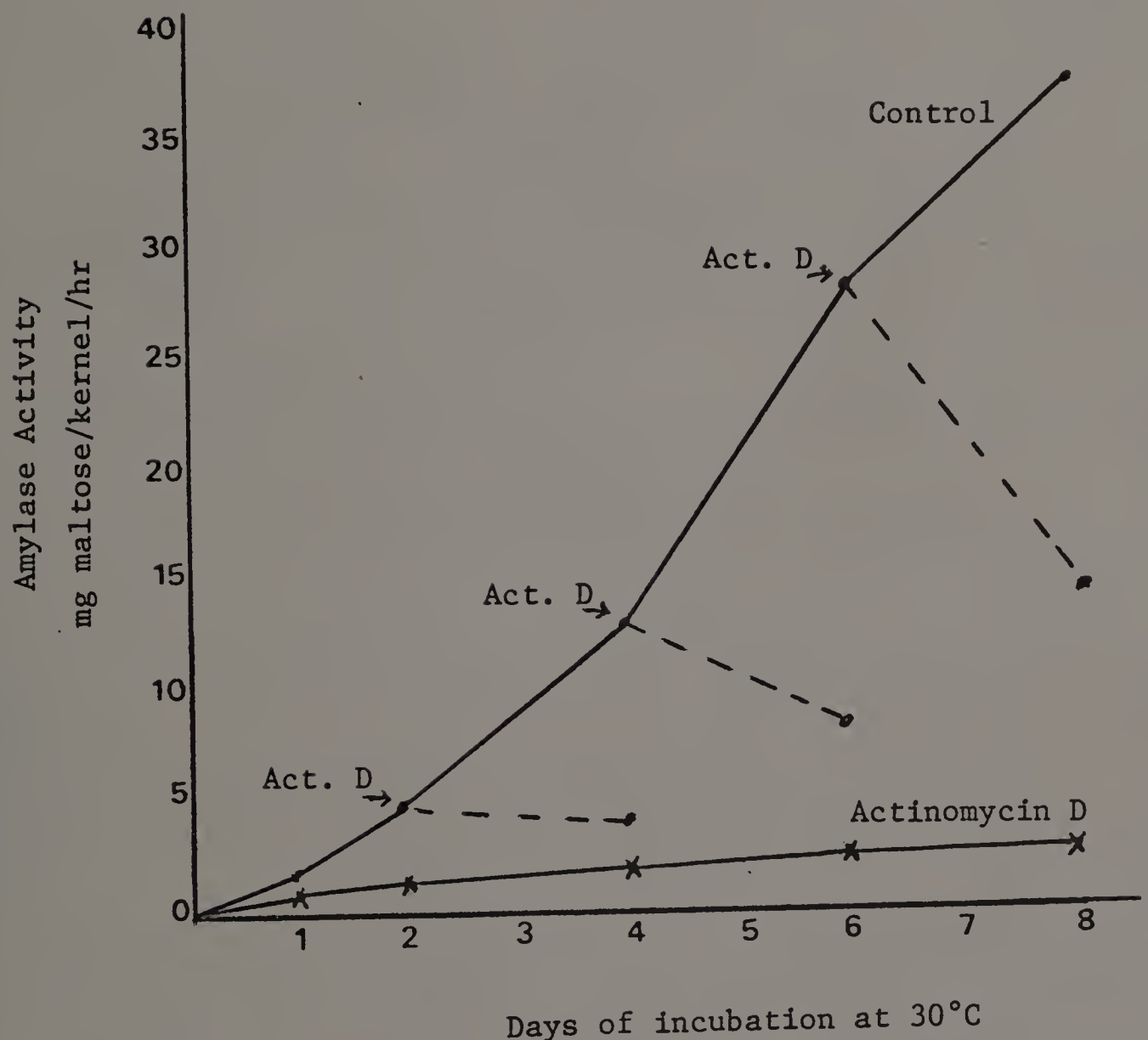


FIGURE 13

Effect of cycloheximide (5 ug/ml) on the time course development of amylase activity in de-embryonated maize kernels.

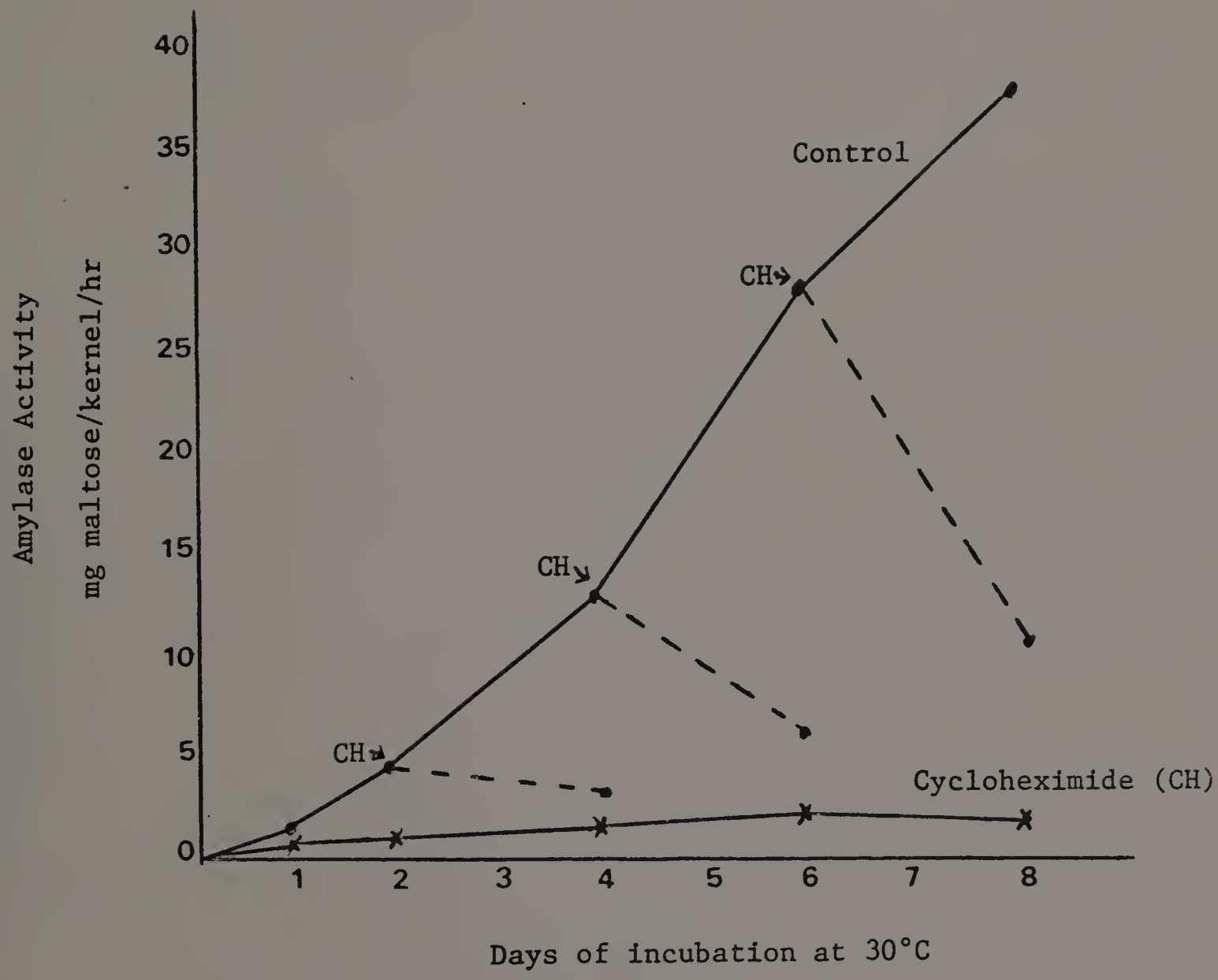
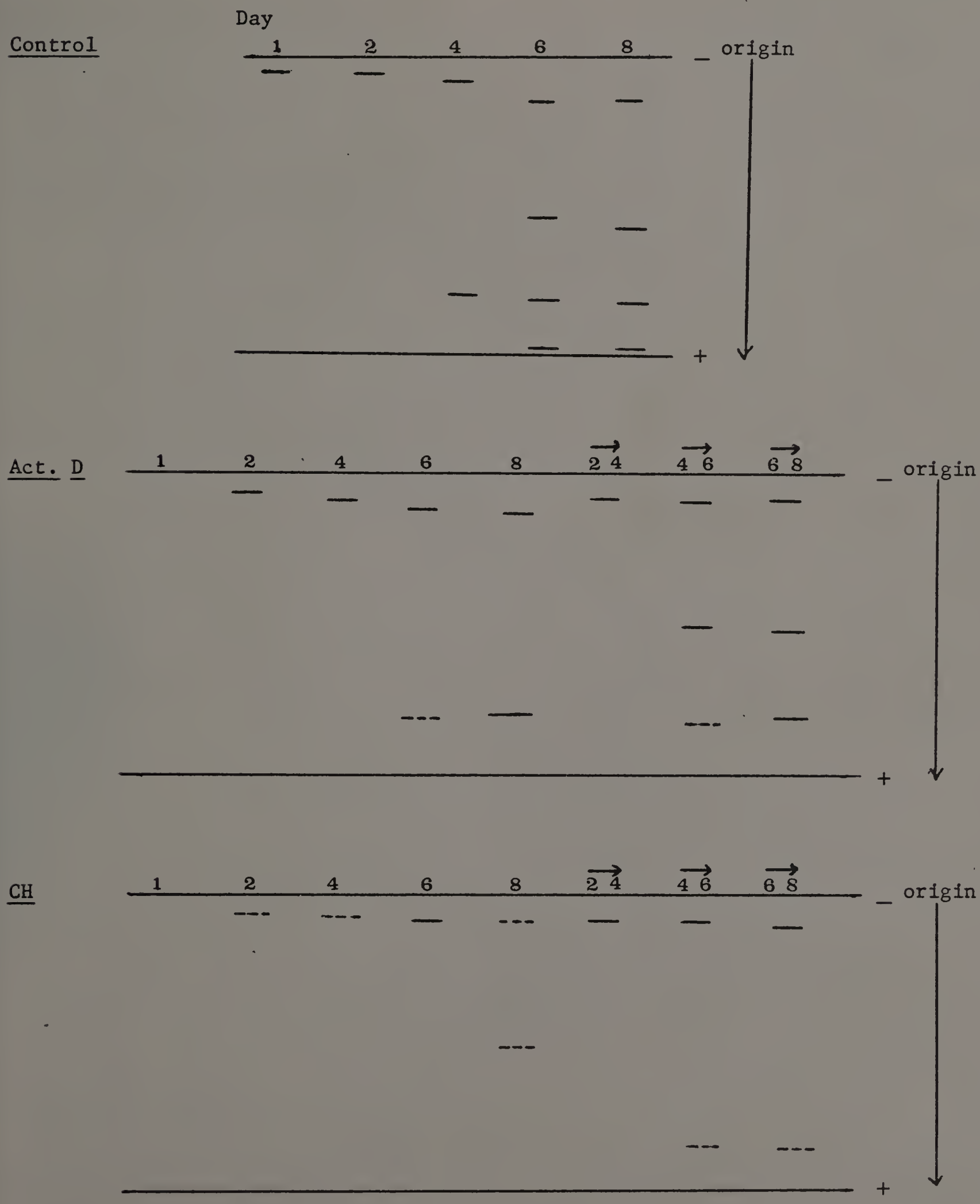


FIGURE 14

PAGE zymograms of the effects of Actinomycin D (20 ug/ml) and Cycloheximide (5 ug/ml) on starch degrading bands appearing during the time course development of amylase activity in de-embryonated maize kernels.



It is interesting to note that inhibitor additions at the start of incubation or later did not affect the appearance of the least anodic band on PAGE analysis (Fig. 14). This observation lends support to the idea that this starch degrading band, which is present after one day of incubation and has activity characteristic of a β-amylase, is present in the maize kernel prior to incubation and does not require RNA or protein synthesis for its expression.

E. Latent Amylase

The presence of a latent or bound form of amylase has been reported to exist in barley (82, 83) and wheat (75, 76). To free the enzyme, numerous physical and chemical extraction techniques have been employed (89, 82, 75), the most effective of these being a combination of a protrolytic enzyme (e.g. papain) and a sulfhydryl reagent (e.g. 2-mercaptoethanol) (82, 83).

To determine if amylase exists in a latent form in the maize endosperm, de-embryonated kernels were incubated for 24 hours, the soluble amylase activity extracted, and the remaining pellet incubated with 0.1% papain and 0.1% cysteine. Results indicate that a bound amylase is present in the de-embryonated maize kernel (Table 4). Activity obtained after one hr of cysteine-papain treatment at 30°C is as great as that obtained with the soluble enzyme in the crude extract. Bound amylase activity was also found at later time of incubation, using a variety of extraction techniques (Appendix).

PAGE patterns of the crude extract and extracts from cysteine-papain treatment were similar and showed the presence of one slow moving band

TABLE 4

Extraction of soluble and latent amylase activity in de-embryonated maize kernels after 24 hours of incubation at 30°C

Treatment	Amylase Activity mg maltose/kernel/hr
1. extraction of soluble amylase	
crude extract	2.13
4th pellet wash with 0.05 M Tris-HCl buffer, pH 7.6	0.00

2. extraction of latent amylase	Control (pellet incubation with 0.05 M citrate buffer, pH 5.4)	Cysteine-papain (pellet incubation with 0.1% cysteine + 0.1% papain in citrate buffer)
hrs of incubation (30°C)		
0.0	0.00	0.00
0.5	0.00	1.24
1.0	0.50	2.09
1.5	0.32	2.22
2.0	0.76	2.50
7.0	0.92	2.69

(results not presented). Physicochemical analysis indicated that this latent activity is characteristic of β-amylase, reacting much like the soluble activity appearing in early incubation (Table 5, Figs. 9, 11). Results are consistent with the report of β-amylase in the mature, resting maize seed (17).

Some activity was also obtained in the control sample upon incubation of the pellet. This activity may be due to the action of maize protease on the bound amylase at incubation temperature (30°C), or to the gradual freeing of amylase due to the aqueous environment. A maize protease has been reported to exist in the endosperm (33).

TABLE 5

Physicochemical analysis of latent amylase activity in de-embryonated maize kernels after 24 hours of incubation at 30°C.

