ABSTRACT

BIOCHEMICAL GENETICS AND DEVELOPMENTAL STUDIES OF STARCH-DEGRADING ENZYMES IN MAIZE

Eddie Su-En Chao

Maize starch-degrading enzymes were studied because of their significance in seed germination and the fact that there are electrophoretic variants. The multiple molecular forms of the starch-degrading enzymes in immature kernels were separated into three zones by horizontal polyacrylamide gel electrophoresis; one phosphorylase zone and two amylase zones. One electrophoretic variant in each one of these three zones was found among fourteen strains investigated.

Genetic studies of the two amylase isozymes, one in each amylase zone, showed that they are independently controlled by two distinct loci *Amy-1* and *Amy-2*, both with codominant dialleles. The product of *Amy-1* is regulated by temporal and spatial mechanism(s) which suggest differential expression of this gene product in the course of development. *Amy-2* is linked with *Ct* (catalase), but unlinked with *AcP* (acid phosphatase). Genedosage effects were apparent with *Amy-1* but not observed with *Amy-2*.

Amy-1 amylase was purified and identified as an alpha-amylase by several criteria. The molecular weight of Amy-1 amylase was estimated by gel-filtration and sucrose gradient centrifugation to be 12,000. The two allelic forms of Amy-1 were found identical in molecular weight and antigenic specificity, but differ in their isoelectric points by 0.4 pH units. Amy-2 amylase, though not purified, was shown to be analogous to Amy-1 amylase in degrading beta-limit-dextrins and possesses a molecular weight of approximately 14,000.

Tissue distribution of amylase activity in germinating seedlings of three maize strains were studied both quantitatively and zymographically. The peak of amylase activity reached at 8-days of germination and coincided with the timing of the appearance of new amylase bands and the strength of Amy-1 amylase. The tissue origins and the possible protein-protein interactions of the amylases are discussed.

BIOCHEMICAL GENETICS AND DEVELOPMENTAL STUDIES OF STARCH-DEGRADING ENZYMES IN MAIZE

By

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DEDICATION

To my wife, Sheila and my

daughter, Grace

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I wish to express my sincere appreciation to Dr. John G. Scandalios for his guidance, encouragement, and constructive criticisms during the course of these investigations. I am grateful for many invaluable discussions with Dr. Joseph E. Varner concerning the chemical characterization of amylases. My sincere thanks to Dr. Elmer C. Rossman and Dr. Anton Lang for their enthusiastic support throughout this research project.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

An isozyme is defined as an enzyme existing in multiple molecular forms having identical or similar catalytic activities, and occuring within the same organism (Markert, 1968; Scandalios, 1969). In practice, these isozymes are discerned from each other on the basis of their molecular sizes, charges, and conformations as differentiated by means of electrophoresis, immunochemistry, chromatography, and solubility. This molecular diversity of an enzyme is generally found in nearly all organisms and can be classified into the following categories on the basis of their origin: (1) allelic isozymes, (2) allelic hybrid isozymes, (3) conjugated isozymes, and (4) conformational isozymes. Of these, allelic and hybrid isozymes due to allelic or non-allelic interactions can be considered as the primary products of their encoding genes. Conjugated and conformational isozymes are considered as secondary gene-products which are derived from one common portein moiety differing either in their tertiary and quarternary structures or their conjugated groups which can be carbohydrates, lipids, or non-enzymic proteins. Such alterations of a protein *in vivo* can be either genetic or epigenetic as artifacts *in vitro* are also

possible. Indeed, artifacts often cannot be readily distinguished from the results of epigenetic control in vivo. However, artifacts can be ruled out if the mode of inheritance of variant isozymes is demonstrated.

The significance of isozymes does not lie in the multiplicity of an enzymes per se, but their role in cellullar physiology and their adaptation in the organisms that carry the isozymes along the course of development. To study the significance of an isozyme system, it is essential to know which isozyme is a primary gene-product. An isozyme, once genetically defined, can be a useful intracellular marker, whose activity reflects the activity of its encoding gene. By tracing this marker during the development of the organism carrying it one can relate its cellular content, catalytic activity, and cellular location to its functional role at a given developmental stage of the organism.

There are several advantages for having chosen maize as the experimental material in these investigations. Maize has a relatively short life cycle well established genetic information, and is amenable to conditions for controlled pollination. Of particular interest is the fact that maize offers a system with monoploid tissue (pollen), diploid tissues, and triploid (endosperm); with which one can study gene dosage effects on their enzymes and the possible interactions between allelic and non-allelic isozymes throughout the life cycle of maize.

Seed germination is characterized by dramatic changes in the activity of enzymes, e.g. starch-degrading enzymes, several glycolytic cycle enzymes, proteases, ribonuclease increase (cf. Filner et al., 1969). The key enzymes in degrading starch are alpha-glucan phosphorylase (alpha-1, 4-glucan: orthophosphate glucosyltransferase, E.C. 2.4.1.1.), alpha-amylase (alpha-1,4-glucan-4-glucanohydrolase, E.C. 3.2.1.1), and beta-amylase (alpha-1,4-glucan maltohydrolase, E.C. 3.2.1.2). The degradation of seed starch during germination has two pathways (Pazur, 1965): (1) Phosphorylase degrades starch into dextrins and glucose-1-phosphate. The latter, mediated by UDP pyrophosphorylase, reacts with UTP to form UDP-glucose which is then condensed with fructose 6-phosphate by UDP-glucose-fructose glucosyl transferase to form sucrose. (2) Alpha-amylase takes starch to glucose, maltose, and maltodextrins which beta-amylase converts to maltose and dextrins. Branched dextrins (alpha-(1,4), (1,6)-glucans) are further debranched by the so-called R-enzyme and digested by amylases. Maltose from amylolytic degradation of starch is converted to glucose by maltase. Thus, the net result of starch degradation in germinating seeds is the formation of sucrose and glucose. Glucose is utilized locally as an energy source for growth. Sucrose is mobilized from starch storage tissues to non-starch storage tissues where it is converted by invertase to

fructose and glucose to be consumed in the glycolytic cycle. (Koller et al., 1962).

This thesis is concerned with the elucidation of the following aspects of maize amylase:

(1) The mode of inheritance of electrophoretic variants of maize amylases.

(2) Developmental changes of maize amylases in developing kernels and germinating seedlings.

(3) Chemical characterization of maize amylases that are genetically defined.

CHAPTER II

GENERAL MATERIALS AND METHODS

The materials and methods described in this chapter were used throughout these studies. Specific experimental procedures and methods will be given in the subsequent chapters as necessary.

The Maize Strains

The following fourteen maize strains were obtained from Dr. E. C. Rossman, Crop and Soil Science Dept., Michigan State University: M14, W64A, 58-3-6, Oh51A, 38-11, 58-3-9, Hawaiian Sugar, 58-3-5, Golden Cross Bantam, In-2, MS 206, MS 215, CMD 5, and A 509. These strains are all adapted to mid-western states and have been used for breeding dent-corn varieties; some have been selfed for at least 7 generations, some for 20 generations. Maize strains were grown either at the MSU Crop Science Nursery, E. Lansing, Michigan in summers, or at Goulds, Florida during the winter months. Ears were harvested generally 16-20 days after controlled pollination was made. Fresh ears were frozen as soon as possible in -50 C deep freezers (Virtis) or within 3 days if they were harvested in Florida. For developmental studies, adult plants were grown on vermiculite-gravel-sand

mixtures in the green house with 8 hours of darkness and 16 hours of light (supplemented with Gro-Lux lamps after sunset). Temperature in the green-house ranged from 80-60 F. Liquid endosperm of immature kernels was used for the initial screening of electrophoretic variants of several isozyme systems.

Acrylamide Gel Electrophoresis

There have been a number of established gel-electrophoresis procedures for separating amylase isozymes prior to this study. Agar gel-electrophoresis has been successfully used for separating amylase isozymes in the housefly (Ogita, 1968), barley (Frydenberg and Nielsen, 1965; Jacobsen et al., 1970), mouse (Sick and Nielsen, 1964), and cultured tobacco crown gall tissues (Jaspars and Veldstra, 1965). The above authors all used essentially the same method except Jacobsen et al. (ibid) who employed, instead of microslides, a horizontal agar slab with a potassium phosphate buffer (0.004M, pH 7.3). This method has a distinct advantage in that it can analyze multiple samples on the same gel. This method was initially applied for the separation of maize starch degrading enzymes. However, after several attempts using varying concentrations of phosphate buffers (0.004M-0.2M) all at pH 7.3 the results were poor. The maize starch degrading enzymes either did not move out from the origin in short

runs (2 hours) or moved out somewhat toward the cathode in long runs (4 hours) with a considerable amount of diffusion. Attempts to reduce the enzyme diffusion by adding 0.1% hydrozymethyl cellulose (QP-15000, Union Carbide Corporation, New York) to the 1% Difco purified agar gel were not successful.

An alternative to agar gel is polyacrylamide gel. The gel matrix is inert in contrast to that of agar gel which contains carboxyl and sulfate groups. Drosphila amylases have been separated with good resolutions on disc polyacrylamide gel (Doane, 1967). The detection method however is tedious. It involves three steps: (1) a transfer of the amylase zymogram from a polyacrylamide gel to an agar gel; (2) further incubation of the agar gel in starch solution; and (3) staining the gel with iodide solution. This method is good for amylase from Drosophila and probably other biological sources with high specific amylase activity. However, it was desirable in this study to obtain an overall picture of all starch-degrading enzymes in maize. The resolution for the separation of maize starch degrading enzymes using Doane's method was apparently limited by step (2) mentioned above since the diffusion of enzymes from a polyacrylamide gel to an agar gel is dependent on both the enzyme concentration and its specific activity.

In view of the shortcomings in these existing gel-electrophoresis methods, a new technique suitable for separating maize starch-degrading enzymes was developed in this laboratory. Various combinations of gel density and buffer systems were tried initially. A satisfactory procedure was found and is described below.

To prepare a polyacrylamide gel, 15 gm of acrylamide (Cyanogum 41 gelling agent, Fisher Scientific Co.) was dissolved in 150 ml of a desired buffer. The acrylamide solution was filtered through a layer of Miracloth (Chicopee Mills, Inc., New York) to remove the foreign particles. To 150 ml of this acrylamide solution, 1.5 ml of 10% ammonium peroxydisulfate ($[NH_4]_2S_2O_8$, Matheson Coleman and Bell Co.) were added. The mixed solution was poured immediately into a plexiglass tray (20 x 20 x 0.3 cm). A glass plate with a size equal or larger than that of the gel tray was coated with 1% Siliclad solution (Adams and Clay, New York) and was placed over the gel tray. Hence, without oxygen the acrylamide solution polymerized quickly at room temperature and within 5 minutes formed a gel of uniform texture. The gel thus prepared was sealed between the gel tray and the glass plate. The gel was chilled at 5 C for 20 minutes before use to lower the gel temperature which was raised by exothermic reaction in the process of polymerization. Storage of a sealed gel for more than ten hours in the

refrigerator might cause a gradual dehydration of the gel, thus changing the gel density as well as the pore size.

A discontinuous buffer system was found to be desirable for the electrophoretic separation of maize starch-degrading enzymes. It consisted of two buffers; Tris-citrate pH 8.2 and LiOH-borate pH 9.0. The first buffer contains 4×10^{-2}M tris (hydroxymethyl) aminomethane and 8×10^{-4}M citric acid (anhydrous). The second buffer contained 6×10^{-2}M lithium hydroxide and 1.9×10^{-1}M boric acid (anhydrous). The gel buffer was a mixture of 9 parts of Tris-citrate buffer and 1 part of LiOH-borate buffer. The LiOH-borate buffer alone was used in the pair of electrode tanks.

Protein samples absorbed onto 8 x 3 mm heavy absorbent papers (Beckman paper wicks for electrophoresis) were inserted into a slit that was cut across the gel about 5 cm from, but parallel with, one edge of the gel tray. Electrophoresis was carried out under a constant voltage gradient of 2.5 v/cm for 20 hours which was best for resolving Amy-1, or 6 v/cm for 12 hours which gave the best resolution for Amy-2; both at 5 C. After electrophoresis, the gel was incubated in a starch solution (0.5% Lintner starch in 0.04M sodium phosphate buffer, pH7.0) at 25 C for 5 hours.

The gel was then rinsed in distilled water and immersed in 0.01 M KI-I_2 (13 gm KI and 6 gm I_2 per liter of water) solution for 20 minutes. Starch-degrading enzymes were localized wherever the starch in the gel matrix was degraded

to dextrins so that the iodine-dextrin staining appeared to be colorless or pink in contrast to the dark-blue background. This method is generally good for detecting alpha-amylases, beta-amylases, and phosphorylases. After staining, the gel was either photographed with a Polaroid MP-3 camera using 4 x 5 black and white film (Type-52, Polaroid), or preserved in a solution consisting of 5% trichloroacetic acid and 50% ethanol.

Starch Gel Electrophoresis

For acid phosphatase, catalase and alcohol dehydrogenase isozyme systems used as markers in linkage analyses, starch gel electrophoresis techniques were employed. The appropriate buffer systems and staining procedures were as described by Scandalios (1969).

Protein and Amylase Assays

Protein determination was done according to Lowry's method (Lowry et al., 1951). Amylase was assayed with the starch-iodine method according to Filner and Varner (1967). The arbitrary amylase unit was defined as the change of absorbance at 620 mμ caused by one ml of the enzyme in one minute.

CHAPTER III

ELECTROPHORETIC VARIANTS OF STARCH-DEGRADING ENZYMES IN MAIZE LIQUID ENDOSPERM

Three distinct anodic zones of starch-degrading enzyme activities were observed on acrylamide gels following electrophoresis (Fig. 1). The most anodic zone (called Zone-1) consisted of one colorless band and one pink band. Zone-2, the zone with an intermediate migration rate, was characterized by one major and three minor bands all pink, but the minor bands having lesser intensity as judged by starch-iodine color and appearing frequently upon prolonged storage of the kernels. Zone-3, the slowest or least anodal zone, consisted of either one or three very fine and distinct colorless bands. The bands in everyone of these three zones are arbitrarily designated with letters; in each case the most anodal band being classified as (a).

Genetic variants in each of these three zones were found among the 14 inbreds investigated. The representative isozyme patterns of the tested inbreds are shown in Fig. 1, and schematically shown in Fig. 2.

Zone-1 amylase consists of two bands; a fast clear band, 1a, and a pink slow band, 1b. Among 14 maize strains investigated at the immature kernel stage, only one strain,

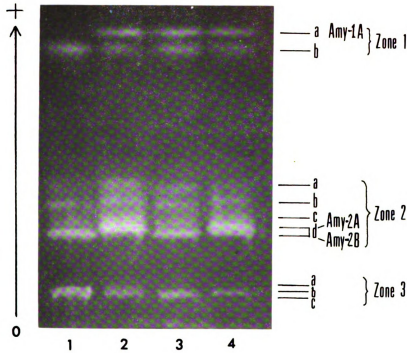


Fig. 1. Zymogram showing the three zones of starch-degrading enzymes of maize liquid endosperm. Numbers on horizontal plane indicate individual kernel samples. With respect to Amy-2, samples 1 and 3 = slow variant (Amy-2B); 2 = fast variant (Amy-2A), 4 = heterozygote. With respect to Amy-1, sampels 2, 3 and 4 = fast variant (Amy-1A); 1 = slow variant (Amy-1B). The activity of Amy-1B is very low in liquid endosperm of immature kernels and is not shown on this photograph. The broad band beneath Amy-1 with identical electrophoretic mobility, was found in immature kernels of all 14 strains investigated. For Zone-3, only the slowest anodal migrating band (c band in Table 1) is shown on this photograph. Details with respect to each zone are to be found in the text. 0 = point of sample insertion.

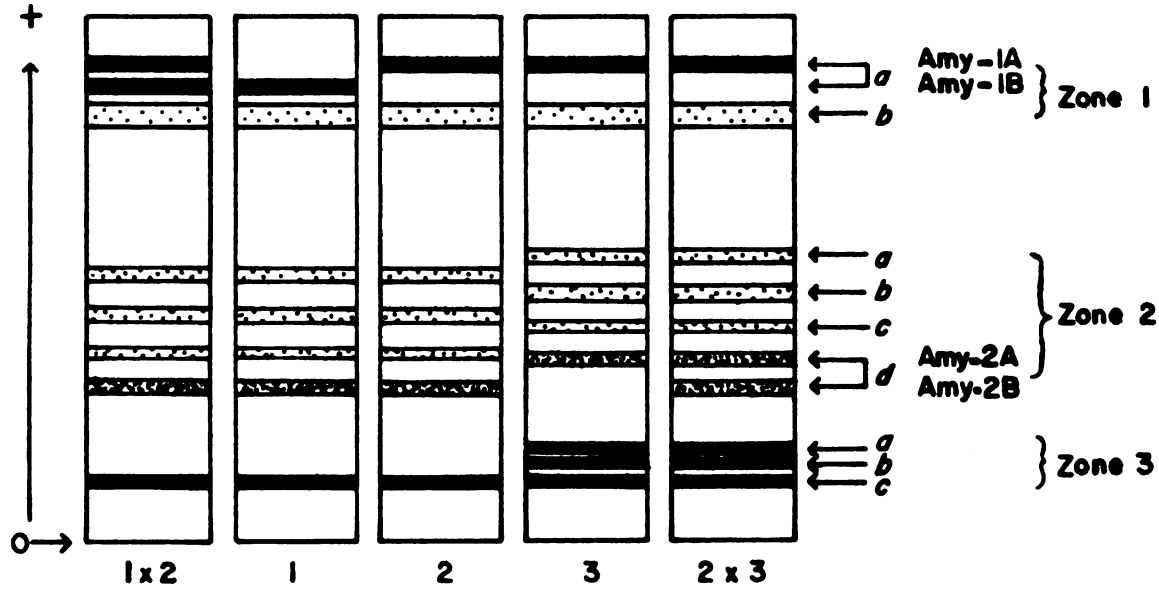


Fig. 2. Schematic drawing of the three starch degrading enzyme zones of maize endosperm. 1, 2, and 3 represent the three electrophoretic variants, 38-11, W64A, and 58-3-6, found among the fourteen inbreds tested. 2 x 3 shows the hybrid resulting from the cross between the inbreds 2 and 3. Similarly, 1 x 2 is the hybrid between the inbreds 1 and 2. There is no difference in the electrophoretic patterns of the hybrids resulting from the reciprocal crosses. The faint minor bands of Zone-2 are found frequently after prolonged storage of the kernels prior to electrophoresis. No electrophoretic variant of the faint band of Zone-1 from immature kernels were found among 14 inbreds investigated. 0 = point of sample insertion.

38-11, has been shown to possess an electrophoretically slow variant of band 1a at Zone-1. The electrophoretic mobility of band 1b at Zone-1 is consistent in all 14 strains. Band 1a is designated as Amy-1 with the fast and slow variants as Amy-1A and Amy-1B respectively.

Zone-2 amylase consists of one major band, 2d, and three minor bands, 2a, 2b, and 2c. Two out of 14 strains (58-3-6 and In-2) were found to possess an identical fast electrophoretic variant in band 2d. The appearance of 2a, 2b, and 2c bands seemed to be associated with band 2d whether in homozygotes or heterozygotes and appeared frequently upon prolonged storage of the kernels at - 50 C. These though having less activity than 2d are not artifacts, since none of these gave rise to others upon re-electrophoresis. The possibility of the bands 2a, 2b, and 2c being genetically unrelated to the major band 2d still exists but has not been explored in depth because of the lack of electrophoretic variants independent from the major band among the strains examined. The major band 2d, at Zone-2 is designated as Amy-2 with the fast and slow variants as Amy-2A and Amy-2B respectively.

The two electrophoretic variants in Zone-3 were identifiable by the fact that some inbreds had three clearly delineated bands in Zone-3; designated as 3a, 3b, and 3c, while other inbreds had only one distinct band in Zone-3 identical to 3c with respect to its electrophoretic mobility.

Table 1. The characteristic starch-degrading enzyme patterns in liquid endosperm tissues of 14 inbred strains of Zea mays.

	Enzymes separated by gel electrophoresis[1]		
Inbred[2]	Zone-1[3]	Zone-2[3]	Zone-3[4]
M14	Amy-1A	Amy-2B	a,b,c
W64A	Amy-1A	Amy-2B	c
58-3-6	Amy-1A	Amy-2A	a,b,c
Oh51A	Amy-1A	Amy-2B	a,b,c
38-11	Amy-1B	Amy-2B	c
58-3-9	Amy-1A	Amy-2B	a,b,c
Hawaiian Sugar	Amy-1A	Amy-2B	a,b,c
58-3-5	Amy-1A	Amy-2B	a,b,c
Golden Cross Bantam	Amy-1A	Amy-2B	a,b,c
In-2	Amy-1A	Amy-2A	a,b,c
MS 206	Amy-1A	Amy-2B	?
MS 215	Amy-1A	Amy-2B	?
CMD 5	Amy-1A	Amy-2B	?
A 509	Amy-1A	Amy-2B	?

[1] A = faster migration; B = slower migration.

[2] The enzyme patterns of the last four inbreds were deduced from their F_2 progeny with W64A, but information on Zone-3 cannot be obtained without knowing its mode of inheritance.