Treatment	Amylase Activity as % of Control
Control (no treatment)	100.
Ca^{+2} (10^{-3} M, 1 hr, 0°C)	95.0
Heat (12 min, 70°C)	12.2
Ca^{+2} + Heat	14.9
Hg^{+2} (10^{-5} M, 15 min, 0°C)	0.00
DTT (1 mM, 15 min, 0°C)	99.1
Hg^{+2} + DTT	44.3
DTT + Hg^{+2}	60.9

Part II. Isolation and Characterization of Amylase Enzymes in Incubated, De-embryonated Maize Kernels.

A. Characterization of Amylase Activity After 5 Days of Incubation.

A preparation of amylase activity from day 5 extracts was purified by gel filtration chromatography and subjected to DEAE-cellulose chromatography (Fig. 1). The activity was resolved into three peaks, labelled I, II and III, according to their order of elution (0.17 M, 0.42 M, and 0.65 M NaCl, respectively). To assure that the separated activities were not artifacts resulting from the procedure used, each peak activity was rechromatographed over the respective ranges of salt concentrations. The original peaks rechromatographed at the same corresponding NaCl concentrations (results not presented). Table 1 indicates the degree of purification obtained for each peak from the crude extract.

PAGE patterns for bulked fractions from each peak are presented in Figure 2. To insure homogeneity of activity, fractions in which preliminary PAGE analysis had indicated overlapping of starch degrading bands from individual peaks, were discarded. Individual and discreet zones of starch degrading activity are indicated for each of the three peaks. Peak I is characterized by one slow moving zone, Peak II by one of intermediate migration, and Peak III has two zones showing the greatest anodic migration.

As a means of determining what type of activity is associated with each peak, the reaction products of the respective amylase activities from a soluble starch substrate were studied. Figure 3 indicates the results of the reaction product analysis. The activity of Peak I is typical of β-amylase. After 10 minutes of reaction with the substrate,

FIGURE 1

DEAE-cellulose chromatographic elution profile of an amylase preparation from de-embryonated maize kernels after 5 days of incubation at 30°C.

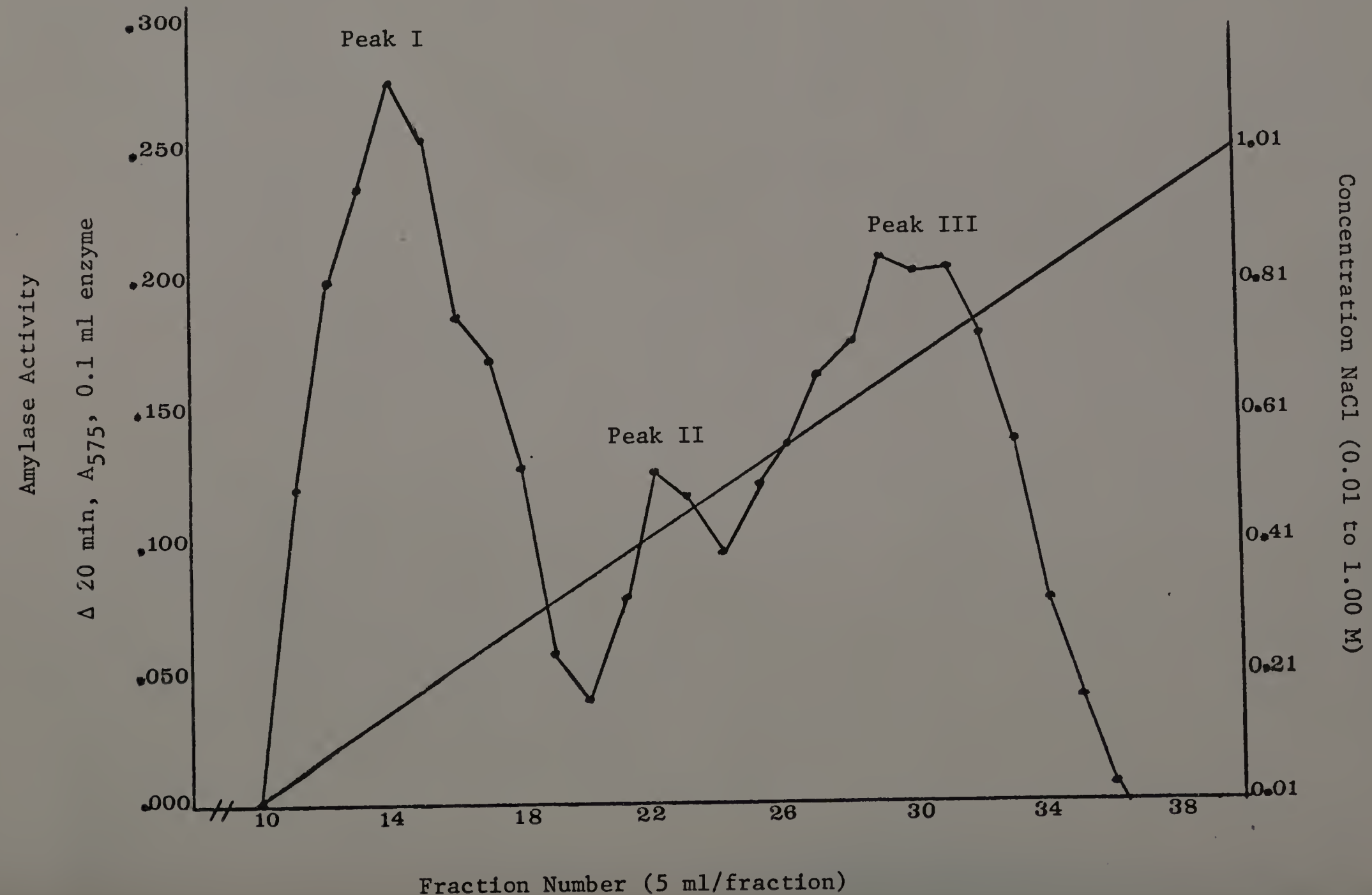


TABLE 1

Purification scheme of amylase activity extracted from de-embryonated maize kernels after 5 days of incubation.

Treatment	Protein (mg)	Specific Activity (*units/mg protein)
Crude extract	1153	1.92
Sephadex G-50	240	6.76
DEAE-Cellulose		
Peak I	14.9	9.81
Peak II	13.2	15.4
Peak III	20.2	12.4

*one unit of activity = Δ 20 min., A_{575} of 0.010 for 0.5 ml of enzyme.

FIGURE 2

PAGE zymograms of starch degrading bands from amylase activities in Peak I, II and III eluted from a DEAE-cellulose column. Amylase activity from de-embryonated maize kernels after 5 days of incubation at 30°C.

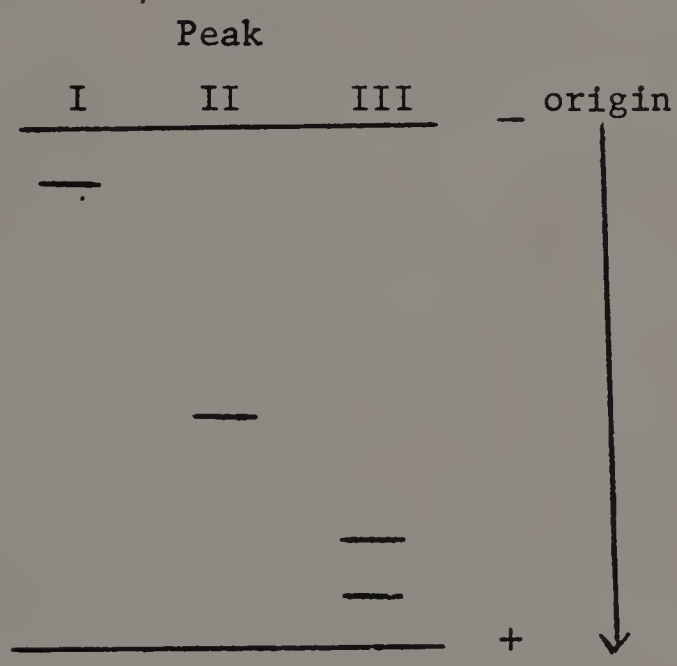
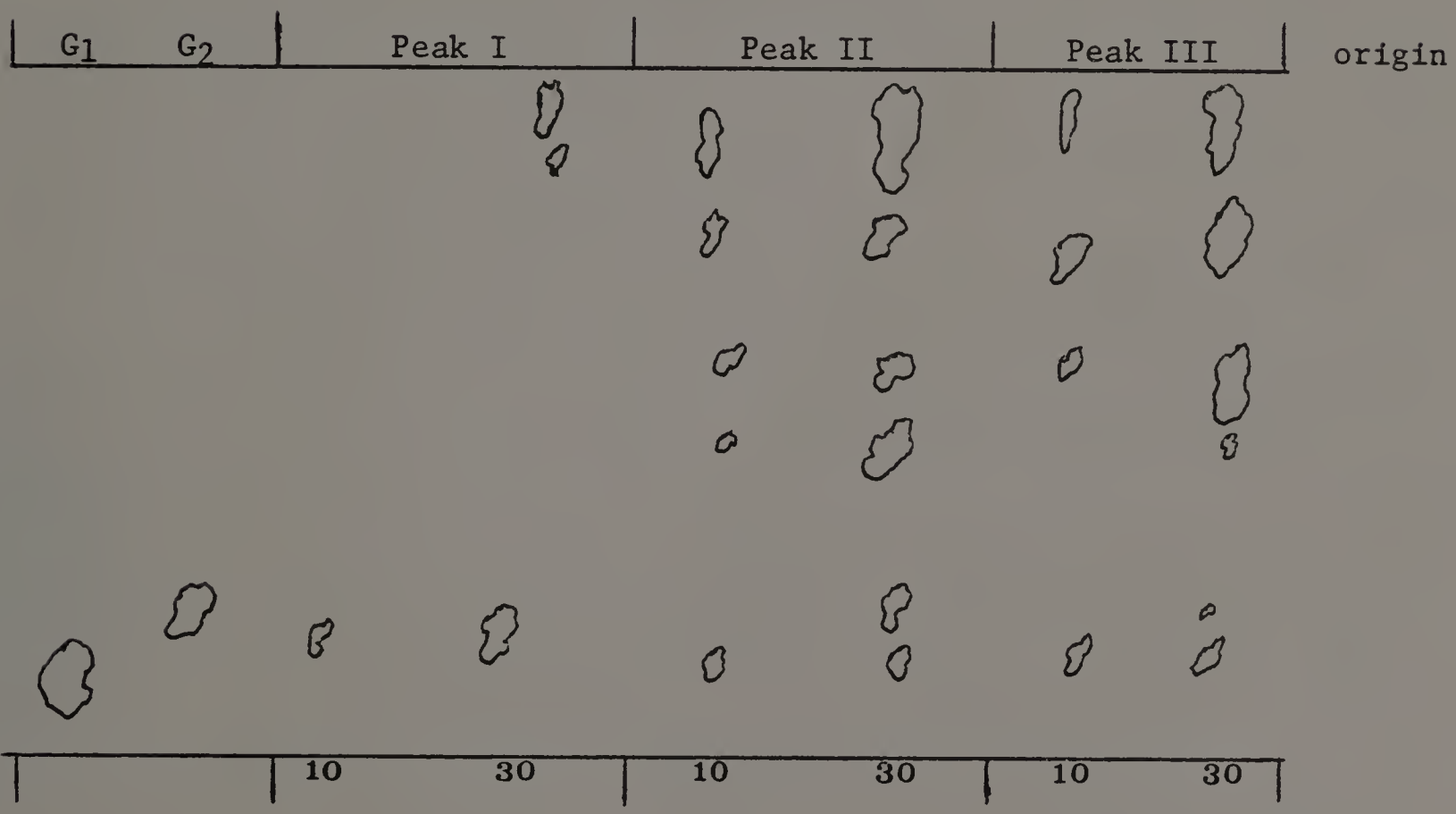


FIGURE 3

Zymogram of paper chromatograms of reaction product patterns from amylase activties in Peaks I, II and III eluted from a DEAE-cellulose column. Amylase activity from de-embryonated maize kernels after 5 days of incubation at 30°C.



G_1- Glucose standard

G_2- Maltose standard

the sole reaction product was maltose (Fig. 3). Further reaction with the substrate (30 min.) shows the appearance of high molecular weight oligosaccharides, formed as additional maltose units are cleaved from the substrate (88). The lack of glucose as a reaction product is additional confirmation of β-amylase activity, as the smallest dextrin which β-amylase hydrolyzes is a four glucose polymer, yielding two maltose units as products (88).

The reaction product patterns from Peak II and Peak III are indicative of α-amylase activities (Fig. 3). Initial products from the two activities include glucose and higher oligosaccharides. Alpha-amylase generally acts very slowly on maltotriose, hydrolyzing it to glucose and maltose, but it cleaves the larger glucose polymers more rapidly (88). Thus, at the end of 30 minutes reaction with the soluble starch substrate, both maltose and glucose are evident, as well as a larger amount of the higher oligosaccharides.

Physicochemical analysis is often used to distinguish between α and β-amylases. Results of various treatments on the activities from the three peaks are presented in Table 2.

The data for Peak I again suggest β-amylase activity. The activity is completely destroyed on application of heat, with no protection afforded by the addition of excess calcium ions prior to heat treatment. No exogenous requirement for calcium ions is shown. The activity is also destroyed by treatment with mercury ions, but is partially restored by additions of DTT either before or after mercury treatment. Dithiothreitol slightly enhanced the activity of Peak I. Protection by the thiol reagent against the effects of the heavy metal ion implicates the

TABLE 2

Physicochemical analysis of amylase activities from Peaks I, II and III eluted from a DEAE-cellulose column. Activity from day 5 preparation of incubated, de-embryonated maize kernels.

Treatment	Amylase Activity as % of Control		
	Peak I	Peak II	Peak III
Control	100.	100.	100.
Ca^{+2} (10^{-3} M, 1 hr, 0°C)	100.	97.1	96.5
Heat (12 min, 70°C)	0.00	25.2	36.6
Ca^{+2} + Heat	0.00	63.0	74.5
Hg^{+2} (10^{-5} M, 15 min, 0°C)	0.00	34.2	45.2
DTT (1 mM, 15 min, 0°C)	108.	96.0	101.
Hg^{+2} + DTT	78.0	32.9	40.5
DTT + Hg^{+2}	84.0	44.6	36.9
EDTA dialysis-24 hr	80.1	23.0	5.00
EDTA dialysis-48 hr	74.2	0.00	0.00

involvement of free sulfhydryl groups known to be present in β-amylase (84).