[3] Only the most anodal clear band in Zone-1 and the major band in Zone-2 are dealt with for reasons given in text.

[4] In Zone-3, bands are arbitrarily designated as a, b, and c in order of descending anodal mobility.

The intensity of these bands as judged by the extent of decreasing starch-iodine color is strong in the 3c band and decreases in the bands of increasing anodal mobility (3c > 3b > 3a). The characteristic isozyme pattern of each of the tested inbreds with respect to Amy-1 of Zone-1, Amy-2 of Zone-2, and Zone-3 is summarized in Table 1. The occurence of electrophoretic variants in each of these three zones appeared not to be associated with one another suggesting independent inheritance of Amy-1, Amy-2 and Zone-3.

CHAPTER IV

GENETICS OF THE TWO AMYLASES IN MAIZE

Introduction

Starch-degrading enzymes of maize liquid endosperm can be separated into three zones by means of horizontal acrylamide gel electrophoresis. Chemical identification of these enzymes (see Chapter VI) showed that Zone-3 is phosphorylase, Zone-1 and Zone-2 are amylases. The present studies dealt only with Amy-1 of Zone-1 and Amy-2 of Zone-2 for which electrophoretic variants were found as described in Chapter III.

Enzymes are gene-products, but genes are not necessarily active concomitantly with their products. The fact that enzyme activities could be events remotely reflecting gene action temporaly and spatially needs not be re-emphasized as it has been discussed extensively by Scandalios (1969); it is particularly true in the case of multiple forms of enzymes where isozymes could be directly encoded by more than one gene or indirectly produced by gene-gene interactions. The task of recognizing isozyme systems of unknown genetic origin is not feasible. Thus, it seemed prudent to me to elucidate the mode of inheritance of an isozyme system

before going into the developmental and physiological studies of the enzymes in question.

Since the genetics of several other isozyme systems in the inbred strains used in these studies are now known (Scandalios, personal communication), their genetic linkage relationship with *Amy-2* were investigated for two reasons: (1) If a close linkage does exist between *Amy-2* and some other isozyme system, it would be of future interest to examine the action of these two linked genes in various developmental stages. (2) The chance of assigning the *Amy-2* locus to one of the ten chromosomes may be increased through its linkage with other marker genes.

Literature Review

Electrophoretic variants of maize starch-degrading enzymes in 3 day-old seedlings were first reported by Scandalios (1966). However, detailed genetic analyses of these variants were not done.

The only genetic study of plant amylase other than maize is barley. Frydenberg and Nielsen (1965) found 9 bands of starch-degrading enzymes in 5-6 day old germinating barley seedling extracts (after removal of sprouts and roots) upon agar gel electrophoresis. The activity of five of these bands was Ca^{++} dependent and was not affected by either Hg^{++} or Cu^{++}. Upon heat treatment (70 C for 15 minutes), these five bands were reduced to 2 heat stable

bands. Among 61 two-row barley varieties investigated, varieties possessing one or two heat stable amylases were found to be of equal frequencies. The mode of inheritance of these two amylases was deduced from their breeding pedigree. New varieties resulting from crosses between varieties of one heat stable amylase and two heat stable amylase bands were shown to resemble either one of the two parental types. Frydenberg and Nielsen (ibid.) suggested that these two heat-stable amylases are alpha-amylases and are inherited in two-row barley varieties as a monogenic trait.

Materials and Methods

Three maize strains, W64A, 58-3-6, and 38-11 used in making the desired genetic crosses, were characterized for several other known isozyme systems in maize and are tabulated in Table 2. The genetic analysis of Amy-1 involves W64A and 38-11. The genetic analysis of Amy-2 involves W64A and 58-3-6.

The electrophoretic patterns of Amy-1 and Amy-2 in young kernels were found to be consistent from 7 - 30 days post-pollination; except that Amy-1B activity is low in immature kernels of 38-11, and sometimes even not detectable at all on zymograms. Hence, the liquid endosperm is not suitable for the genetic analysis of Amy-1. When maize seeds of any one of the three strains were germinated, Amy-1

Table 2. Genotypes of several enzyme systems in three maize strains used in genetic analyses for *Amy-1* and *Amy-2*.

Inbred	Known Genotypes of Amylases and Other Enzymes in the Inbreds Used					
	AcP	*Adh*^{1}	*Adh*^{2}	*Amy-1*	*Amy-2*	*Ct*
W64A	*AcP*^{A}	*Adh*^{1F}	*Adh*^{2F}	*Amy-1*^{A}	*Amy-2*^{B}	*Ct*^{S}
58-3-6	*AcP*^{B}	*Adh*^{1F}	*Adh*^{2F}	*Amy-1*^{A}	*Amy-2*^{A}	*Ct*^{F}
38-11	*AcP*^{A}	*Adh*^{1F}	*Adh*^{2F}	*Amy-1*^{B}	*Amy-2*^{B}	*Ct*^{S}

N.B. The isozyme systems indicated by the gene symbols and the references to their genetics are the following: *Acp*; Acid phosphatases (Scandalios and Chao, in preparation). *Adh*^{1} and *Adh*^{2}, Alochol dehydrogenases (Scandalios, 1967). *Ct*, Catalases (Scandalios, 1968).

activity was notably enhanced in the endosperm tissues with no change of its electrophoretic mobility. The Amy-1 band was also detected in all other tissues of the young seedlings namely; scutellum, leaf, stem and root. In the same tissue extracts, Amy-2 is no longer seen, but a new band of greater anodal mobility, similar in appearance to Amy-2, is observed. Whether this band is Amy-2 which has undergone a shift in mobility due to altered developmental conditions or is a new gene product, is not as yet resolved.

Genetic analysis of Amy-1 and Amy-2 requires the gene products in question to be stable in a developmental stage of maize with a considerable length of time so that the analysis of tissue extracts are amenable. Hence, 8-12 day-old seedlings were used for the genetic analysis of Amy-1, and liquid endosperm of 16-22 day old kernels were used for genetic analysis of Amy-2.

For linkage studies, the three inbreds W64A, 58-3-6, and 38-11 were initially checked for their genotypes of acid phosphatase *(AcP)* alcohol dehydrogenases *(Adh^1* and *Adh^2)*, catalase *(Ct)*. Since the three inbreds used in studying Amy-2 genetics are also genetic variants with respect to *Ct* and *AcP* (Table 2), the analysis was focused on their linkage relationship with *Amy-2*.

Crude tissue extracts were made by crushing the tissue from single seeds directly using a procelain spot-plate and a small procelain pestle in a few drops of Tris-HCl buffer

(0.05M, pH 7.5) and 1mM $CaCl_2$. The crude extracts were used for electrophoresis without centrifugation since no difference was observed with or without centrifugation of the tissue extracts prior to electrophoresis. Electrophoresis for Amy-2 resolution was conducted 12 hours at 5 C with a voltage gradient 6 v/cm; for the best resolution of Amy-1 the voltage gradient was 2.5 v/cm for 20 hours at 5 C.

Segregation analysis of each genetic cross was made on immature kernels or mature seeds originating from the same ears.

Results

Genetic Control of Amy-2 Amylase

Two strains of maize, W64A and 58-3-6, were used in the genetic analysis. Zone-2 amylase in strain W64A migrates slower than that in strain 58-3-6. The phenotypes of W64A and 58-3-6 as obtained from gel assays, are designated as B^2B^2 and A^2A^2, respectively. F_1 heterozygotes resulting from crosses between W64A and 58-3-6 have both parental bands in this zone. Eleven possible genetic crosses were made; self pollination of the two parents, the two reciprocal F_1s, the five backcrosses, and the two F_2s as shown in Table 3. A typical gel with $9F_2$ segregants is shown in Fig. 3. Altogether, 1,398 kernels were assayed. The five sets of backcrosses with permutations of the

Table 3. Results showing the mode of inheritance of Amy-2 amylase variants in maize liquid endosperm. X^2 tests were based on the hypothesis of monogenic inheritance.

Genetic cross		Amylase patterns in offspring				X^2	P
Female	Male	AA	AB	BB	Total		
A^2/A^2	A^2/A^2	42	0	0	42		
B^2/B^2	B^2/B^2	0	0	119	119		
A^2/A^2	B^2/B^2	1*	40	0	41		
B^2/B^2	A^2/A^2	0	61	0	61		
A^2/A^2	$(B^2/B^2 \times A^2/A^2)$	55	50	0	105	0.02	>0.80
A^2/A^2	$(A^2/A^2 \times B^2/B^2)$	59	61	0	120	0.03	>0.80
B^2/B^2	$(B^2/B^2 \times A^2/A^2)$	0	115	120	235	0.12	>0.70
$(A^2/A^2 \times B^2/B^2)$	A^2/A^2	48	46	0	94	0.04	>0.80
$(A^2/A^2 \times B^2/B^2)$	B^2/B^2	0	59	44	103	1.18	>0.20
$(A^2/A^2 \times B^2/B^2)$	$(A^2/A^2 \times B^2/B^2)$	62	112	50	224	1.29	>0.50
$(B^2/B^2 \times A^2/A^2)$	$(B^2/B^2 \times A^2/A^2)$	73	123	88	284	5.77	>0.05

*This unexpected phenotype in the F_1 is likely due to the result of accidental pollen contamination.

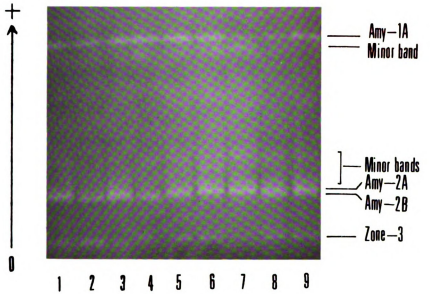


Fig. 3. Electrophoretic analysis of Amy-2 in individual kernels from a F_2 cross between W64A *(Amy-2^B/Amy-2^B)* and 58-3-6 *(Amy-2^A/Amy-2^A)*. 0 = point of sample insertion. Samples 1, 6, and 7 are A^2A^2; 2 and 4 are B^2B^2; 3, 5, 8, and 9 are A^2B^2.

parentage, all segregated in good statistical agreement with 1:1 ratios of either $A^2B^2:A^2A^2$ or $A^2B^2:B^2B^2$. Chi-square tests for fitness to single-gene Mendelian inheritance, made for each one of the crosses and presented in Table 3, do not deviate significantly from expectation. The results from the crosses involving the same genetic components are additive. For instance, the expected F_2 segregation ratio of 1 : 2 : 1 becomes more strikingly obvious when the observed data from the F_2 of (W64A x 58-3-6) and its reciprocal F_2 are pooled together. Thus, among a total of 508 F_2 kernels assayed, there were 135 A^2A^2, 235 A^2B^2, and 138 B^2B^2 and the segregation ratio has a Chi-square value, $X^2 = 2.11$; $P>0.30$.

No gene dosage effects were observed in the liquid endosperm of hybrids with either *Amy-2*^{A}/*Amy-2*^{A}/*Amy-2*^{B} or *Amy-2*^{B}/*Amy-2*^{B}/*Amy-2*^{A} genotype. This finding suggests that the endosperm tissue may not be the site for the synthesis of Amy-2 amylase.

Linkage Analysis of Amy-2 Amylase, Catalase, and Acid Phosphatase

I have attempted to examine the possibility of genetic linkage of Amy-2 amylase with several other genetically well defined isozyme systems in maize, namely, alcohol dehydrogenase (ADH), catalase (Ct), and acid phosphatase (AcP, for which two codominant alleles were found recently: Scandalios and Chao, in preparation). This part of the

investigation was undertaken for reasons that will be given in the discussion. The two strains, W64A and 58-3-6 were found to possess identical alleles with respect to ADH (Adh^1 and Adh^2), and thus rendering linkage tests for these two enzymes impossible in this material (Table 2). The two strains did, however, differ in catalase and acid-phosphatase. Individual kernels obtained from the backcrosses:

W64A X (W64A X 58-3-6) i.e., $\frac{Ct^S\ Amy\text{-}2^B\ AcP^A}{Ct^S\ Amy\text{-}2^B\ AcP^A} \times \frac{Ct^S\ Amy\text{-}2^B\ AcP^A}{Ct^F\ Amy\text{-}2^A\ AcP^B}$

and 58-3-6 X (58-3-6 X W64A) i.e., $\frac{Ct^F\ Amy\text{-}2^A\ AcP^B}{Ct^F\ Amy\text{-}2^A\ AcP^B} \times$

$\frac{Ct^F\ Amy\text{-}2^A\ AcP^B}{Ct^S\ Amy\text{-}2^B\ AcP^A}$ and the F_2 (W64A X 58-3-6) X (W64A X 58-3-6)

were employed to assay their zymogram phenotypes; amylase on acrylamide gel, catalase and acid phosphatase on starch gel. Thus, each kernel represented a segregant and was assayed for its genotype with respect to amylase Amy-2, catalase and acid phosphatase. If there was no linkage among the three loci, one would expect to find equal frequencies of parental and recombinant phenotypes in the progenies of both backcrosses and the F_2. Conversely, if any two of these three loci were linked on a chromosome within 50 map units, the resulting recombinant frequency, with respect

to the pair of loci in question, would be expected to be less than 0.50.

I have scored approximately 300 kernels in the F_2s and 100 in each backcross. The results are given in Table 4. For any two of the three loci considered, there are two possible recombinant types resulting from each of the two backcrosses. These recombinants are homozygous for one locus while heterozygous for the other. For instance, the two possible recombinants from the backcross, $\frac{Ct^S\ Amy\text{-}2^B}{Ct^S\ Amy\text{-}2^B} \times \frac{Ct^S\ Amy\text{-}2^B}{Ct^F\ Amy\text{-}2^A}$, are $\frac{Ct^S\ Amy\text{-}2^B}{Ct^F\ Amy\text{-}2^B}$ and $\frac{Ct^S\ Amy\text{-}2^B}{Ct^S\ Amy\text{-}2^A}$. These recombinants can be easily discerned from parental types which are either doubly homozygous $\frac{Ct^S\ Amy\text{-}2^B}{Ct^S\ Amy\text{-}2^B}$ or doubly heterozygous $\frac{Ct^F\ Amy\text{-}2^A}{Ct^S\ Amy\text{-}2^B}$. A similar relationship can be obtained when *Amy-2* and *AcP* are considered pairwise.

Unlike the backcrosses, the recombinants among the F_2 cannot be readily picked out by the gel assay. The segregating genotypes in the F_2 progeny are tabulated in Table 5. The non-crossover gametes are represented by the genetic constituents: $Amy\text{-}2^B$, Ct^S and $Amy\text{-}2^A$, Ct^F whereas the crossover gametes are $Amy\text{-}2^B$, Ct^F and $Amy\text{-}2^A$, Ct^S. For simplicity, the genotypes of the F_2 progeny are abbreviated

Table 4. Distribution of parental and recombinant segregants in progenies of genetic crosses concerning *Amy-2, Ct* and *AcP*.

Genetic Cross	Loci pair	No. kernels assayed	Parental type*	Recombinant type**	Estimated recombination freq***	± S.D.
W64A X (W64A X 6)	*Amy-2, Ct*	180	164	16	0.09	±0.02
	Amy-2, AcP	100	47	53	0.53	±0.05
	Ct, AcP	101	47	54	0.53	±0.05
6 X (6 X W64A)	*Amy-2, Ct*	118	112	6	0.05	±0.02
	Amy-2, AcP	120	55	65	0.54	±0.05
	Ct, AcP	119	60	59	0.50	±0.05
(W64A X 6) X (W64A X 6)	*Amy-2, Ct*	278	252	26	0.05	±0.01
	Amy-2, AcP	312	159	153	0.49	±0.03
	Ct, AcP	298	153	145	0.49	±0.03

*For F_2, as a whole, the fraction $p^2 + q^2$ (see Table 5) are scored and given in this column.

**For F_2, as a whole, the fraction 2pq (see Table 5) are scored and given in this column.

***The recombination frequencies of *Amy-2:AcP* and *Ct:AcP* do not significantly deviate from 0.50 as evident from the small values of their standard deviations.

Table 5. Progeny resulting from the selfing of (W64A X 6), whose genotype is *Amy-2*^{B}*Ct*^{S}/*Amy-2*^{A}*Ct*^{F}.

	Gametes	Non-crossover (freq.=p)				Crossover (freq.=q)			
		Amy-2^{B}*Ct*^{S}		*Amy-2*^{A}*Ct*^{F}		*Amy-2*^{B}*Ct*^{F}		*Amy-2*^{A}*Ct*^{S}	
Non-Crossover (freq. = p)	*Amy-2*^{B}*Ct*^{S}	B/B	S/S	A/B	F/S	B/B	F/S	A/B	S/S
	Amy-2^{A}*Ct*^{F}	A/B	F/S	A/A	F/F	A/B	F/F	A/A	F/S
Crossover (freq. = q)	*Amy-2*^{B}*Ct*^{F}	B/B	F/S	A/B	F/F	B/B	F/F	A/B	F/S
	Amy-2^{A}*Ct*^{S}	A/B	S/S	A/A	F/S	A/B	F/S	A/A	S/S

as (B/B, F/S) for ($Amy\text{-}2^{B}/Amy\text{-}2^{B}$, Ct^{F}/Ct^{S}), etc. Assuming that the frequencies of non-crossover and the crossover gametes are p and q respectively, and p + q = 1, then the recombinant frequency in the F_2 progeny is $q^2 + pq$. However, according to the gel assay, there is one genotypic class, $Amy\text{-}2^{A}/Amy\text{-}2^{B}$, Ct^{F}/Ct^{S}, common to both the p^2 fraction of parental types and the q^2 fraction of the recombinant type progeny. This overlapping class makes a direct estimation of recombinant frequency, $q^2 + pq$, impossible. To circumvent this problem, I scored for types of F_2 progeny that are homozygous for one locus while heterozygous for the other. The overall frequency of the four types scored equals 2pq. Hence, from the relationship, p + q = 1, it is possible to estimate the crossover frequency (q). Table 4 summarizes the pair relationships among the three markers, Amy-2, Ct and AcP. It appears that Amy-2 and Ct are linked with a recombinant frequency of about 5 map units. No linkage was detected between AcP and Amy-2 or Ct.

Genetic Control of Amy-1 Amylase

Amy-1 amylase was detected by gel assay in all developmental stages from seedling to mature seeds. The two electrophoretic variants, Amy-1A and Amy-1B are each represented by W64A (or 58-3-6) and 38-11 respectively. The phenotypes of individual plants carrying Amy-1A is

designated as A^1A^1 whereas the phenotype of those carrying Amy-1B is designated as B^1B^1.

There are some peculiarities to Amy-1 amylase obtained from the liquid endosperm of F_1 immature kernels. When liquid endosperm of individual heterozygote kernels was subjected to gel assay, the resulting Amy-1 patterns always resembled that of the maternal parent. Thus, $A^1A^1 \times B^1B^1$ gave rise to A^1A^1; $B^1B^1 \times A^1A^1$ gave rise to B^1B^1. This result cannot be explained by the relative abundance of each isozyme since pooled endosperm extract of 20 F_1 immature kernels also failed to show the paternal Amy-1 band on gels. Therefore, it is not a matter of Amy-1 concentration but perhaps the complete absence or the enzymatic inactivity of the paternal form of Amy-1 amylase in heterozygote kernels. This phenomenon is not unique to liquid endosperm of immature heterozygote kernels but it is also observed in endosperm and scutellar tissues of 2-8 day old F_1 seedlings of $A^1A^1 \times B^1B^1$. In contrast to this, both parental Amy-1A and Amy-1B are detectable in leaf tissue extracts of the same F_1 seedlings 8-22 days old. This interesting aspect was pursued further and will be presented later in the section of developmental studies of maize amylases.

The latent expression of *Amy-1*^{B} allele in A^1B^1 seedlings compounded part of the genetic analysis of Amy-1 initially when endosperm extracts (triploid tissue with

two maternal A^1 and one paternal B^1) of 8-12 day old individual seedlings of all crosses were used. To circumvent this problem, the later part of the genetic analysis was made with leaf extracts of 8-22 day-old seedlings. It was found that leaf extract is particularly suitable for Amy-1 gel-assays, since Amy-1 is the only amylase present in this tissue at all developmental stages of the adult plant. In contrast, endosperm extract of the same age seedlings has both the Amy-1 band and the broad pink band right beneath it; both are located in Zone-1.

Ten genetic crosses were analyzed: the two parental inbred strains, the two reciprocal F_1s, the four out of eight possible backcrosses, and the two reciprocal F_2s. A total of 857 seedlings were assayed individually for their Amy-1 patterns in the endosperm tissue. The results are shown in Table 6a. The heterozygotes produced only two parental bands, Amy-1A and Amy-1B, indicating the two Amy-1 forms do not hybridize to form an enzymatically active hybrid form of amylase with a predictable intermediate migration rate between the two parental bands. The intensity of the two reciprocal F_1 endosperm tissues were different. F_1 hybrids of $A^1A^1 \times B^1B^1$ with A^1A^1 as the maternal parent produced strong Amy-1A and weak Amy-1B. This hybrid pattern is denoted as A^1B^1. When B^1B^1 was the maternal parent in the reciprocal cross, the resulting hybrids produced both parental bands with equal intensities,

Table 6a. Results showing the mode of inheritance of Amy-1 amylase variants in the endosperm of 8-12 day-old maize seedlings. A^1B^1 and B^1A^1 heterozygotes were discernible in the triploid tissue.