Peak II and III exhibited α-amylase activities. Although requiring no exogenous source of calcium ions for full activity, both activities were partially protected against heat inactivation when calcium ions were added prior to the heat treatment. Reports indicating increased thermostability of α-amylase in the presence of high levels of calcium ions substantiate the α-amylase character of Peaks II and III (25, 32, 88).

All α-amylases investigated appear to contain calcium, the removal of which may result in either reversible or irreversible loss of activity (23). Dialysis against a calcium chelator, such as EDTA, has been employed to inactivated α-amylases (86). Loosely bound calcium is easily removed by this treatment, while the one gram atom of calcium/mole remaining is only released by more exhaustive dialysis (88).

Peak II exhibits α-amylase activity with the complete loss of activity after 24 hours of dialysis against EDTA (Table 2). Peak III has some activity remaining after 24 hr of EDTA dialysis, but is almost completely inactivated after 48 hours of the treatment. In contrast, Peak I retains the greater portion of its activity, even after the 48 hr treatment. Loss of some activity by this peak could be due to instability of the enzyme over time, or sensitivity to the physical conditions of the dialysis treatment.

Addition of mercury ions to amylases from Peaks II and III resulted in substantial decrease in activity. In contrast to the data from Peak I, no protection against the reduction in activity on mercury treatment was shown by addition of DTT either before or after treatment with the heavy metal ion. Also, DTT alone did not enhance the activities from Peaks

II and III. The irreversible denaturation of α-amylase activity due to mercury effects has been described for α-amylases from other sources, as well (31, 37).

Data from DEAE-cellulose column chromatography shows the separation into three distinct amylase activities of the full complement of starch degrading bands extracted from de-embryonated maize kernels after 5 days of incubation. Peak I data suggests the presence of a β-amylase, while Peaks II and III exhibit α-amylase characteristics.

B. Characterization of Amylase Activity After 24 hr of Incubation.

A preparation of amylase activity from day 1 extracts was purified by gel filtration chromatography and subjected to DEAE-cellulose column chromatography (Fig. 4). The activity was eluted as one sharp peak at 0.11 M NaCl concentration. Rechromatography of the active fractions resulted in the same sharp peak at 0.13 M NaCl (results not presented).

The activity purification scheme is shown in Table 3. Approximately a six fold increase in specific activity was obtained from the DEAE fractions purified from the crude extract. The amount of proteinaceous material from the day 5 crude extract was much higher than that of the day 1 crude extract (Tables 1 and 3), reflecting an increase in soluble proteinaceous material in the de-embryonated maize kernels, as they are incubated. However, quantities of bound amylase were still found at later times of incubation (Appendix), as well as in the pellet from the day 1 preparation (Part I, Table 4).

Figure 5 shows a comparison of the electrophoretic banding patterns for the day 1 crude extract, the day 1 bulked DEAE fractions, and a

FIGURE 4

DEAE-cellulose chromatographic elution profile of an amylase preparation from de-embryonated maize kernels after 24 hr of incubation at 30°C.

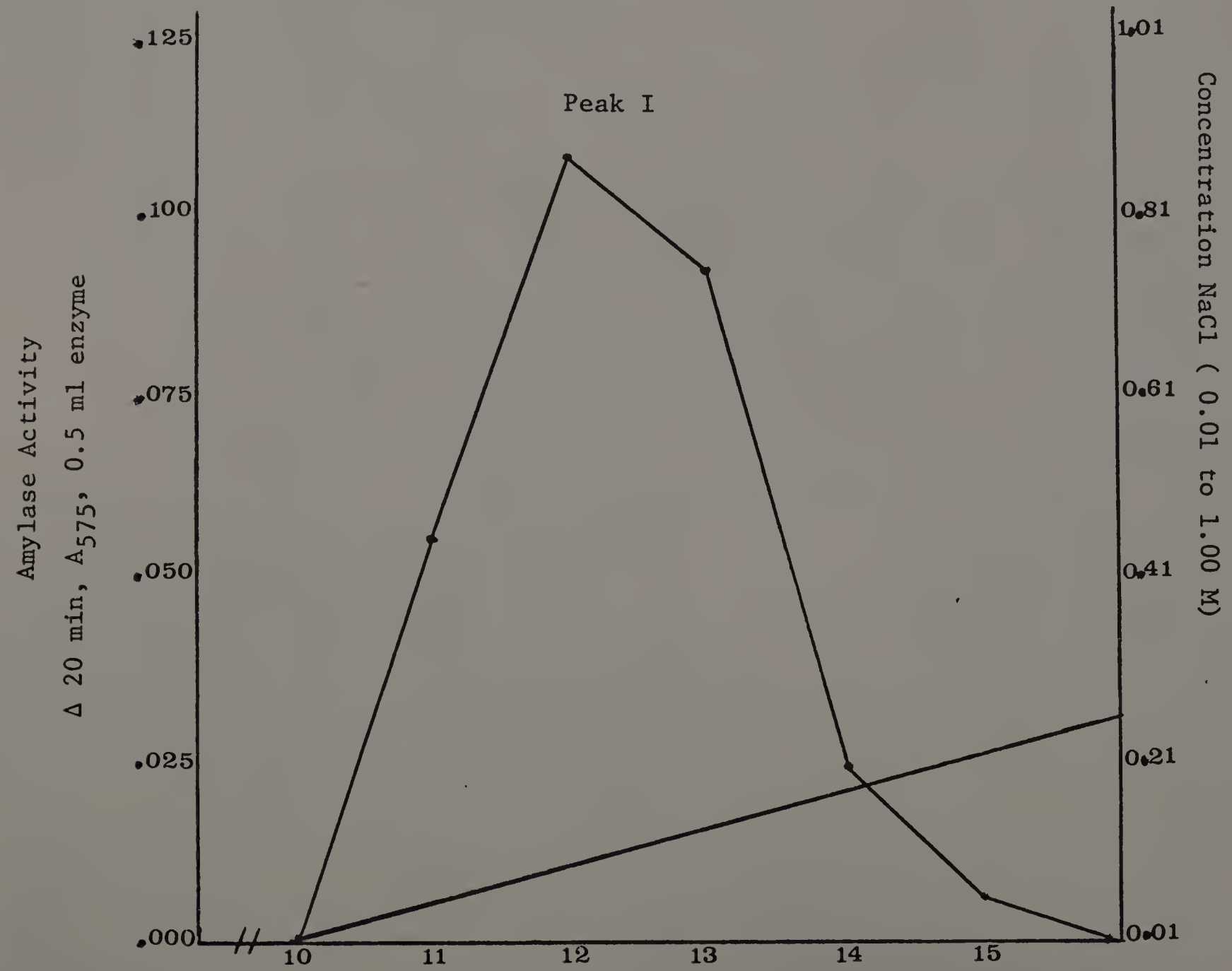
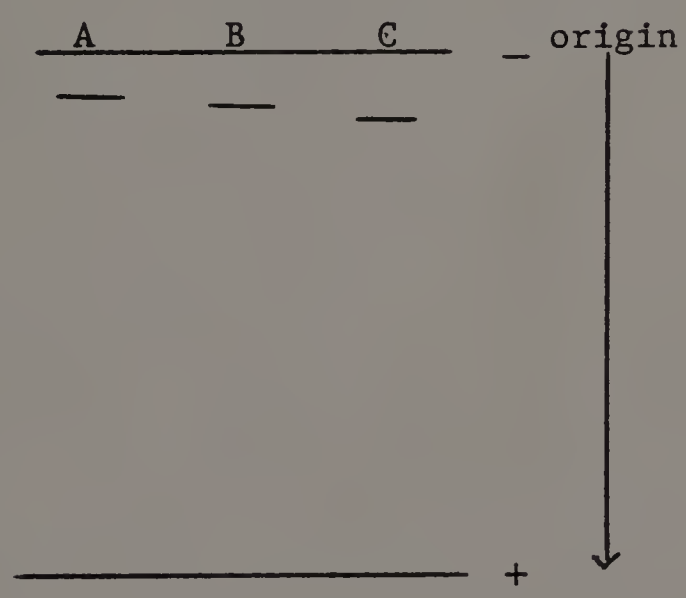


FIGURE 5

PAGE zymograms of starch degrading bands from activity in de-embryonated maize kernels incubated for 24 hr, and from activity of a commercial β-amylase.



A- Day 1 crude extract

B- Day 1 bulked DEAE fractions from Peak I

C- Commercial β-amylase

TABLE 3

Purification scheme of amylase activity extracted from de-embryonated maize kernels after 1 day of incubation.

Treatment	Protein (mg)	Specific Activity (*units/mg protein)
Crude extract	105	2.03
Sephadex G-50	31.5	6.29
DEAE-cellulose		
Peak I	12.2	12.9
Peak I (rechromatographed)	11.5	11.8

*one unit of activity = Δ20 min., A_{575} of 0.010 for 0.5 ml of enzyme

commercial β-amylase. The presence of one slow moving starch degrading zone in both of the day 1 preparations indicates that, indeed, only one amylase was present in the early incubation of the de-embryonated kernel, and that this activity was not altered during the purification of the crude extract. Further support for the β-amylase character of this activity was shown by the similarity of the PAGE patterns for the day 1 preparations to that for the commercial β-amylase.

Data for physicochemical treatments and PAGE patterns for the treated DEAE fractions are presented in Table 4 and Figure 6, respectively. The presence of one slow moving starch degrading zone is evident in the control (bulked DEAE fractions with no treatment) and in all treated samples where the major portion of activity remains after physicochemical treatment. No alteration in the mobility or the number of bands was apparent.

Physicochemical data for the crude extract and the bulked DEAE fractions was similar and indicates β-amylase activity (Table 4). Calcium ions did not enhance activity, nor protect against the loss of activity on heat treatment. Dialysis against EDTA did not result in complete loss of activity as would be expected with an α-amylase (88). Mercury ions eliminate activity entirely, but additions of DTT either before or after mercury treatment partially reverses this inhibition such that the activity remains about 50% of the control in the bulked DEAE fractions.

Higher activities for the combined mercury and DTT treatments in the crude extract (Table 4) may indicate that the enzyme is more stable before purification, or that the presence of other substances in the crude extract may have a protective effect on the enzyme. Data for

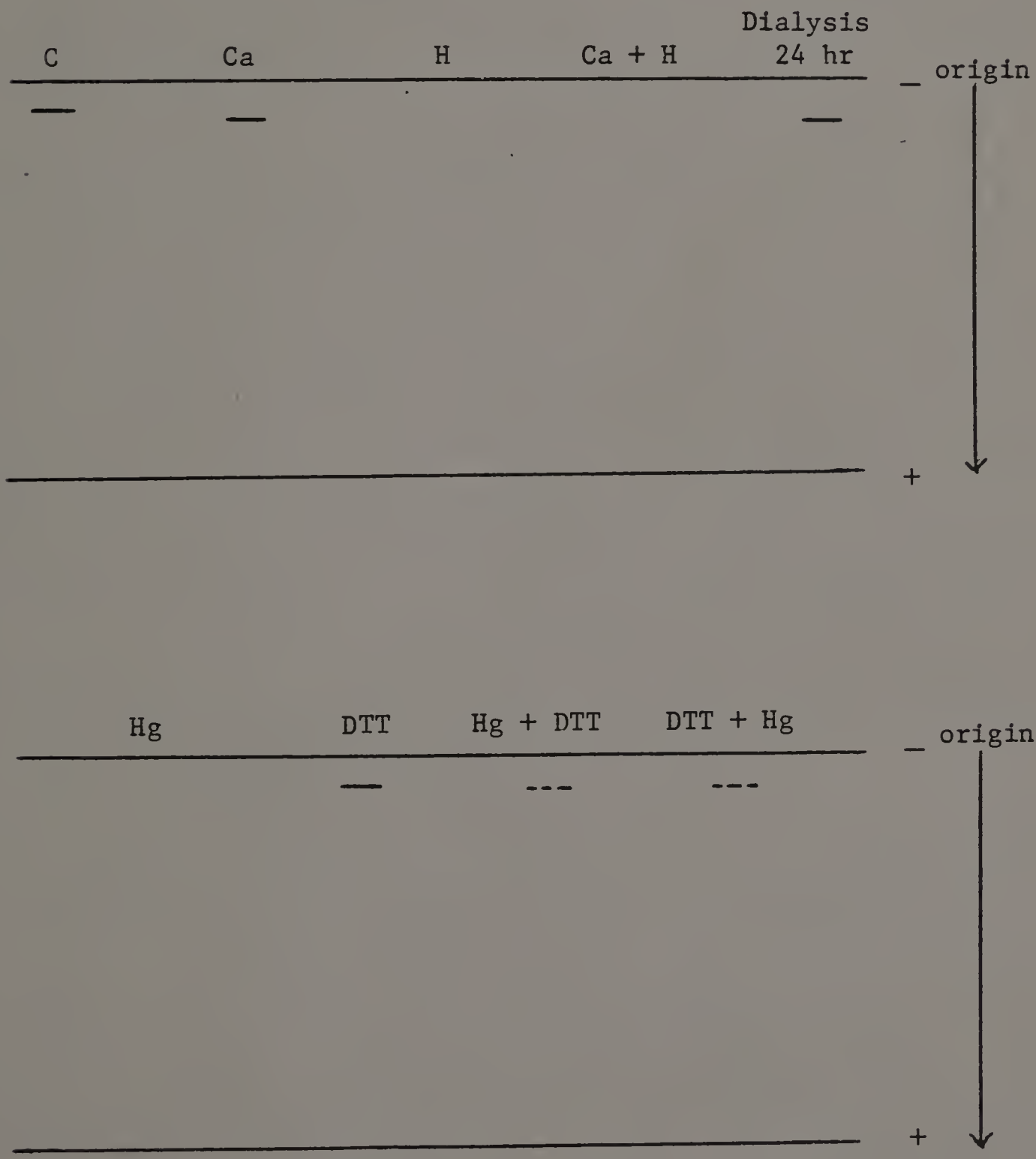
TABLE 4

Physicochemical analysis of amylase activity in the crude extract and in Peak I eluted from a DEAE-cellulose column. Amylase activity from de-embryonated maize kernels, incubated for 24 hr at 30°C.