Genetic cross		Amy-1 patterns in offspring					X^2	P
Female	Male	A^1A^1	A^1B^1	B^1A^1	B^1B^1	Total		
A^1A^1	A^1A^1	50	0	0	0	50		
B^1B^1	B^1B^1	0	0	0	50	50		
A^1A^1	B^1B^1	0	30	0	0	30		
B^1B^1	A^1A^1	0	0	30	0	30		
$(A^1A^1 \times B^1B^1)$	B^1B^1	0	34	0	32	66		
$(B^1B^1 \times A^1A^1)$	A^1A^1	49	0	56	0	105	0.46	>0.30
$(B^1B^1 \times A^1A^1)$	B^1B^1	0	43	0	44	87	0.02	>0.80
$(A^1A^1 \times B^1B^1)$	$(A^1A^1 \times B^1B^1)$							
		75	44	68	51	238	10.50	>0.01
		75	112		51	238	6.07	>0.02
$(B^1B^1 \times A^1A^1)$	$(B^1B^1 \times A^1A^1)$							
		64	31	55	51	201	11.66	>0.01
		64	86		51	201	5.90	>0.05

and this Amy-1 pattern is designated as B^1A^1. The A^1B^1 and B^1A^1 patterns were also distinct in the backcrosses, for instance A^1B^1 from $(A^1A^1 \times B^1B^1) \times B^1B^1$ and B^1A^1 from $(B^1B^1 \times A^1A^1) \times A^1A^1$. Likewise, four types of F_2 progeny were scored; A^1A^1, B^1B^1, A^1B^1, and B^1A^1. A typical zymogram of F_2 segregation is shown in Fig. 4. The hypothesis that Amy-1 is controlled by a pair of alleles was tested by Chi-square calculations. The X^2-values and the probabilities of chance errors for each backcross and F_2 segregations are given in Table 6a. The three backcrosses analyzed all yielded segregation ratios in good agreements with 1 : 1 ratio. However, the segregation in two F_2 progeny yielded deviations from 1:1:1:1 ratio which situated on the borderline of statistical significance; thus it cannot be accounted by chance errors. On close examination of the X^2-values contributed by the four types of F_2 segregants, it is found that they do not attribute equally to the total X^2-value. About 70% of the total X^2-value in F_2 of $(A^1A^1 \times B^1B^1)$ and 95% of the total X^2-value in F_2 of $(B^1B^1 \times A^1A^1)$ were from the segregant types A^1A^1 and A^1B^1. There is one possible source of error which could not be eliminated from the analyses where endosperm tissues were used in the gel-assays. That is, the latent expression of Amy-1B in heterozygotes that possess two maternal A^1 in their triploid endosperm genetic make-up. As a result, it is likely that A^1B^1 can be mistaken as A^1A^1. This is particularly true

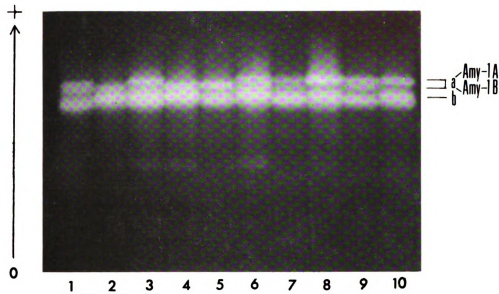


Fig. 4. Electrophoretic analysis of Amy-1 in individual endosperm of 10-day-old F_2 seedlings of 38-11 (*Amy-1^B/Amy-1^B*) and W64A (*Amy-1^A/Amy-1^A*).[2] 0 = point of sample insertion. Samples numbers 2, 4, and 5 are B^1B^1; 8, and 10 are A^1A^1; 3 is A^1B^1; and 1, 6, 7 and 9 are B^1A^1. Electrophoretic variants of the broad band traveling behind the Amy-1 are detected in the endosperm extracts of these F_2 seedlings suggesting that it is genetically independent from Amy-1. The genetics of this particular band of amylase activity, however, was not studied in depth.

with F_2 progeny. Indeed, it is shown in Table 6a that the A^1A^1 segregants are in excess while A^1B^1 segregants are deficient in the same progeny.

In view of this complication in scoring Amy-1 using endosperm tissue, about half of the F_2 progeny of (B^1B^1 x A^1A^1,) were assayed with both endosperm and leaf tissue from the same individual seedlings. The two tissues were assayed either simultaneously (see Table 6b F_2 data [1]) or independently (see Table 6b F_2 data [2]) with respect to the age of the seedlings. Among 113 F_2 seedlings of (B^1B^1 x A^1A^1) analyzed, there were 36 A^1A^1, 50 hetrozygotes (A^1B^1 and B^1A^1 were not discernable), and 27 B^1B^1 according to the leaf assays. A typical zymogram of the F_2 leaf assays is shown in Fig. 5. It can be seen that Amy-1 alone was detected in leaf extracts. The broad pink band usually present together with Amy-1 in endosperm extracts (Fig. 4) is not detectable in the leaf extracts. The results of the endosperm assay show there were 40 A^1A^1, 27 B^1B^1, 16 A^1B^1, and 30 B^1A^1. In comparing the two sets of results obtained from the same samples of F_2 segregants, it is found that the F_2 segregation ratio is in good agreement with a 1 : 2 : 1 ratio according to the data obtained from leaf assays (X^2 = 2.89, 0.30>p>0.20), while it is in rather poor agreement with 1 : 1 : 1 : 1 ratio according to the data obtained from endosperm assays (X^2 = 10.37, 0.02>p>0.01). The discrepancy between the two sets of data can be accounted for

Table 6b. Results showing the mode of inheritance of Amy-1 amylase variants in leaf tissue of 8-22 day old maize seedlings. No gene dosage effect was observed in the diploid tissue. Consequently A^1B^1 and B^1A^1 could not be distinguished from each other.

Genetic cross			Amy-1 patterns in offspring				X^2	P
Female	Male		A^1A^1	A^1B^1 & B^1A^1	B^1B^1	Total		
B^1B^1	(A^1A^1 X B^1B^1)		0	46	41	87	0.28	>0.50
(B^1B^1 X A^1A^1)	(B^1B^1 X A^1A^1)	(1)	36	50	27	113	2.89	>0.20
		(2)	26	53	27	96	2.73	>0.20
		(1)+(2)	62	103	44	209	3.23	>0.10

In F_2 data (1) leaf tissue and endosperm of the same individual seedlings were assayed simultaneously at 8-10 days of germination. In F_2 data (2) leaf tissue was taken from 14-22 day-old seedlings. Those endosperm had been excised and assayed on the 12th day of germination.

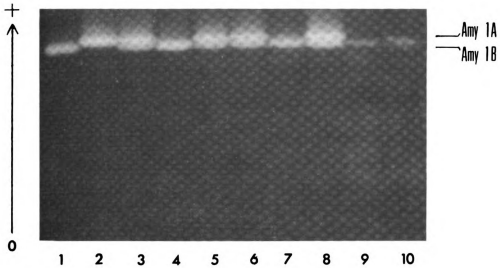


Fig. 5. Electrophoretic analysis of Amy-1 in leaf extracts of 14 day-old F_2 seedlings of 38-11 *(Amy-1^B/Amy-1^B)* and W64A *(Amy-1^A/Amy-1^A)*. 0 = point of sample insertion. Sample numbers 1, 4, 7, and 9 are B^1B^1; 2 is A^1A^1; 3, 5, 6, 8, and 10 are heterozygotes (A^1B^1 and B^1A^1 are not distinguishable).

by the possible over scoring of A^1A^1 at the expense of A^1B^1 in the endosperm assays. To make certain this was the case, an additional 96 leaf samples from F_2 progeny of (B^1B^1 x A^1A^1) were subjected to gel-assays and the scores of the three segregant types were added to those obtained from the 113 seedlings which were derived from seeds shelled from the same ear. The results are given in Table 6b. It can be seen that those 209 seedlings segregated into a ratio with a fairly good agreement with 1 : 2 : 1 ratio as would be expected from monogenic inheritance.

As another independent source of genetic data, 87 seedlings of the backcross progeny of B^1B^1 x (A^1A^1 x B^1B^1) were scored with leaf assays. The data presented in Table 6b indicates these seedlings fall into two categories, heterozygotes and homozygotes, with an apparent ratio of the two as 1 : 1.

In conclusion, the mode of inheritance of Amy-1 is controlled by a pair of alleles at one locus. The two alleles are acting in a codominant manner although their expression as detected via the active gene products in the endosperm tissue, seemed to be controlled differently from those in the leaf tissues.

Discussion

I have presented data to show that the two Amy-1 variants are controlled genetically by what first appeared to be a case of maternal inheritance in endosperm tissue

of immature kernels, but later it was proved to be controlled by a pair of codominant alleles in leaf tissues. There are two possible explanations for this: (1) Amy-1 is encoded by two redundant genes. One gene is located in cytoplasmic DNA which is transmitted to progeny via maternal gametes and is only active in liquid endosperm of young kernels. The other gene is located in the nucleus (one of the 10 chromosomes) and only becomes active after seed germination. (2) Amy-1 is controlled by one locus with two alleles having differential activity which is under a temporal control. Thus a heterozygote, either A^1B^1 or B^1A^1, would possibly have only one of the two gene products in the liquid endosperm stage but as the kernel is brought to its maturity and grown to seedling stage, the other gene product is either activated or synthesized and thus becomes detectable. If the explanation of two-redundant genes is true, we would expect that both diploid embryo (scutellum and embryo axis) and triploid endosperm tissues in immature kernels would show only the maternal Amy-1. If the explanation of differential allelic expression is true, there are two possible consequences with equal probability that might be observed when liquid endosperm and young embryo tissues in immature heterozygotic kernels are assayed separately; the same allelic products (either Amy-1A or Amy-1B) present concomitantly in two tissues. Alternatively, two different allelic products would appear with one detectable in the endosperm and the

other detectable in the embryo of the same immature heterozygote kernels. The experimental finding with pooled tissue extract of 20 heterozygote immature kernels of A^1A^1 x B^1B^1 shows only Amy-1A was present in both endosperm and embryo tissues. However, in the case of B^1B^1 x A^1A^1, Amy-1B was found in endosperm tissue while Amy-1A was found exclusively in embryo tissue. This result presents strong evidence supporting the explanation that there is a differential allelic expression of the *Amy-1* locus, which is both under temporal and spatial control.