Treatment	Amylase Activity as % of Control	
	Crude extract	DEAE fractions
Control	100.	100.
Ca^{+2} (10^{-3} M, 1 hr, 0°C)	84.9	92.3
Heat (12 min, 70°C)	0.00	0.00
Ca^{+2} + Heat	10.1	0.00
Hg^{+2} (10^{-5} M, 15 min, 0°C)	0.00	0.00
DTT (1mM, 15 min, 0°C)	103.	93.3
Hg^{+2} + DTT	69.0	47.4
DTT + Hg^{+2}	83.1	53.4
EDTA dialysis-24 hr	80.8	84.0
EDTA dialysis-48 hr	77.1	75.4

FIGURE 6

PAGE zymograms of the effects of physicochemical treatments on starch degrading bands from amylase activity in Peak I eluted from a DEAE-cellulose column. Amylase activity from de-embryonated maize kernels incubated for 24 hr at 30°C.



physicochemical treatments for the day 1 purified preparation (Table 4) parallel the data obtained for Peak I of the day 5 purified preparation (Table 2).

The increased thermostability of α-amylase in the presence of high concentrations of calcium ions has been described previously. To further examine the nature of the amylase present in Peak I of the day 1 preparation, the thermostability of the enzyme was studied. Results are presented in Figure 7. One hour pre-incubation with calcium ions was ineffective in protecting the amylase against thermal inactivation at at 70°C. Both the control and the $CaCl_2$ treated sample were similarly inactivated within 6 minutes, a typical β-amylase response. In contrast, Peaks II and III from the day 5 preparation, which had exhibited α-amylase characteristics, had over 50% of the activity remaining when $CaCl_2$ (10^{-3} M) was added prior to a heat treatment at 70°C for 12 minutes (Table 2).

Further evidence for the presence of β-amylase was gained by a study of reaction product patterns (Fig. 8). Ten minutes of enzyme reaction with the soluble starch substrate yielded maltose as the sole reaction product. With longer times of incubation, the appearance of higher oligosaccharides is evident. The reaction product pattern for the activity from Peak I in this study bears a striking similarity to that from Peak I of the day 5 preparation (Fig. 3).

To investigate the effects of temperature on enzyme activity routing assays were carried out in 5°C increments over a range of 15°C to 55°C (Fig. 9). Optimum temperature for amylase activity under these conditions was 45°C, with greater than 50% of the optimum activity occurring in the range of 30 to 55°C. Energy of activation (E_a) was

FIGURE 7

The effect of calcium ions (10^{-3} M, 1 hr incubation) on the thermostability of amylase activity from Peak I eluted from a DEAE-cellulose column. Amylase activity from de-embryonated maize kernels after 24 hr of incubation at 30°C.

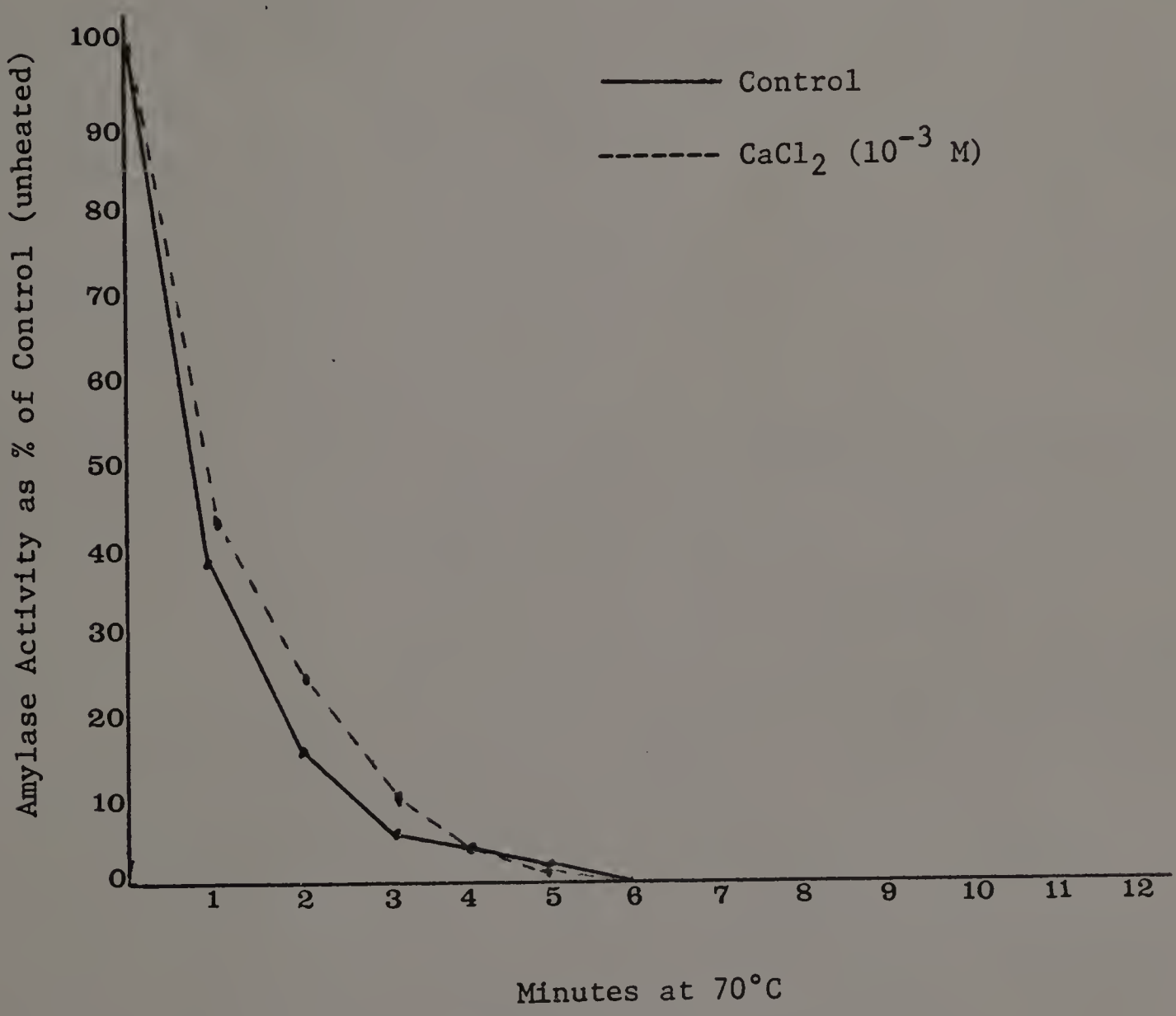
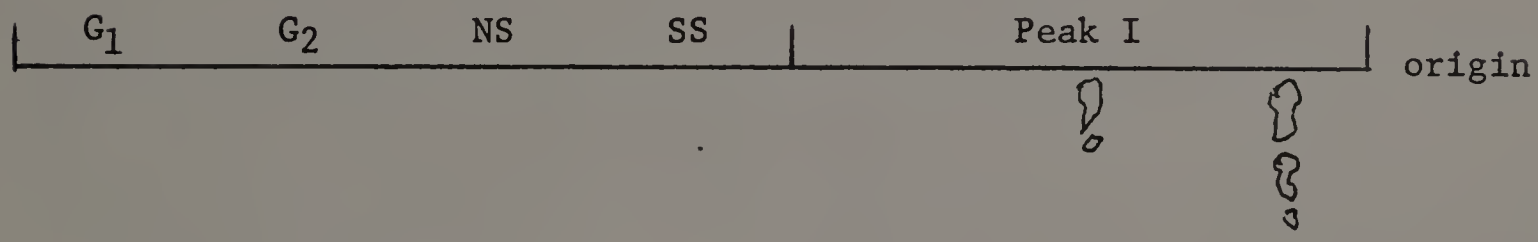
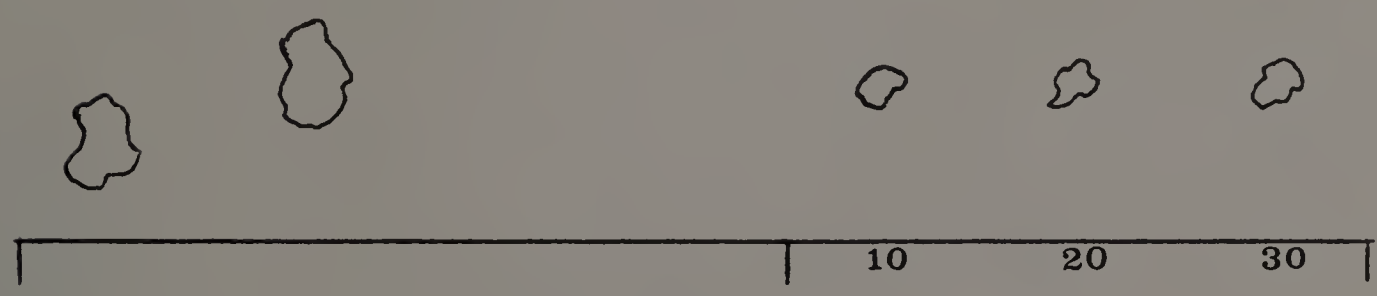


FIGURE 8

Zymogram of paper chromatograms of reaction product patterns from amylase activity in Peak I eluted from a DEAE-cellulose column. Amylase activity from de-embryonated maize kernels after 24 hr of incubation at 30°C.





minutes of enzyme reaction with soluble starch substrate at 30°C

G_1- Glucose standard

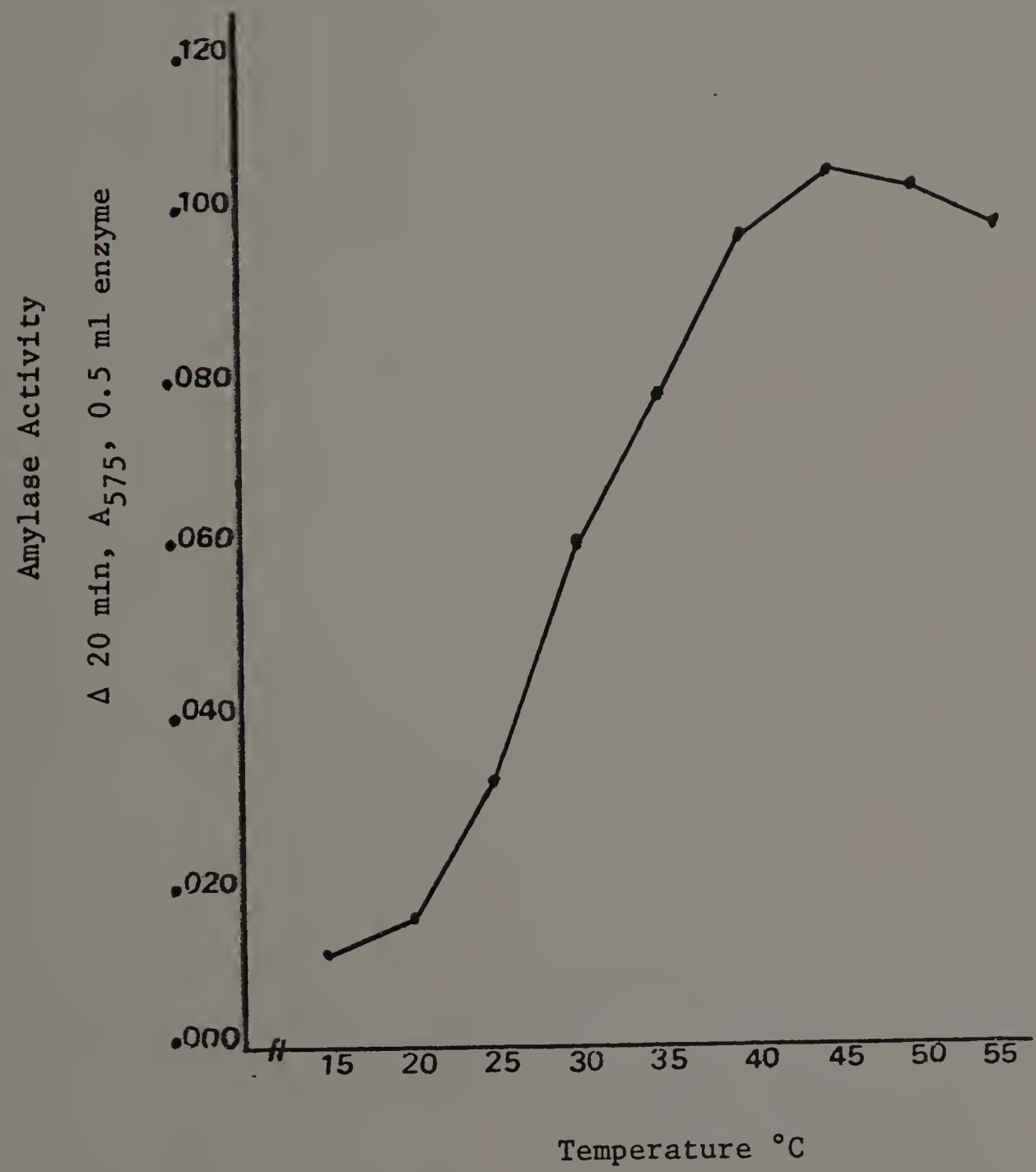
G_2- Maltose standard

NS- No substrate

SS- Soluble starch

FIGURE 9

Temperature activity profile of amylase activity from Peak I eluted from a DEAE cellulose column. Amylase activity from de-embryonated kernels after 24 hr of incubation at 30°C.



calculated from an Arrhenius plot of amylase activity, derived from the temperature optima data (Fig. 10). E_a was found to be 14.6 Kcal/mole between 20 and 30°C, decreasing to 8.3 Kcal/mole between 30 and 40°C. These values are somewhat higher than the 14.0 Kcal/mole at 10°C reported for amylases in the literature (51, 88).

The pH activity profile of the amylase is presented in Figure 11. Activity was determined by routine assay over a range of pH 2.5 to 8.0. Buffers in the appropriate pH ranges were prepared according to the method of Gomori (1955). As pH stabilitiy of an amylase is a function of protein and salt concentrations (88), all assays were prepared using 0.5 ml of the purified enzyme in a 0.025 M buffer solution. The anions of the buffers used (citrate-phosphate, acetate, Tris-HCl) are reported to have no deleterious effects on amylase activity (60).

The pH optimum of 4.8 is similar to other reports for β-amylases (24). Greater than 50% of the optimum activity was realized in the pH range of 3.4 to 6.6. The stability of an amylase in an acid environment (pH3.5) is one criteria for β-amylase activity (25, 87).

The Michaelis-Menten constant, K_m, was determined from a Lineweaver-Burke plot of amylase activity (Fig. 12). The range of soluble starch concentrations in the complete reaction mixture varied from 2 to 40 mg. The K_m determined was found to be 3.5×10^{-3} grams soluble starch/ml, and is similar to those found for β-amylases from other sources (24, 32).