There are some intrinsic difficulties that make a direct linkage test between *Amy-1* and *Amy-2* loci virtually impossible despite the fact that the two strains--one homozygous for $Amy\text{-}1^A$ and $Amy\text{-}2^A$ and the other homozygous for $Amy\text{-}1^B$ and $Amy\text{-}2^B$--necessary for the linkage test are available as they are represented by 58-3-6 and 38-11 respectively. A few genetic crosses involving these two strains were made in the greenhouse. Nevertheless, the genetic analysis of Amy-2 could only be made by using liquid endosperm tissue and Amy-1 would have to be assayed at the seedling stage. Hence, it was impossible to assay one amylase isozyme on individual segregants without sacrificing the resolution of the other amylase isozyme. When 14 maize strains were screened for electrophoretic variants of Amy-1 and Amy-2, three distinct strains were found, namely; $Amy\text{-}1^A/Amy\text{-}2^A$; $Amy\text{-}1^A/Amy\text{-}2^B$; and

Amy-1^{B}/*Amy-2*^{B}. The variation of one amylase isozyme seems to be independent of the other. This is taken as indirect evidence to indicate that *Amy-1* and *Amy-2* loci are not linked.

With a similar gel-assay technique Finnegan (1969) has independently obtained some genetic data on maize amylase based on the segregation of one backcross and one F_2 cross. This amylase by his descriptions appears to be identical with Amy-2. His data, though scanty, support my conclusion on the genetics of *Amy-2*^{A}.

In comparing the genetics of Amy-1 and Amy-2, two distinctions are noted. First, the phenomenon of differential allelic expression is unique to the *Amy-1* locus and is not observed in the *Amy-2* locus. Second, there is an apparent effect of gene dosage with Amy-1 but it is not observed with Amy-2 in endosperm tissues; suggesting that Amy-1 is synthesized in triploid endosperm tissue and Amy-2 is synthesized in diploid embryo tissue. This is contradictory to Dure's finding (1960) who suggested that maize alpha-amylase is synthesized exclusively in scutellar tissue of germinating seedlings. His evidence was based on data of non-specific quantitative determinations of reducing sugars released from starch after selective destruction of beta-amylase in crude tissue extracts with heat treatment.

The *Amy-2* and *Ct* (catalase) linkage is of interest since the genetic analysis of *Amy-2* can most conveniently be done by using 16 day old kernels, at which stage none of the morphological characteristics of the kernels can be distinguished. Catalase, on the other hand, can be easily assayed by using either 16-day-old kernels (Scandalios, 1965) or scutellar tissues dissected from 3-day imbibed seeds (Scandalios, unpublished). Therefore, the task of locating *Ct* on a chromosome is likely to be easier than that for *Amy-2*. However, once *Ct* has been assigned to a chromosome, the *Amy-2* locus can be located.

CHAPTER V

DEVELOPMENTAL STUDIES OF MAIZE AMYLASES

Introduction

Increase of total amylase activity occurs during seed germination of several cereal crops. It is difficult to attribute the level of amylolytic activity to the specific increase of alpha-amylase and/or beta-amylase because of the lack of a quantitative assay for distinguishing the two amylases when present in a mixture. Nor can one be certain of the number of genes involved in the synthesis of amylases once seed germination has been triggered.

Since phosphorylases and amylases in maize can be discerned by gel electrophoresis (see Chapter VI), it is possible then to relate the total amylolytic enzyme activity with the zymogram patterns. Furthermore, Amy-1 and Amy-2, the two major amylases, are both present in endosperm and scutellar tissues of immature kernels and mature seeds. The results of genetic analyses of Amy-1 and Amy-2 suggest that the former is synthesized in the endosperm tissue and the latter is synthesized in the scutellar tissue. Evidence has also been presented to show there is a temporal control of Amy-1 amylase activity in developing kernels and germinating seedlings. The purpose of this study is to

investigate quantitative changes of amylolytic activity of the enzymes with the concomitant qualitative changes which occur during maize kernel development. Such information will hopefully enable us to determine whether the two amylases encoded independently by two genes are activated differentially in various tissue and at various developmental stages.

Literature Review

The amylases in maize, in certain respects, are different from those in barley, wheat, and rye. The level of beta-amylase in the ungerminated cereal grains is high in barley, wheat, and rye (Kneen, 1944), but low in maize (Bernstein, 1943; Kneen, 1944). In germinated cereals, high levels of both alpha and beta-amylases are characteristic of barley, wheat, and rye; whereas in maize alpha-amylase accounts for 90% of the total peak amylolytic activity, and only 10% is attributed to beta-amylase (Dure, 1960). Barley alpha-amylase in crude malt extract can be completely inactivated in 30 minutes at pH 3.3 without destroying the beta-amylase in the same extract (Kneen et al., 1943). On the other hand, while maize alpha-amylase in crude tissue extracts can be completely inactivated at pH 3.4, 50% of the beta-amylase activity is also lost (Dure, 1960). Barley beta-amylase can be selectively inactivated by 5 minutes at 70 C (Kneen, 1943) whereas in

maize 1/3 of the alpha-amylase in addition to beta-amylase is also inactivated (Dure, 1960).

Dure (1960) studied the amylase activity in scutellar and endosperm tissues of maize seedlings. He found that after 3 days of germination, the total amylolytic activity in intact endosperm increased linearly with time and reached its peak at 10 days which is about a 20-fold increase of the initial activity in dry seeds. The amylolytic activity secreted from scutellar tissue was estimated by those secreted from excised scutella into gelatin; and it was found to be parallel with the increase of total amylolytic activity in intact endosperm tissues. The secreted amylolytic activity was attributed to alpha-amylase alone. On the other hand, there was only a slight increase of amylolytic activity in excised endosperms during the same period. This activity was attributed to beta-amylase. Nevertheless, the assay method he employed was increase in reducing sugars and the possibility that some of the reducing power might be due to glucose released by maltase was not excluded. Hence, it appears not to have been clearly established that copious amounts of alpha-amylase were secreted into the endosperm during maize seed germination. It is also questionable whether alpha-amylase is synthesized in scutellum alone and beta-amylase originates exclusively in endosperm before and after seed germination. It is clear though from Dure's data that there must be some

interactions between the scutellum and the endosperm to allow for the maximal increase rate of amylolytic activity in intact endosperm during seed germination.

Materials and Methods

Amylases in Developing Maize Kernels

The inbred strain Golden Cross Bantam was grown in a growth chamber which was kept at 60-75 C, illuminated daily with 2,000 foot-candles for 16 hours. Two kernels in the mid-section of an ear were sampled every day starting from 6 days after pollination and ending at 27 days. The kernel samples were stored at -50 C and thawed shortly before assays were to be made. Liquid endosperm of one freshly thawed kernel of each sample was used for gel assays while the liquid endosperm of the remaining kernel was used for quantitative assays for amylase and protein. Each kernel was pierced at its non-embryo end with a needle and 10 µl of the liquid endosperm was taken out for amylase assays, another 10 µl sample was assayed for protein using the method of Lowry, et al., (1951).

Amylase in Germinating Maize Seedlings

Three maize strains, W64A, 58-3-6 and 38-11 were used. Seeds of each strain were surface sterilized with 1% hypochlorite for 15 minutes, washed twice with sterilized deionized water and were then soaked for 48 hours. After soaking the seeds of each strain were divided into two

halves, and transfered into two moisture chambers. The moisture chamber is a plastic box (30 x 25 x 10 cm) with a plastic cover. The box was paved with a layer of sterilized fine vermiculite which was covered by several sheets of sterilized paper towel. The box was irrigated with sterilized deionized water. Soaked seeds were sown on the wet paper towels and the box was kept closed to retain the moisture. One set of seeds was germinated in the dark and the other set of seeds was germinated in the light, with 12 hours of light supplemented with two Sylvania Gro-Lux lamps (Instrumentation Specialities Co., Nebraska) hanging 2 feet above the seeds with 210-240 foot-candle illumination. The temperature for both light and dark germination was 23 C. Ten light-grown and ten dark-grown seedlings of each strain were sampled at 2 day intervals. Sample seedlings were dissected into endosperm, scutellum, shoot and root, and extracted in a pH 7.5 Tris-HCl buffer (0.05 M Tris-HCl and 1mM $CaCl_2$) and the tissue extracts were spun at 10,000g in a Sorvall RC2-B refrigerated centrifuge for 20 minutes. The supernatants were collected in volumetric conical centrifuge tubes. And the total volume of each tissue supernatant was recorded. The concentration of protein and amylase activity was measured by Lowry's method and the starch-iodine method respectively. The supernatants of each tissue extract were also subjected to gel electrophoresis to obtain a

qualitative pattern of amylase isozymes present in each tissue extract.

Results

Amylases in Developing Maize Kernels

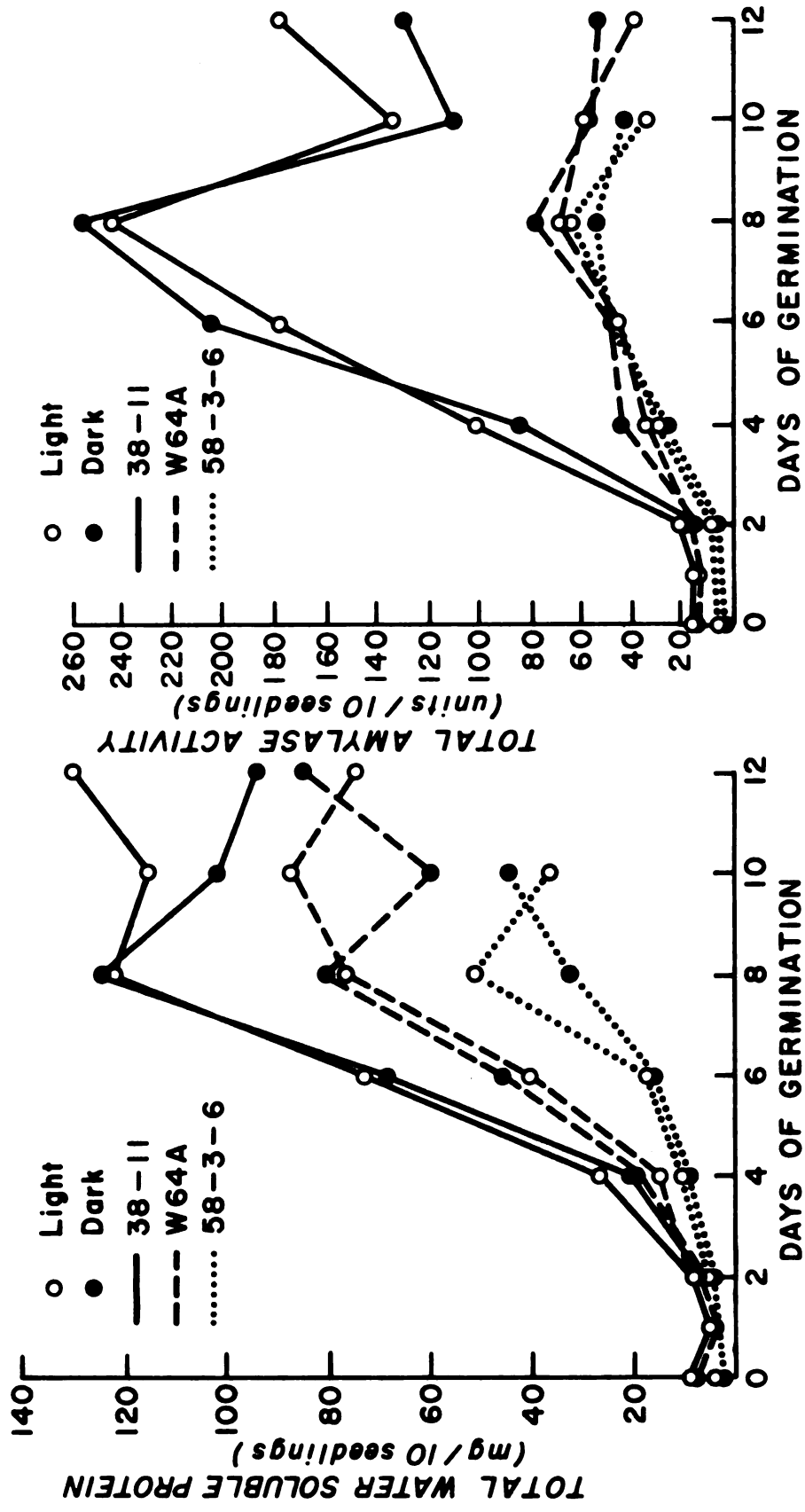
The specific activity of amylase in liquid endosperm of immature kernels fluctuated between 7 units/mg protein to 10 units/mg protein from 6-27 days after pollination, while the concentration of protein doubled. The patterns of amylases and phosphorylases were found to be consistent throughout the period (7-30 days) of kernel development in the strain, Golden Cross Bantam. No systematic studies were made on other strains due to the problem of poor development of selfed ears in the growth chamber. Results of sporadic sampling of immature kernels of strains W64A, 58-3-6 and 38-11 from various ears and plants were found to be in conformity with Golden Cross Bantam.