FIGURE 10

Arrhenius plot of amylase activity from Peak I eluted from a DEAE-cellulose column. Amylase activity from de-embryonated maize kernels after 24 hr of incubation at 30°C.

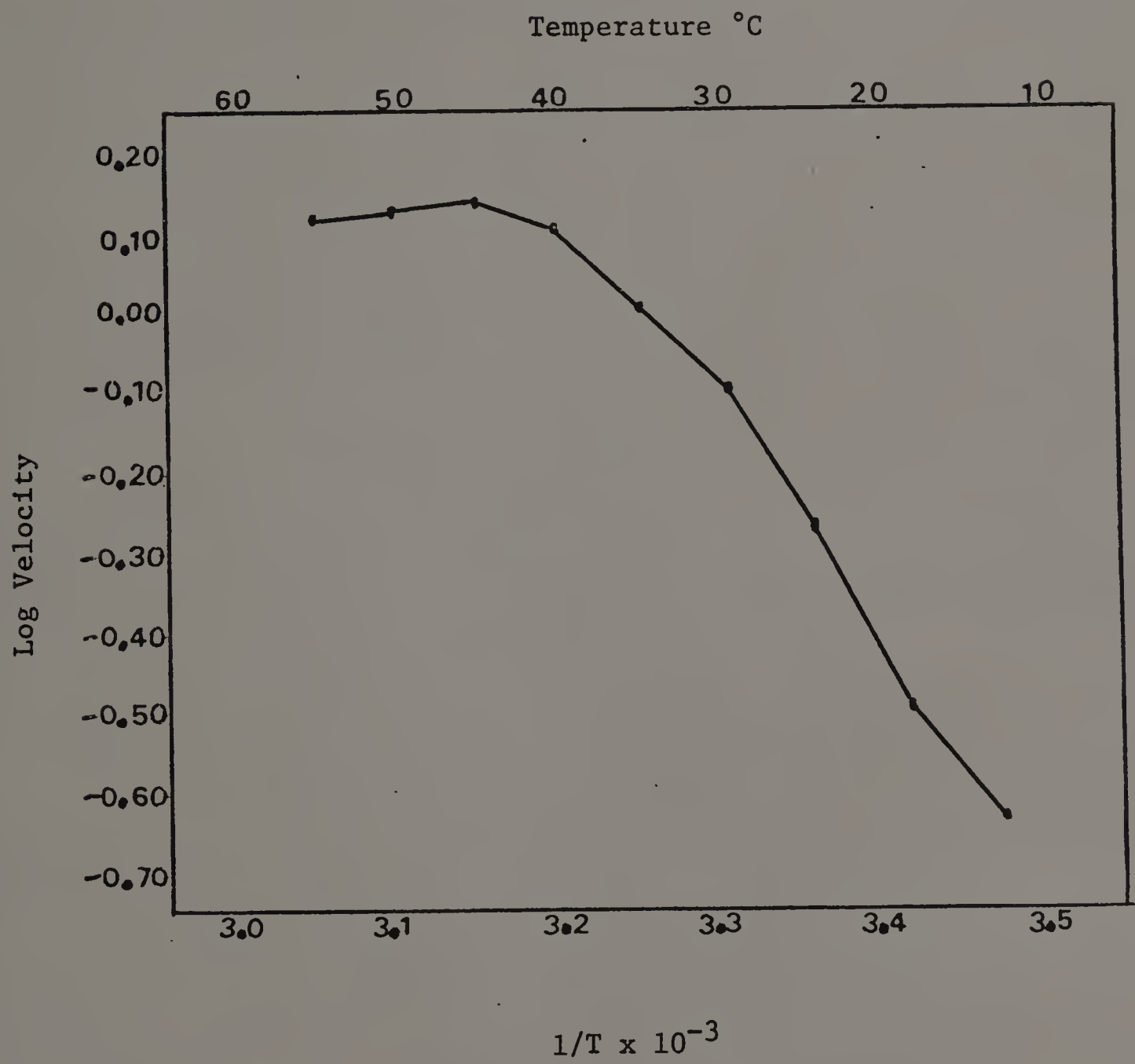


FIGURE 11

pH activity profile of amylase activity from Peak I eluted from a DEAE-cellulose column. Amylase activity from de-embryonated kernels after 24 hr of incubation at 30°C.

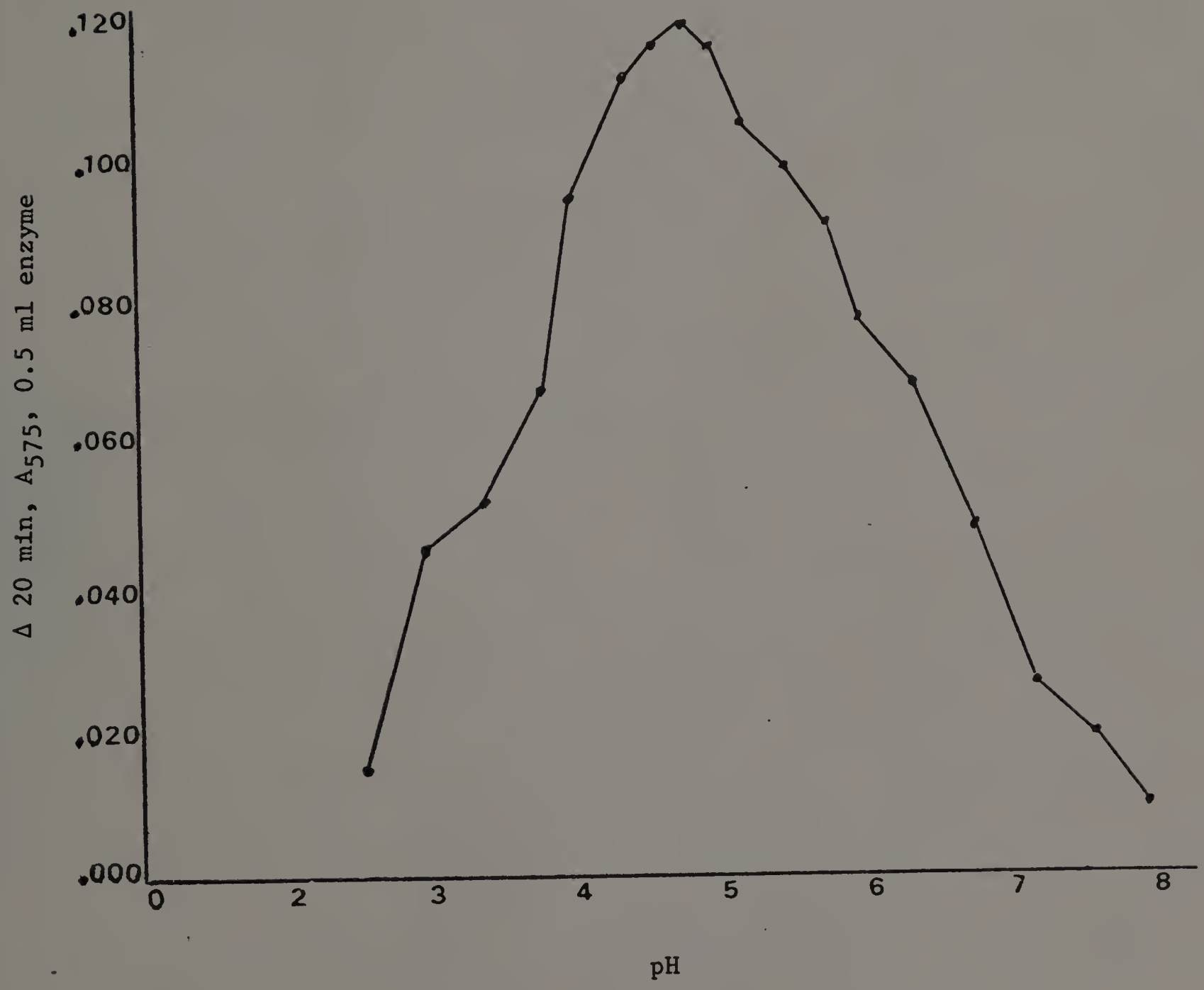
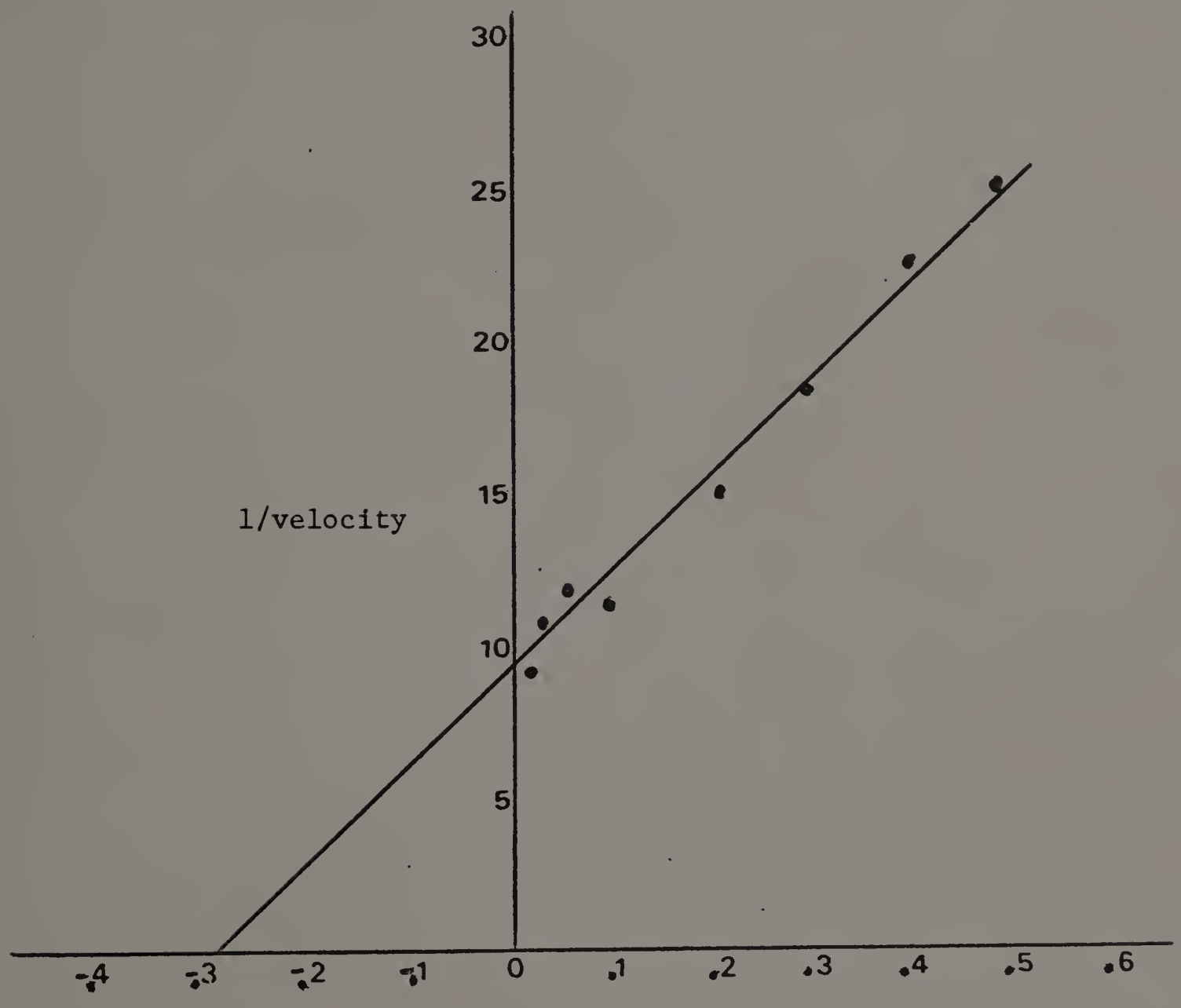


FIGURE 12

Lineweaver-Burke plot of amylase activity from Peak I eluted from a DEAE-cellulose column. Amylase activity from de-embryonated maize kernels after 24 hr of incubation at 30°C.



$K_m = 3.5 \times 10^{-3}$ grams soluble starch/ml

DISCUSSION

Part I. Occurrence and Development of Amylase Activity in Maize Kernels.

The data indicate that amylase activity is present in three different maize tissue systems; the developing kernel, the germinating kernel, and the incubated de-embryonated kernel,

A. Amylase Activity in Developing Kernels.

Physicochemical data for amylase activity in the endosperm of the developing maize kernel suggests the presence of both α and β-amylase activities (Table 3). Both enzymes have been reported in the endosperm of developing maize kernels (9, 10, 22). Chao and Scandalios (1969) have reported three zones of starch degrading activity in the endosperm of 16 to 20 day old kernels, while Finnigan (1969) found two zones of activity in kernels harvested 30 to 35 days after pollination. Four zones of starch degrading activity were found in this study with the endosperm from kernels 24 days after pollination (Fig. 6). The least anodic of these zones contained multiple bands, a situation observed in other studies, as well (9, 22). Variations of the isozymic complement in different studies might be the result of the different maize cultivars used for experimentation or from differences in times of sampling.

A comprehensive time course study of the nature of the amylase activity in maize kernels during the entire developmental stage was beyond the scope of this investigation. However, it does appear that both α and β-amylase activities are present at at least one time during kernel ontogeny. Chao and Scandalios (1969) report that the amylase

zymogram of activites from the maize endosperm did not vary from 7 days to full maturity in the maize cultivars which they examined.

Moisture content for 24 day old developing kernels was found to be 59.8% versus the 11.9% for fully mature kernels from a humid environment (Table 1). Whereas four starch degrading zones were identified by PAGE analysis from the endosperm of developing kernels (Fig. 6), only one zone was present in the endosperm from mature kernels during the early stages of incubation (one day) (Fig. 2). Correlating with a reduction in the number of electrophoretic zones, total amylase activity in extracts from mature, de-embryonated kernels during early incubation was less than one third of that from the endosperm of developing kernels (Table 2).

A number of researchers have reported a decrease in amylase activity in developing grains upon maturation (5, 13, 15, 16, 50, 67). Finnigan (1969) found maximum activity in the maize endosperm at 30 to 35 days after pollination, thereafter activity decreased. This decrease in amylase activity has been attributed in part to decreasing moisture levels of the cereal grain upon maturation, which either reversibly inactivates or denatures the amylases present (13, 67, 82, 83).

Assay for bound amylase activity in the endosperm from 24 day old developing kernels yielded negative results (Appendix). In the mature de-embryonated kernel during early incubation, however, as much activity was found in the latent form as in the soluble, active form (Table 4). Numerous researchers have reported the presence of bound β-amylase in mature cereal grains (71, 82, 82, 91). In addition, work by Gibson and Paleg (1972) supports the presence of a structurally latent form of α-amylase in mature wheat aleurone cells. After treatment with GA_3,

membrane rupturing chemical or mechanical treatments were required to release the amylase into its full active form. These researchers suggest that α-amylase exists within lysosomes, distinct membrane enclosed organelles, containing acid hydrolases.

The following results with the maize kernel would suggest that developmental changes in α and β-amylases during the ripening of the kernel are similar to those found for other cereal grains. 1. decrease in moisture level upon maturation, 2. decrease in soluble amylase activity and zones of starch degrading activity, 3. increase in latent activity upon maturation. It would appear therefore, that α and β-amylases present in the maize endosperm and/or aleurone layers during early development are reversibly inactivated or denatured with increasing moisture loss. Activity in the mature kernel is thus a function of both soluble and latent forms of the amylases. The finding of little (17) or no (79) amylase activity in the mature, resting maize seed strengthens this argument.

B. Amylase Activity in Germinating Kernels.

The total amylase activity from the endosperm of germinating kernels is higher than that from de-embryonated kernels, most notably during early incubation (Fig. 3). Correlating with this faster increase in activity is the earlier appearance of starch degrading zones upon PAGE analysis (Fig. 4). Reaction product patterns for amylase activity from the endosperm of germinating kernels suggest that at least part of the activity was due to α-amylase (Fig. 5). The presence of α-amylase activity has been previously reported in the germinating maize kernel (80), and specifically in the endosperm of the germinating maize seed (17).

During germination, the embryo which is in contact with the endosperm also shows an increase in activity over time (Fig. 3), as well as a complex banding pattern on PAGE analysis (Fig. 4). The increased activity from the endosperm during germination could be due to interactions between the embryo and the endosperm. Similar to the biochemical events occurring in barley (45), amylase synthesis in the germinating maize endosperm may be enhanced by the action of gibberellins from the embryonic axis or scutellum (72, 73). In fact, the increase in amylase activity over time from the endosperm of the intact, germinating maize kernel parallels closely the enhanced activity of incubated, de-embryonated maize kernels when the latter are treated with 1 uM GA_3 at the start of incubation (Fig. 3, Fig. 7).

However, the presence of the embryo in the intact, germinating maize seed may not be solely responsible for the amylase activity and the complement of starch degrading bands arising from the endosperm during germination. Both of these phenomena also occur in the incubated, de-embryonated maize kernel (Fig. 2, Fig. 3), suggesting the activation of pre-existing amylase or the de novo synthesis of amylase in the endosperm, without the assistance of an embryo factor. Reaction product patterns for amylases from the endosperm of germinating kernels and from the incubated, de-embryonated kernel are similar (Fig. 5), suggesting similar amylase activities. In addition, a maximum of four electrophoretic starch degrading zones was observed in both of the tissues (Fig. 2, Fig. 4).

C. Amylase Activity in De-embryonated Kernels.

1. Physical effects. In de-embryonated kernels, amylase activity

develops during incubation, independent of the embryo or exogenous GA_3. Pretreatment conditions of the mature kernels greatly affect the expression of amylase activity upon incubation. Kernels kept in a desiccator, under dry conditions contain less than half the moisture of kernels kept under conditions of 75% R.H. (Table 1). Upon incubation, the dry stored kernels exhibited less activity and fewer starch degrading bands in comparison to the humid stored kernels (Fig. 1, Fig. 2). Moisture level in relation to the constancy of amylase expression was stressed by Dure (1960). He found that a median fresh weight range for maize experimental material must be maintained in order to achieve reproducible results for amylase activity.

It has been suggested that the viability of a seed is related to its moisture level, and increase in moisture level will increase metabolic rates, and hence may alter the response of the seed upon imbibition and germination (96). With wheat, Olered and Jonsson (1970) found decreased activity of α-amylase in developing grain subjected to dry conditions. A temporary increase in humidity stimulated an increase in α-amylase activity, during any time of the wheat development. Drying the kernels subsequent to harvesting also decreased the α-amylase activity in the ungerminated grain.

Olered and Jonsson (1970) also found that the expression of β-amylase activity was not dependent upon hydrolytic processes. Significantly, the only starch degrading activity consisitantly present in preparations from the endosperm of dry stored kernels was the slow moving band characterized as a β-amylase (Fig. 2). The more anodic bands, which exhibited α-amylase activity on physicochemical analysis (Fig. 9, Fig. 11)

were only sporadically present in the dry stored kernels and then only after 5 to 6 days of incubation.

Removal of the embryo prior to pretreatment of the kernels did not affect moisture losses from the kernels stored in either dry or humid environments (Table 1). It would appear that the embryo is not a determining factor in the moisture level of the mature maize kernel. Water held by maize kernels has been shown to be a function of the colloidal compounds of the endosperm as well as a function of the pericarp (62).

It would seem then that the moisture level of the mature maize kernel is a function of both the environmental humidity and the physical characteristics of the endosperm and pericarp. In turn, the expression of amylase activity upon incubation of the de-embryonated kernel is dependent at least partially upon the moisture level of the mature seed.

On removing the pericarp and aleurone layers from the de-embryonated kernels, a complete absence of amylase activity and a lack of starch degrading zones on PAGE analysis was noted throughout the incubation period. This negative observation would support the role of the cereal aleurone or sub-aleurone layers as a storage site for bound β-amylase (15, 18) and as a site for de novo amylase and protease synthesis as reported for barley (21, 93, 95).

However, the aleurone layers have been shown to function in other physiological processes, as well, which would be altered on the removal, subsequently affecting amylase expression. In barley the aleurone cells by their selective permeability and osmotic concentration function in the secretion and release mechanisms for the hydrolytic enzymes (44, 46, 94).

Also, the aleurone layers may serve as the site of synthesis or action of endogenous gibberellins. Similar to the GA-controlled synthesis of α-amylase in barley (45, 93), endogenous gibberellins in maize may play a role in the expression of α-amylase. Gibberellins have been reported in the aleurone layers of barley (11), as well as in the barley embryo (72, 73). Finally, the synthesis or action of proteases, which have been shown to be GA_3 controlled in the barley aleurone layers (38), and active in liberating bound β-amylase (33, 78) could be altered by removal of the aleurone layers, thus affecting the expression of β-amylase.

Critical analysis of the results would thus indicate that, although the aleurone layers most probably play a primary role in amylase expression as a site of origin or synthesis, secondary factors must also be taken into account. One result is clear. without the aleurone layer, the expression of soluble amylase activity in the incubated, de-embryonated kernel is nil.

2. Gibberellic acid effects. With aleurone layers intact, amylase activity in de-embryonated kernels increases as a function of incubation, without the addition of GA_3 (Fig. 7). From a low initial level on the first day of incubation, amylase activity increased to a maximum at about 7 days of incubation, with the presence of four starch degrading zones on PAGE analysis (Fig. 8). Additions of GA_3 to the de-embryonated kernels at the start of incubation did not alter the maximum activity reached or the number of starch degrading zones present at the time of maximum activity (Fig. 7, Fig. 8). Rather, the effect of exogenously applied GA_3 is seen as a quantitative one. The rise in amylase activity and the appearance of starch degrading zones occurred sooner than in the control.

The quantitative effect of GA_3 was further borne out by the response of the de-embryonated kernels to different levels of the hormone (Fig. 7). At concentrations less than 1 uM GA_3, the hormone enhanced amylase activity was less. Maximum GA_3 enhancement of amylase activity was reached at 1 uM GA_3, and a higher concentration did not bring about greater amylase activity.

Analysis of physicochemical data for amylase activity in GA_3-treated samples compared to controls over time of incubation further support the quantitative aspect for GA_3 (Fig. 9, Fig. 11). For both samples amylase activity was predominanatly β-amylase in character early in the incubation period. Later appearance of α-amylase activity and additional starch degrading bands on PAGE analysis occurred in both cases (Fig. 9, Fig. 10, Fig. 11). The response of total activity to physicochemical treatments for the GA_3-treated sample was similar to that of the control, only occurring sooner in the incubation period.

The development of amylase activity in the incubated, de-embryonated maize kernel which is partly α-amylase in character, independent of an embryo, and only quantitatively affected by exogenous GA_3 is contrary to the results from several researchers (17, 36).

Dure (1960) concluded that α-amylase activity arises only from the scutellum of the maize kernel and is secreted into the endosperm to form the bulk of the amylase activity during germination. Ingle and Hageman (1965) maintained that exogenous GA_3 replaced a component produced in the embryonic axis and received by the endosperm on germination, the initiation of sugar production being completely dependent upon this embryo factor in the germinating seed or upon exogenous GA_3

in the incubated, de-embryonated kernel. Though both researchers (17, 36) used de-embryonated maize kernels for their studies, the intact kernels were allowed to imbibe water for several hours before dissection.

Biochemical events initiated by early imbibition may, however, involve interaction between the embryo and endosperm, altering the amylase response of the subsequently de-embryonated kernel. Maize embryos are capable of early response upon imbibition. Embryos dissected from germinating kernels in this study showed amylase activity and a number of starch degrading bands within the first 24 hr of incubation (Fig. 3, Fig 4). Marcus et al. (1960) demonstrated that the wheat embryo is capable of protein synthesis within the first 30 minutes of imbibition.

In agreement with results from this study, other work indicates that the maize endosperm is capable of digesting its own starch reserves (27, 34, 63). Goldstein (1974) reported both α and β-amylase activities arising from the de-embryonated maize kernel upon incubation. Exogenous GA_3 enhanced early amylase activity, although total maximum activity in GA_3-treated and control samples was similar. Harvey and Oaks (1974) suggest a de novo synthesis for α-amylase from de-embryonated maize kernels, from evidence of inhibitor studies. These researchers did not find enhanced amylase activity on addition of GA_3, but suggest that gibberellins are instrumental in the development of activity in overcoming the inhibition of amylase synthesis by abscisic acid (ABA) (34).

The quantitative effect observed for amylase expression on application of GA_3 to de-embryonated maize kernels in this study, lends itself to several interpretations. Upon imbibtion the presence of endogenous

gibberellins could bring about the activation of de novo synthesis of amylases in the endosperm or adhering aleurone layers. There is some indication that cold treatment such as used in this study (4 to 6°C for at least two months prior to experimentation) will result in higher endogenous levels of gibberellins (69). Higher concentrations of gibberellins were found in a winter wheat variety after vernalization (69).

Enhancement of amylase activity on GA_3 additions during early incubation may indicate that initially the maize endosperm, itself, does not contain the level of endogenous gibberellins needed to effect full amylase expression. The maize embryo may be another source of these gibberellins. Gibberellins are known to be synthesized in the scutellum of germinating barley (72, 73).

Alternatively, the endosperm or aleurone may possess the capacity to synthesize gibberellins during incubation or to release them from a bound form (45), attaining the level of GA needed for full amylase expression over time. It would appear that by some mechanism the full amylase activity is realized in the intact germinating seed. As previously noted, the total amylase activity from endosperm excised from germinating kernels paralleled that activity found when incubated, de-embryonated kernels were treated with exogenous 1 uM GA_3 (Fig. 3, Fig. 7).

Another explanation for the lower activity in non-GA_3 treated, de-embryonated kernels is the possible interaction of gibberellins with other hormones or inhibitors. In barley abscisic acid (ABA) inhibits GA_3 induced α-amylase synthesis (61). The inhibitory effects of ABA on α-amylase expression in maize can be overcome by exogenous GA_3 (34). Enhanced amylase synthesis with exogenous GA_3 in this study could thus

be due to the exogenous hormone overcoming the inhibitory effects of other hormones such as ABA. As only initial activities differed, while maximum activity levels for both GA_3-treated and control de-embryonated kernels were similar (Fig. 7), endogenous levels of gibberellins and substances antogonistic to their action could be changing in relation to each other over the time of incubation. Inhibitory substances may be leached out or inactivated, or the level of endogenous gibberellins may be increased over the incubation period.

3. Inhibitor Effects. Amylase activity in the incubated, de-embryonated kernel may arise from two sources: activation of latent forms of amylase residing in the ungerminated kernel, or de novo synthesis of new enzyme upon incubation of the kernel.

A series of starch degrading zones on PAGE analysis is evident upon the development of amylase activity in incubated, de-embryonated kernels (Fig. 8). These electrophoretic variants may only be tentatively indentified as amylase isozymes, as structural and genetic studies have not yet extablished their true identity as such. However, isozymes of α and β-amylases based upon genetic work have been identified in the endosperm of developing maize (9, 10, 22) and in maize seedlings (80).

Additions of cycloheximide or actinomycin D at the start of incubation restricted total amylase activity to a low, initial level and prevented the appearance of the full electrophoretic complement of starch degrading enzymes. Additions of the inhibitors at later times in incubation dramatically decreased activity from the level of the control and also reduced the number of electrophoretic variants (Fig. 12, Fig. 13, Fig. 14) after two days of incubation with the inhibitors. The results suggest

that development of amylase activity and the occurrence of starch degrading bands are related processes, partially dependent upon the continual synthesis of RNA and proteins. Similar results for the involvement of protein synthesis in amylase development were reported by Goldstein and Jennings (1975). They found a similar inhibition of amylase activity and the appearance of starch degrading bands during early incubation of de-embryonated maize kernels with cycloheximide (5 ug/ml) and actinomycin D (20 ug/ml).

Inhibitor additions at the start of the time course or later did not affect the appearance of the least anodic starch degrading zone in this study, characterized as having β-amylase activity (Fig. 14). Because this enzyme is present during early incubation and does not require continual RNA or protein synthesis, it would appear to exist preformed in the maize endosperm prior to incubation. The presence of β-amylase in the endosperm of the resting maize kernel has been reported previously (17).

The more anodic amylase electrophoretic variants which show a continual requirement for RNA and protein synthesis (Fig. 14), may be synthesized de novo in the maize endosperm. Turnover of these amylases seems apparent from the reduction in both activity (Fig. 12, Fig. 13), and the appearance of the more anodic starch degrading bands (Fig. 14) after two days of incubation with the inhibitors. These starch degrading zones appear later in the time course of incubation (Fig. 8). They have been shown to be predominantly α-amylase in character, as evinced by preliminary studies of physicochemical treatments on the crude extracts (Fig. 9, Fig. 11) and studies of physicochemical treatments and reaction

product analysis for these purified electrophoretic variants on day 5 of incubation (Part II, Fig. 3, Table 2).