Quantitative Changes of Amylases in Germinating Maize Seedlings

The amount of water soluble proteins and amylase totaled from tissue extracts of endosperm, scutellum, shoot, and root are plotted against the age of seedlings as shown in Fig. 6 and Fig. 7. The quantitative changes of protein and amylase can be generally described as follows:

Fig. 6. The increase of water-soluble protein in germinating maize seedlings. Each point on the graph represents the pooled amount of protein in the tissue extracts of endosperm, scutellum, root, and shoot, obtained from 10 seedlings of the same age. Due to the relative poor growth of 58-3-6, the sampling was discontinued after 10 days of germination.

Fig. 7. The increase of soluble amylase in germinating maize seedlings. Each point on the graph represents the pooled amount of protein in the tissue extracts of endosperm, scutellum, root, and shoot, obtained from 10 seedlings of the same age. Due to the relative poor growth of 58-3-6, the sampling was discontinued after 10 days of germination.


TOTAL WATER SOLUBLE PROTEIN
(mg / 10 seedlings)
TOTAL AMYLASE ACTIVITY
(units / 10 seedlings)
DAYS OF GERMINATION
DAYS OF GERMINATION
Light
Dark
38-11
W64A
58-3-6
Light
Dark
38-11
W64A
58-3-6

(1) There is a sharp increase of total soluble protein and amylase during seed germination. The peaks of protein and amylase were reached at the 8th day of germination. Until then the seedlings grown in light and dark are alike in terms of total soluble protein and amylase activity.

(2) After 8 days of germination, the level of soluble protein remained high in light-grown seedlings. Whereas it dropped 30% of the peak amount in dark-grown seedlings (Fig. 6).

(3) The quantitative amylase profiles in germinating seedlings did not differ significantly for those grown under light and those grown in dark (Fig. 7).

(4) The growth rates of the three strains were quite different, and were reflected in the amounts of soluble protein and amylase. At the 8th day, when amylase and protein reached their peaks, the ratio of the total soluble protein in 58-3-6 extract to those in W64A extract and 38-11 extract was roughly 5:8:12 (Fig. 6). Whereas the ratio of total amylase was 6:8:25 (Fig. 7). The increase of amylase in 38-11 is not in proportion with the increase of soluble protein but at a higher specific rate as compared to that of W64A and 58-3-6. It may be recalled, that 38-11 unlike the other two strains, has very low activity in Amy-1 of immature kernels that is hardly detectable upon gel electrophoresis. In germinating seedlings of 38-11 however, Amy-1 as part of Zone-1 amylase activity, became

very prominent and appeared to be the main fraction of increased amylase during seed germination although direct quantitative determination of Amy-1 alone was not possible. The high specific rate of amylase synthesis or activation during the germination of 38-11 was apparently associated with the appearance of this particular Zone-1 amylase isozyme.

(5) The specific activity of amylase in the tissue of the germinating seedlings are shown in Fig. 8. If protein degradation was negligible in those seedlings, the rise of specific activity of amylase in the endosperm tissue might signify the synthesis or activation of amylase at a rate higher than the synthesis of non-amylase proteins.

(6) The peaks of amylase specific activity in endosperm tissues appeared at the 6th day of germination of 38-11 and 58-3-6 and at the 8th day of that of W64A. This difference is possibly due to the initial rate of germination being relatively slow in W64A and fast in 38-11 and 58-3-6.

(7) In contrast to endosperm amylase, the specific amylase activity in scutellar tissue decreased quickly in the first 6 days of germination (Fig. 8) and slowly afterwards. Although the total amylase in the scutellar tissue was increasing with days of germination up to the 8th day, the amylase specific activity was dropping. This loss of amylase in the scutellar tissue may be due to either a quick turn over rate within the tissue or due to amylase

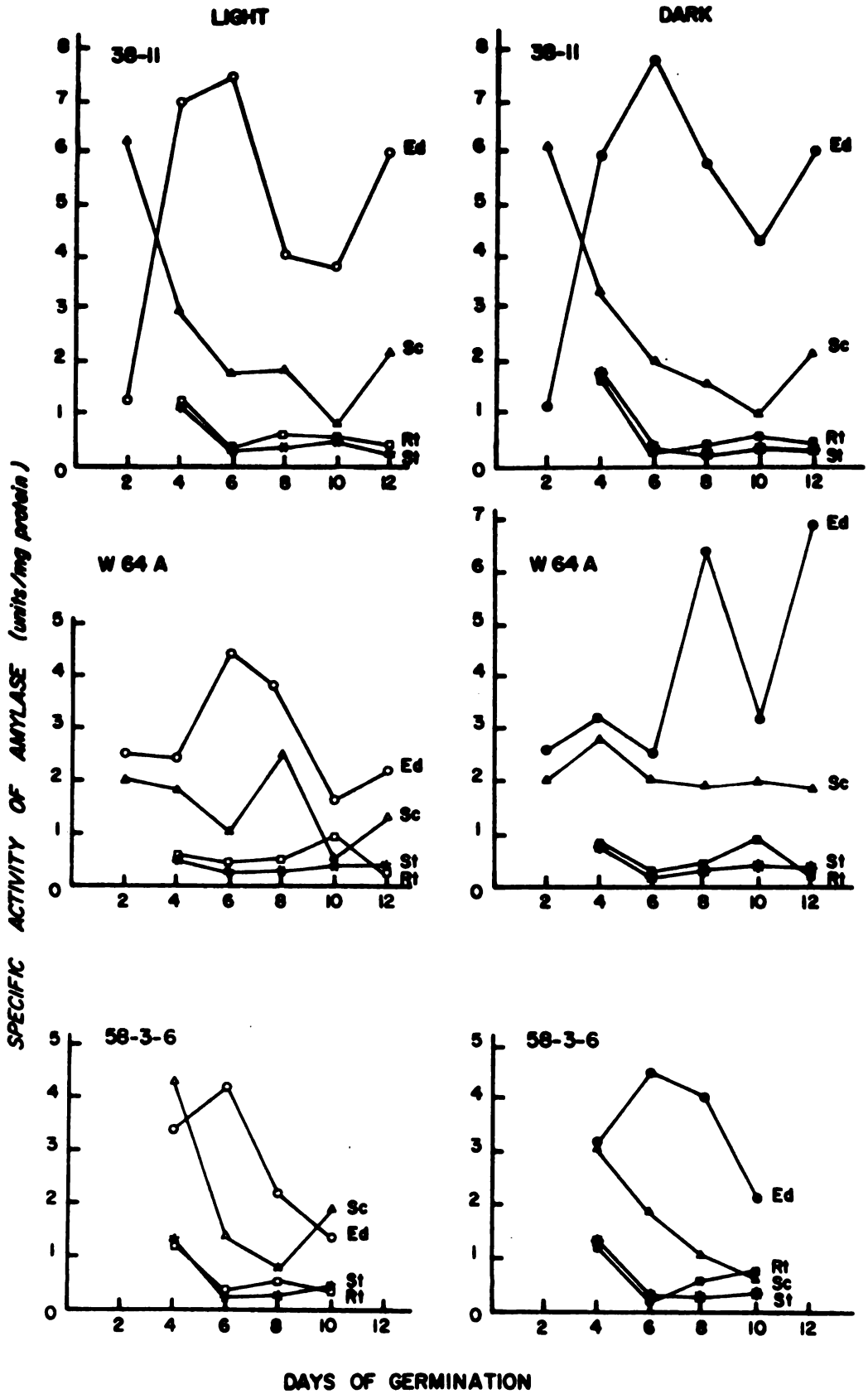


Fig. 8. The specific activity of amylase in the tissues of germinating maize seedlings of 38-11, W64A and 58-3-6 grown in light and dark.

being secreted externally to the surrounding endosperm tissue.

Qualitative Changes of Amylase in Germinating Maize Seedlings

The results of gel assays are shown schematically in Fig. 10 accompanied by photographs (Fig. 9). Four maize strains were investigated. They were W64A, 58-3-6, 38-11 and Golden Cross Bantam. Despite the fact that they represent genetic variants of Amy-1, Amy-2 and phosphorylase, the qualitative changes and tissue distribution of these enzymes during seed germination are identical from strain to strain. The zone of phosphorylase activity was detected in scutellar extract throughout 12 days of germination but was absent from endosperm, shoot, and root tissues after four days of germination. Zone-1 consists of a clear band (band 1a) which has been genetically defined and a pink band (band 1b). The activity of this zone was enhanced greatly in tissues of later germinating stages. In addition to 1a and 1b of Zone-1, a new pink band (band 1c) appeared in scutellar and endospermic tissues about 4 and 6 days of germination, respectively. This new band in Zone-1 though being the slowest of the three bands it migrates closely behind the band 1b. This new band was not detected in either the shoot or the root tissue.