Working with barley aleurone layers, Goodwin and Carr (1972) have shown a high degree of specificity for actinomycin D inhibition of GA_3 enhanced α-amylase development. Inhibition of GA_3 controlled α-amylase synthesis by cycloheximide and actinomycin D occurs in barley (11, 95), rice (70), and wheat (49). Consistent with the results obtained in this study, cycloheximide inhibition of α-amylase production, and prevention of substrate mobilization in the maize endosperm, suggested to Harvey and Oaks (1974b) that α-amylase was synthesized de novo in the maize endosperm.

Part II. Isolation and Characterization of Amylase Enzymes in Incubated, De-embryonated Maize Kernels.

Contrary to findings of other researchers (17, 36), it has been established that amylase activity arising from the incubated, de-embryonated kernel and accompanied by a series of starch degrading bands, does not require the presence of an embryo factor or additions of GA_3. Furthermore, at least part of this amylase activity from both GA_3 and non-GA_3 treated samples appears to be α-amylase in character. Implication for possible de novo synthesis of part of this activity comes from the sequential appearance of amylase electrophoretic variants over the time course of incubation. The later appearing variants display α-amylase characteristics upon physicochemical treatments and require continual RNA and protein synthesis for their expression.

The amylase activities arising during early and midcourse incubation were further investigated. The respective activties were purified, fractionated, and characterized as to type of activity.

A. Criteria for the Validity of Separated Activities.

As artifacts arising during enzyme purification are known to occur (32), several steps were taken to avoid this problem or the alteration of enzyme properties. Experimentation with two protein precipitants, cold acetone and ammonium sulfate, indicated that the latter was less harmful to the amylases. Ammonium sulfate at 75% saturation yielded the highest level of total amylase activity in the purified precipitate (results not presented). As some plant enzymes are known to be short-lived (39), all purification procedures were carried out at 0 to 4°C,

usually within one week, to maintain activity of the enzyme.

Proteases known to be present in the maize endosperm (33, 66), may modify the amylases. However, the crude extract assayed for protease activity according to Yomo and Varner (1972) yielded negative results. Invertase, which hydrolyzes sucrose, would yield inaccurate results for an amylase assay based upon reducing sugar power. No invertase activity was found in the crude extract, as assayed by the method of Jaynes and Nelson (1971). In addition, invertase has been shown to have little activity at the low temperatures used for the purification procedures (39).

Verification of the separated amylase activities as distinct and real entities was based upon several criteria. The enzyme banding patterns monitored by PAGE analysis at each step in the purification procedure were seen to be constant. Only fractions from a DEAE-cellulose peak with the same PAGE pattern were used for further study, i.e., fractions collected between peaks were discarded. Finally, each peak was rechromagraphed to insure its homogeneity. The procedures used for fractionation of amylase activities in this study were previously employed in another study with incubated, de-embryonated maize kernels of the Seneca Chief variety (27). Goldstein (1974) obtained excellent resolution of the amylase complement into three separate activities on DEAE-cellulose chromatography. Fractionation of the activities was verified using PAGE analysis and isoelectric focusing (27).

The ability of the purified enzyme preparations to satisfy these criteria and the excellent stability of the extracts over the time period of purification is good evidence that artifacts or changes in enzyme properties were not introduced during purification.

B. Activity After 5 Days of Incubation.

To investigate the nature of amylase activity occurring in midcourse incubation (5 days), the amylase complement was purified according to Materials and Methods (sec. 8, 9). The fifth day of incubation was chosen as the kernels exhibited an amylase activity level conducive to purification, and the complete complement of starch degrading zones was present at this point (Part I, Fig. 1, Fig. 2). Amylase activity obtained from day 5 extracts was resolved into three separate activities on DEAE-cellulose chromatography (Fig. 1).

Peak I eluted at the lowest salt concentration (0.17 M NaCl). PAGE analysis indicated that the activity in this peak was solely due to one slow moving band (Fig. 2). The amylase exhibited classical β-amylase activity on physicochemical analysis (Table 2). It was afforded no protection by calcium ions at high temperatures and reaction with mercury ions completely inactivated the enzyme. However, additions of DTT, either before or after Hg^{+2} treatment showed that the thiol reagent would protect the major activity of the enzyme against effects of the heavy metal ion. Dialysis with EDTA did not dramatically decrease the enzyme activity, as is reported for α-amylase activity (88). Finally, maltose was the sole reaction product for the amylase during early hydrolysis (Fig. 3). A β-amylase with characteristics similar to the above has been isolated from incubated, de-embryonated maize kernel (27).

Peaks II and III eluted at 0.42 M and 0.65 M NaCl concentration, respectively (Fig. 1). On PAGE analysis Peak II contained one band of medium mobility, and Peak III consisted of two faster migrating bands (Fig. 2). The electrophoretic variants corresponded with the starch

degrading zones appearing in the crude extract at later times in the incubation period (Part I, Fig. 2).

Both peaks exhibited α-amylase characteristics (Table 2). Physico-chemical analysis showed a marked dependence upon added calcium ions for thermostability. Failure to retain all activity of the control sample on heat treatment in the presence of calcium ions may indicate that irreversible denaturation of the enzyme was beginning to occur at the high temperature after 12 minutes of incubation, resulting in rupture of hydrogen bonds and unfolding of the enzyme molecule (51).

Surprisingly deleterious effects on activity were noted on addition of Hg^{+2} (10^{-5} M) with or without DTT treatments (Table 2). It would appear that in this case the heavy metal ion had a non-specific effect on amylase activity, with the ability to reversibly inactivate the β-amylase from Peak I, and irreversibly inactivate α-amylase activities from Peaks II and III. An α-amylase sensitive to Hg^{+2} has been reported in purified extracts from incubated, de-embryonated, maize kernels (27), and from the broad bean (31).

Alternatively, α-amylase activities isolated from Peaks II and III may possess properties intermediate between those for classical α and β-amylases. Such has been found to be the case for two GA_3 enhanced α-amylases from the barley aleurone, which are stable at pH 3.7, inactivated by Hg^{+2}, but not inactivated by EDTA dialysis (37). The authors suggest that the calcium ion is bound very tightly in these amylases (37). In germinating rye seed, two enzymes classified as modified α-amylases were only partially inactivated at pH 3.3, were resistant to EDTA dialysis, and exhibited the thermolability of β-amylases (97). Reaction product

analysis, however, showed a typical α-amylase activity (97).

Activities from Peaks II and III were both effectively inactivated by dialysis against EDTA (Table 2). Reaction product patterns showed glucose and oligosaccharides of varying size, characteristic of α-amylase hydrolysis (88) (Fig. 3).

C. Relationship of Amylase Activities After 1 and 5 Days of Incubation.

Analysis of DEAE Peak I activity from 5 day incubated kernels strongly suggests that this activity is the same as that extracted from 1 day incubated kernels. In both cases PAGE analysis indicated the presence of one slow moving starch degrading zone (Part I, Fig. 2, Part II, Fig. 2). Physicochemical analysis of both activities were similar and indicated classical β-amylase activity (Part I, Fig. 9, Fig. 11, Part II, Table 2). Furthermore, all other activity isolated from 5 day incubated kernels demonstrated α-amylase characteristics (Table 2).

Data previously discussed for the enzyme appearing in early incubation support the fact that it existed preformed in the endosperm or aleurone layers of the resting kernel prior to germination (Part I, Table 4). Numerous researchers have cited the presence of β-amylase in the ungerminated cereal grain, which is present in both active and zymogen insoluble forms (16, 75, 76, 82, 83). To verify whether this was true for maize, de-embryonated kernels were assayed for bound β-amylase. After one day on incubation, the activity liberated from the kernels by cysteine activated papain was at least as great as the activity in the soluble form, determined by routine preparation of the enzyme extract (Materials and Methods, sec. 4b) (Part I, Table 4). This latent activity also showed β-amylase characteristics (Part I, Table 5). PAGE analysis of the latent

activity indicated again the presence of one slow moving band, similar to that found for the soluble β-amylase counterpart.

D. Amylase Activity After 1 Day of Incubation.

The correlation between the β-amylase activity appearing early in incubation and β-amylase activity in Peak I of the day 5 preparation was further investigated. A day 1 enzyme extract was prepared and purifiied under the same conditions as that for the day 5 preparation.

The day 1 DEAE-cellulose chromatographic profile resulted in one peak, (Peak I) (Fig. 4). As in Peak I from the day 5 preparation, the activity from Peak I from the day 1 preparation contained one slow moving band (Fig. 2, Fig. 5). In addition, the activities from Peak I of both preparations were found to be eluted over the same range of salt concentration (0.11 M to 0.17 M NaCl) (Fig. 1, Fig. 4). Physicochemical analysis and reaction product patterns for activity from Peak I of the day 1 preparation were similar to those for activity from Peak I of the day 5 preparation, again indication β-amylase activity (Table 4, Fig. 8. Table 2, Fig. 3).

Similarity of elution profile, activity characteristics, and PAGE analysis for Peak I for both times of incubation would indicate that both peaks contain the same β-amylase activity: an enzyme which is present in both soluble and latent forms during early incubation. The electrophoretic pattern for activities from the two peaks were similar to that for a commercial β-amylase preparation (Fig. 5).

E. Characterization of Activity After 1 Day of Incubation.

The amylase activity from the purified day 1 preparation was further

characterized. Temperature optimum for the enzyme was 45°C under the conditions tested (Fig. 9). Greater than 50% of the optimum activity was present in the temperature range of 30 to 55°C. Activity only gradually diminished at temperatures greater than 45°C. Temperature optima for amylases have been reported to be in the range of 50 to 65°C, in the proximity of the results obtained in this study.

Caution must be used, however, in placing too much emphasis on temperature optima, as the data could also reflect effects due to denaturation of the enzyme (88). In addition, the differential temperature dependence of the several different steps present in any enzyme mechanism must be taken into account. Duration of the assay (20 min.) may have an effect. Finally, the affinity of the enzyme for its substrate (soluble starch) may change with temperature, or the enzyme may be afforded thermal protection by the substrate (88). The latter case seems unlikely at a higher temperature (70°C), however, as the enzyme activity from Peak I of the day 1 preparation was reduced to less than 10% of its initial level in three minutes (Fig. 7).

An Energy of Activation (E_a) of 14.6 Kcal/mole for the temperature range of 20 to 30°C, decreasing to 8.3 Kcal/mole at 30 to 40 °C, was determined from an Arrhenius plot of amylase activity (Fig. 10). Activation energies for amylases are usually about 14 Kcal/mole at 10°C, decreasing to a few Kcal/mole at 50°C (88). Although a decrease in E_a was apparent with increasing temperature, the E_a for the β-amylase in this study is somewhat high for the temperature ranges indicated. The higher E_a may be an inherent property for this particular amylase. Also, a higher E_a may reflect less dissociation of the enzyme-substrate complex or a slower

denaturation of the enzyme at the higher temperatures of the assay. Data for the Arrhenius plot is taken from data for the temperature optimum study (Fig. 9). At 50°C the enzyme exhibited almost maxiumum activity, an indication that little denaturation occurred at temperatures slightly higher than the temperature optimum. Finally, the E_a calculated may reflect the experimental conditions, dependent upon the substrate used, or the conditions of the assay.

The pH optimum for the β-amylase was pH 4.8 (Fig. 11). Greenwood and Milne (1968) report pH optima for β-amylases to be between pH 4.0 and pH 6.0. Using a soluble starch substrate, Trachuk and Tipples (1966) found maximum activity for 3 β-amylases to occur at pH 5.4, pH 4.6, and pH 5.2 to 6.2.

The optimum pH for α-amylases (pH 5.0 to pH 6.0) is usually higher than that for β-amylases (32, 86). In addition, α-amylase is usually denatured at a low pH (pH 3.5) (17, 25, 87, 97). In this study, the amylase exhibited a broad pH activity profile, with greater than 50% of the optimum activity in the range of pH 3.4 to 6.6; a characteristic of β-amylase activity (25).

The K_m of 3.5×10^{-3} grams of soluble starch/ml (Fig. 12) agrees favorable with values of 2.0×10^{-4} to 5.0×10^{-3} grams soluble starch/ml reported for amylases (27, 32, 86). Goldstein (1974) reported a K_m of 5.0×10^{-3} grams of soluble starch/ml for a β-amylase isolated from incubated, de-embryonated maize kernels. The K_m values for β-amylases are usually higher than those for α-amylases (51). Rye α-amylase has a K_m of 2.78×10^{-3} grams soluble starch/ml (51), while the K_m for an α-amylase isolated from germinating peas is 2.0×10^{-4} grams soluble starch/ml (86).

A low K_m indicates a high enzyme affinity for the substrate in question. Alpha-amylase thus has a higher affinity toward soluble starch than β-amylase (51). One explanation for the differences in K_m values for the two types of amylases would be their differences in mechanisms of action on the starch molecule. As an endoamylase, α-amylase randomnly attacks internal and external α-1,4 linkages. Beta-amylase, an exoamylase, is capable of attacking polysaccharides only from the outer chains. Thus, the frequency of attachment of α-amylase to the polysaccharide is much greater than that for β-amylase.

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SUMMARY AND CONCLUSIONS

Based upon the results of this study, the following observations can be made concerning amylase activity in the maize kernel:

Part I. Occurrence and Development of Amylase Activity in Maize Kernels.

1. Amylase activity exists in the embryo and endosperm of the developing maize kernel, 24 days after pollination; in the embryo and endosperm of the germinating kernel; and in the incubated, de-embryonated kernel.

2. Incubated, de-embryonated maize kernels and the endosperm from developing and germinating kernels contain both α and β-amylase activities.

3. Amylase activity in the endosperm of the developing kernel is greater than that found in the endosperm of mature kernels during early incubation with or without the intact embryo: an indication that a reduction in amylase activity during maturation of the maize grain has taken place.

4. Upon incubation, total amylase activity increases in both the endosperms from germinating and de-embryonated kernels. PAGE analysis indicates the sequential appearance of four electrophoretic zones with starch degrading activity on development of amylase activity in both samples, pointing to a mutual relationship between the two phenomena.

5. The development of amylase activity in incubated, de-embryonated kernels is not dependent upon the presence of an embryo factor or exogenous GA_3.

6. The development of amylase activity in the incubated, de-embryonated maize kernel is markedly dependent upon the relative humidity of the kernel environment prior to incubation.

7. No amylase activity in the de-embryonated kernel is realized as a function of incubation when the aleurone and pericarp layers are removed prior to incubation.

8. Amylase activity in the incubated, de-embryonated kernel is present in both soluble and latent forms during early incubation (1 day). The latent form exhibits β-amylase characteristics.

9. Addition of GA_3 to de-embryonated kernels has a quantitative effect during early incubation, as evidenced by the higher initial level of amylase activity and the earlier appearance of starch degrading zones upon PAGE analysis

10. In de-embryonated kernels during incubation, amylase activity reaches its maximum enhanced level with the addition of 1 uM GA_3, paralleling the rise of activity from the endosperm of germinating kernels.

11. Early and late additions of actinomycin D or cycloheximide during the time course incubation of de-embryonated kernels do not effect the appearance of the slow moving starch degrading zone present during early incubation and characterized as a β-amylase.

12. Early and late additions of actinomycin D or cycloheximide during the time course incubation of de-embryonated kernels prevented the appearance of starch degrading zones with α-amylase activity occurring

later in the time course and reduced the total amylase activity. This inhibitor action suggests that RNA and protein synthesis are necessary for the full expression of amylase activity in the de-embryonated kernel.

Part II. Isolation and Characterization of Amylase Enzymes in Incubated, De-embryonated Maize Kernels.

1. The purified amylase activity from de-embryonated kernels after 5 days of incubation was resolved into three separate activities on ion-exchange chromatography with a linear salt gradient. Reaction product patterns and physicochemical analysis indicated that Peak I (0.17 M NaCl) has classical β-amylase activity. Peaks II and (0.42 M NaCl) and Peak III (0.64 M NaCl) contained α-amylase activity.

2. The resolution of purified amylase activity from de-embryonated kernels during early incubation (1 day) showed one peak (Peak I) eluting at 0.13 M NaCl. Activity from Peak I was characterized as β-amylase. Similarity of physicochemical analysis, reaction product patterns, and DEAE-cellulose elution profile for Peak I activities of early and mid-course incubation preparations suggest that the same amylase is present in both peaks. The amylase activity in both peaks migrates as a single starch degrading band on PAGE analysis.

3. Physicochemical analysis indicated that the β-amylase activity from Peak I of the day 1 preparation is afforded no protection against thermal inactivation by the additions of calcium ions. Mercury ions completely inactivate the enzyme, although the major activity is restored upon treatment with DTT either before or after treatment with the heavy metal

ion. Extended dialysis against EDTA does not inactivate the enzyme. The sole initial reaction product for Peak I was maltose.

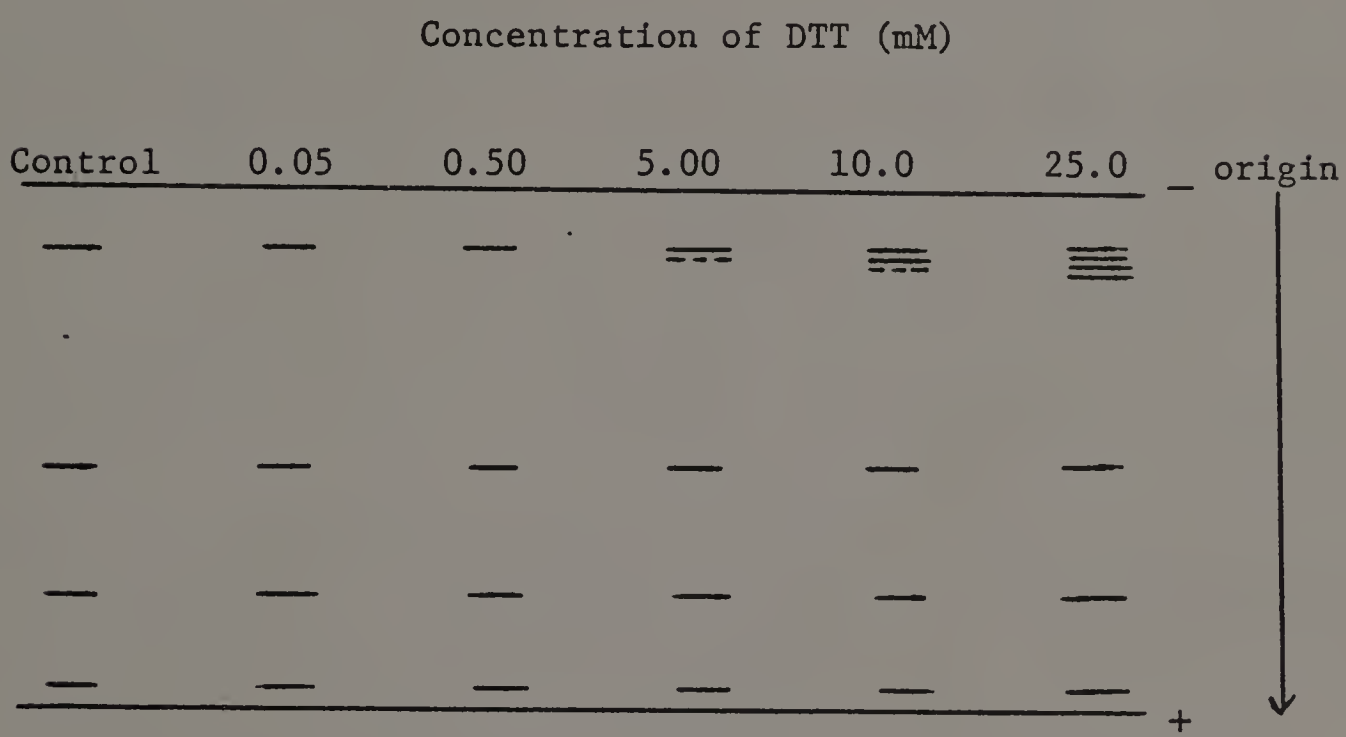
4. Characterization of Peak I activity from the day 1 preparation is as follows:

pH optimum	pH 4.8
pH range*	pH 3.4 to pH 6.6
temperature optimum	45°C
temperature range*	30°C to 55°C
Energy of Activation	14.6 Kcal/mole (at 20 to 30°C)
K_m	3.5×10^{-3} grams soluble starch/ml

*greater than 50% of the optimum activity

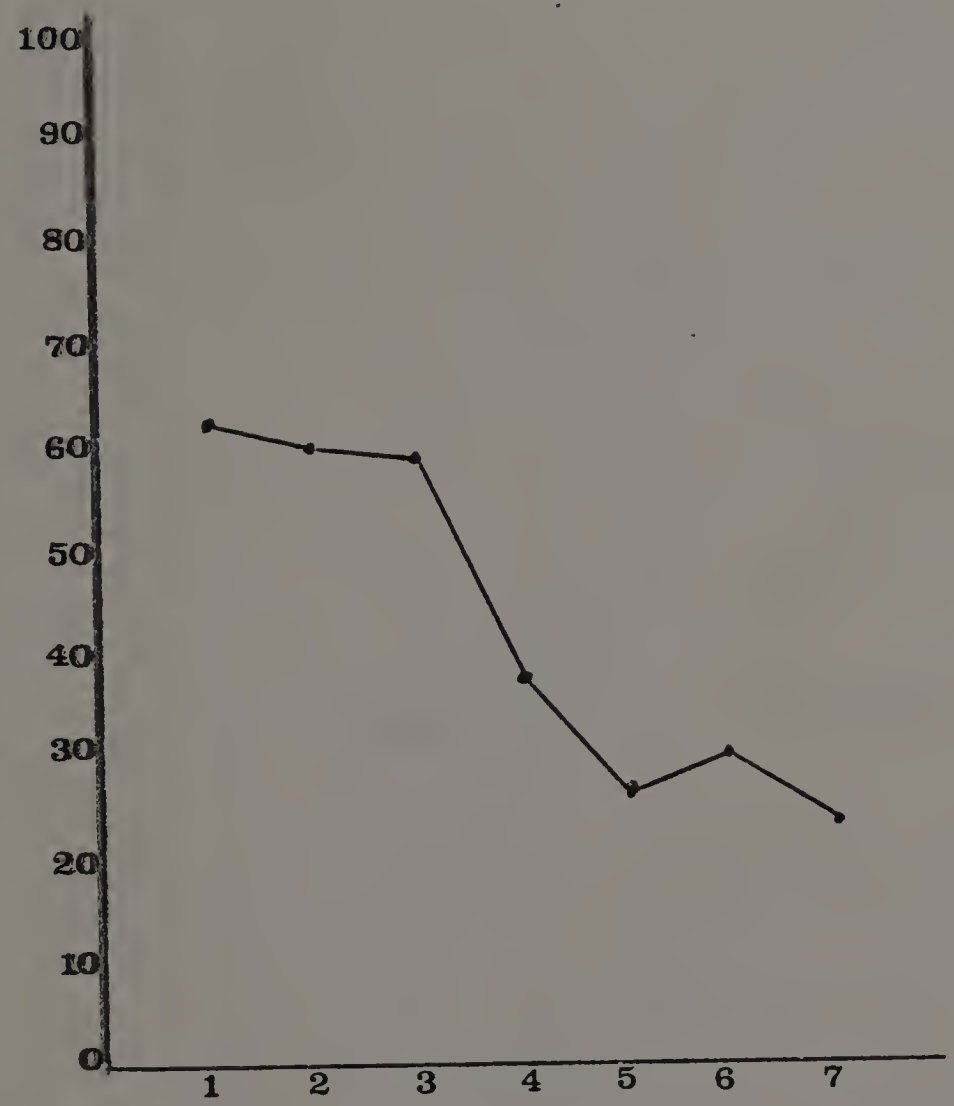
APPENDIX

PAGE zymograms of starch degrading bands from amylase activity treated with concentrations of dithiothreitol. Amylase activity from de-embryonated kernels after 7 days of incubation at 30°C.



Procedure- Aliquots of crude extract from incubated, de-embryonated maize kernels after 7 days of incubation treated with final concentrations of DTT from 0.05 mM to 25.0 mM. The enzyme extracts were incubated with the DTT for 30 min. at 0 to 4°C prior to PAGE analysis.

The effect of low pH (pH 3.5) assay on the time course development of amylase activity from de-embryonated maize kernels incubated at 30°C.



Procedure- Crude extracts prepared during the time course development of amylase activity in incubated, de-embryonated maize kernels. Control assayed using the standard reaction mixture with 0.05 M acetate buffer, pH 5.0 (Materials and Methods, sec. 5). Low pH samples assayed using standard reaction mixture with 0.025 M citrate-phosphate buffer, pH 3.5.

Extraction of soluble and latent amylase activity in de-embryonated maize kernels after 7 days of incubation at 30°C.

Treatment	Amylase Activity mg maltose/kernel/hr
1. extraction of soluble amylase	
crude extract	40.1
1st pellet wash with 0.05 M Tris-HCl buffer, pH 7.6	39.7
2nd wash	15.2
3rd wash	3.91
4th wash	0.00

2. extraction of latent amylase	Control	Cysteine-papain
hrs of incubation (30°C)		
0.0	0.00	0.00
1.0	8.10	31.2
2.0	7.65	28.4

Procedure- A crude extract was prepared from de-embryonated maize kernels after 7 days of incubation (Materials and Methods, sec. 4b). The resulting pellet was washed 4 times with 10 ml of 0.05 M Tris-HCl buffer, pH 7.6. The control pellet was resuspended with 10 ml of 0.025 M citrate-phosphate buffer, pH 5.4. Cysteine-papain pellet was resuspended in 10 ml of the citrate-phosphate buffer, containing 0.1 % cysteine and 0.1 % papain. Pellets were incubated for 1 and 2 hr at 30°C with frequent stirring. The latent amylase activity was determined using standard assay (M + M, sec.5).

The effects of successive extraction procedures on latent amylase activity from de-embryonated maize kernels after 5 days of incubation.

Treatment	Amylase Activity mg maltose/kernel/hr
1. extraction of soluble amylase	
crude extract	23.0
1st pellet wash with 0.05 M Tris-HCl buffer, pH 7.6	15.4
2nd wash	9.20
3rd wash	4.24
4th wash	0.00
2. extraction of latent amylase	
pellet treatment I	successive treatments
0.5% deoxycholate	4.92
0.2 M NaCl	4.62
0.6 M NaCl	5.21
1.0 M urea	10.9
0.1% papain + 0.1% cysteine (1 hr)	11.4
pellet treatment II	successive treatments
1.0 M urea	10.3
1.0 M urea	9.31
0.1% papain + 0.1% cysteine (1 hr)	16.7

Procedure- A crude extract was prepared from de-embryonated kernels after 5 days of incubation at 30°C (M + M, sec. 4b). The resulting pellets were washed 4 times with 10 ml of 0.05 M Tris-HCl buffer, pH 7.6. The pellets were subjected to successive treatments with extracting reagents of increasing strengths (10 ml/pellet) for 20 min. with constant stirring in an ice bath. Final extraction was effected by incubating the pellet with 0.1% papain and 0.1% cysteine in 10 ml of 0.025 M citrate-phosphate buffer, pH 5.4 for 1 hr at 30°C. Latent amylase activity was collected in the supernatant after centrifuging at 20,000 g for 15 min. and was determined using standard assay (M + M, sec. 5).

Extraction of soluble and latent amylase activity from the endosperm of developing maize kernels 24 days after pollination.

Treatment	Amylase Activity mg maltose/kernel/hr
1. extraction of soluble amylase	
crude extract	3.82
1st pellet wash with 0.05 M Tris-HCl buffer, pH 7.6	2.20
2nd wash	0.84
3rd wash	0.21
4th wash	0.05

2. extraction of latent amylase	Control	Cysteine-papain
hrs of incubation		
0.5	0.02	0.11
1.5		0.00

Procedure- A crude extract was prepared from de-embryonated, developing maize kernels 24 days after pollination (M + M, sec. 4a). The resulting pellet was washed 4 times with 0.05 M Tris-HCl buffer, pH 7.6 (20 ml/wash). The control pellet was resuspended with 10 ml of 0.025 M citrate-phosphate buffer, pH 5.4. Cysteine-papain pellet was resuspended in 10 ml of the citrate-phosphate buffer containing 0.1% cysteine and 0.1% papain. The pellets were incubated for 0.5 and 1.5 hr at 30°C with frequent stirring. The latent amylase activity was collected in the supernatant after centrifuging at 20,000 g for 15 min., and was determined using the standard assay (M + M, sec. 5).

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