Zone-2 amylase of liquid endosperm usually has 4 bands, one major band (band 2d) and three minor bands

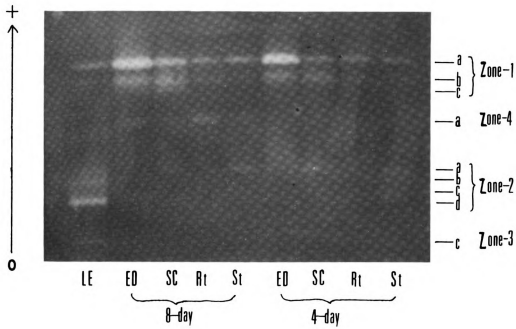
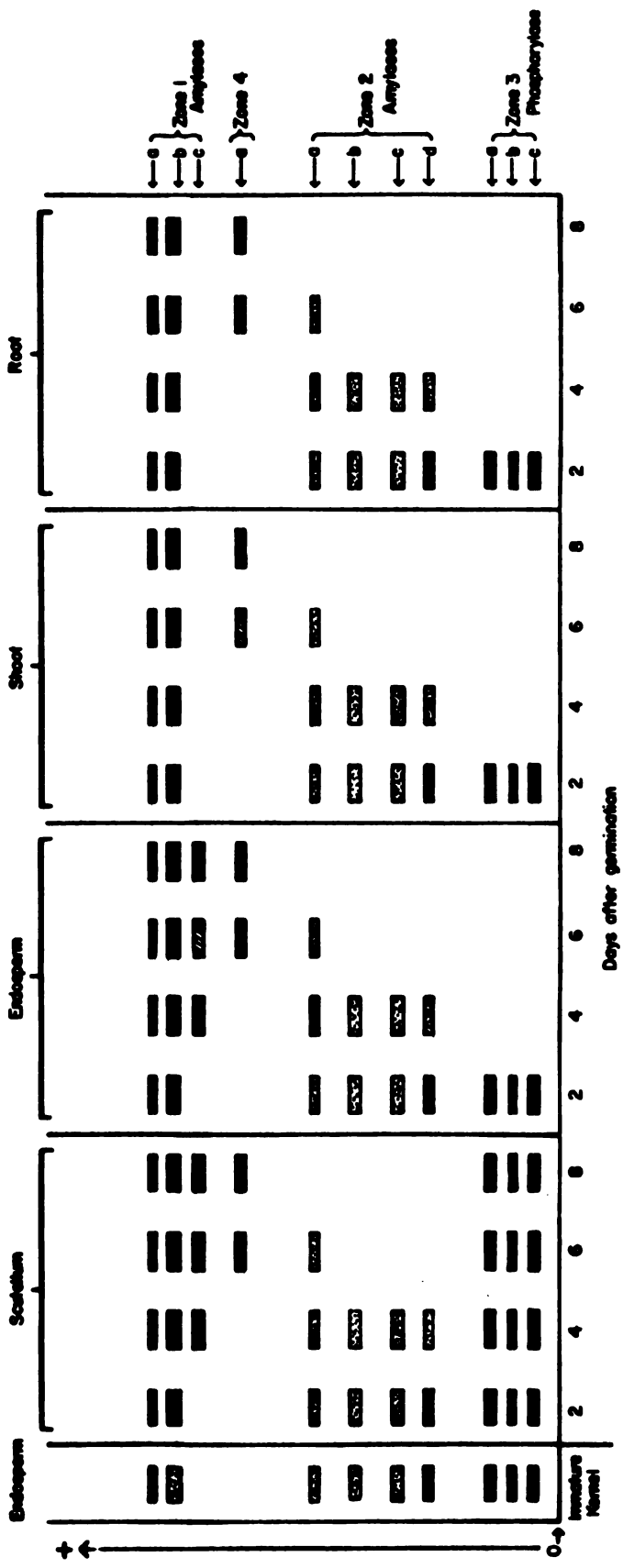


Fig. 9. Maize amylase zymogram of various tissue extracts of the strain W64A that was grown under light and dissected on the 4th and 8th day. LE = 16 day-old liquid endosperm of a immature kernel. ED = endosperm. SC = scutellum. Rt = root. St = shoot.

Fig. 10. Schematic drawing of starch-degrading enzyme patterns resulting from gel assays of tissues in germinating maize seedlings. Zone-1, 2, 3 and 4 are named arbitrarily according to the order of their discovery. For a given inbred strain, the bands in each zone are designated alphabetically with small letters in the order of their decreasing anodal electrophorteic mobility at the specified experimental conditions. The arbitrary designation of the bands and the zones was initially made without knowledge of their genetic basis. However, once variants of a given band were detected and the mode of inheritance was elucidated, a gene symbol was (or will) assigned to this particular band. For example, the a-band of Zone-1 was given the gene symbol *Amy-1*. In order to distinguish the two allelic forms, Zone-1a of W64A was named Amy-1A and that of 38-11 Amy-1B. Likewise, Zone-2d, the major band in Zone-2, was given the gene symbol *Amy-2* whose allelic products were named Amy-2A in 58-3-6 and Amy-2B in W64A. When the information of chemical identification of a given starch-degrading enzyme band or zone becomes available, it can be further specified by the name of the enzyme as Zone-1 amylases, Zone-2 amylases, and Zone-3 phosphorylases.


Zone 1
Amylases
Zone 4
Zone 2
Amylases
Zone 3
Phosphorylase
a
b
c
d
Root
Shoot
Endosperm
Scutellum
Endosperm
2
4
6
8
Days after germination
Imbibed
Kernel
+
0

(bands 2a, 2b and 2c). In tissue extracts, bands 2a, 2b and 2c were no longer distinct except the major band. During seed germination, Zone-2 underwent a seemingly stepwise shift toward the anodal direction of the electric field and eventually a new band was formed in 8-day-old seedlings. The new band (Zone-4) was located half-way between Zone-1 and the initial location of Zone-2. Zone-4 was observed in all tissues except less activity of the new band could be detected in shoot and root tissues. Without knowledge of its relation with Zone-1 and Zone-2, this band is tentatively designated as band a of Zone-4. It is important to note that the disappearance of Zone-2 and the appearance of a new band is a step-wise process in that a delineated band is observed in each anodal shift with constant spacings between the bands phased in and out. Although there is no direct evidence to suggest that the appearance of band 4a is due to the shift of the pre-existing Zone-2, the color of this band and of the intermediates is pink, suggesting their similarity to Zone-2 amylase normally found in liquid endosperm.

The timing of the appearance of these new bands, particularly the one in Zone-1 (1c) and 4a were found to be coincident with the rise of total amylase activity in the germinating seedlings. Phosphorylase was precluded since none of the new bands showed phosphorylase activity

on the basis of gel assays using glycogen and glucose-1-phosphate as substrates.

Tissue Distribution of Amy-1 Amylase in Immature F_1 Kernels and Germinating F_1 Seedlings of Maize

Twenty frozen kernels from four F_1s: (W64-A x 38-11), (58-3-6 x 38-11) and the two reciprocal crosses harvested 20 days after pollination were thawed and the embryos (scutella and embryo axis) were separated from their endosperms. The two tissues were each ground in a procelain mortar with some white sand and 2 ml of pH 7.5 Tris-HCl buffer (0.05 M Tris-HCl with 1mM $CaCl_2$). The tissue homogenate was centrifuged (12,000g) to eliminate the cell debris. The supernatants of the tissue extracts were used directly for gel assays, with special interest in Amy-1 amylase. The results of the gel assays were recorded and are presented in Table 7, where "A" stands for the fast Amy-1 and "B" stands for the slow Amy-1.

Table 7. The pattern of Amy-1 amylase in 20 day-old F_1 kernels.

Tissue	W64A x 38-11	38-11 x W64A	58-3-6 x 38-11	38-11 x 58-3-6
Endosperm	A	B	A	B
Embryo	A	A	A	A

The Amy-1 phenotypes in endosperm and embryo tissues of (W64-A x 38-11) and (58-3-6 x 38-11) are identical: both showing Amy-1A. However, in the two reciprocal crosses, Amy-1B was found in the endosperm tissue while Amy-1A alone was detected in embryo tissue. The result suggests that only one of two potential genes is expressed in these young F_1 kernels. Amy-1A is active in the embryo tissues whereas the activity of Amy-1B in the endosperm tissue is apparently dependent on the gene dosages. Amy-1A is active in endosperm with two dases of *Amy-1A* and one dose of *Amy-1B* (W64A x 38-11, and 58-3-6 x 38-11). Conversely, Amy-1B is active in endosperm with two doses of $Amy\text{-}1^B$ and one dose of $Amy\text{-}1^A$ (38-11 x W64A, and 38-11 x 58-3-6).

Tissue Distribution of Amy-1 Amylase in Germinating F_1 Seedlings

The differential allelic expression of the *Amy-1* locus was traced into the germinating F_1 seedlings. Ten F_1 seeds of W64A x 38-11, 58-3-6 x 38-11 and their two reciprocals were germinated and grown under Gro-Lux lamps. Individual seedlings were sampled at two-day intervals and dissected into four parts; endosperm (with adhered aleurone layer), scutellum, shoot (including stem and leaf) and root. The pericarp was discarded. Each tissue was ground in 0.2 ml of a pH 7.5 Tris-HCl buffer (0.05 M Tris-HCl with 1mM $CaCl_2$) with some sand using a procelain mortar and a pestle. The crude tissue extracts were

centrifuged (10,000 g, 10 minutes) and the supernatants used directly for gel electrophoresis. Table 8 summarizes the results. The letters "A" and "B" indicate the presence of Amy-1A or Amy-1B band, or both. In 2-day and 4-day old seedlings, the embryo axis is represented by the pooled tissue of shoot and root. It is evident from the gel assays that not all the tissues of a seedling gave rise to identical Amy-1 pattern. If a generalization can be made, it seems wherever F_1 was derived from A^1A^1 x B^1B^1 regardless that A^1A^1 being W64A or 58-3-6, both the endosperm and the scutellar tissues possess the Amy-1A isozyme exclusively throughout 12 days of germination but their shoot and root tissues possessed both Amy-1A and Amy-1B isozymes. With the cross B^1B^1 x A^1A^1, the results were different. The endosperm tissues gave rise to both Amy-1A and Amy-1B amylase isozymes throughout the experimental period. The scutellum of the same F_1 seedling showed Amy-1A isozyme in first 8 days and both Amy-1A and Amy-1B after 10 days of germination. Such a concomitant presence of Amy-1A and Amy-1B isozymes was also observed in both shoot and root tissues of the same F_1 seedlings as early as 6 days after germination. This differential allelic activity is apparently both tissue and time dependent, with 38-11 x 58-3-6 (B^1B^1 x A^1A^1) as the only exception in which both allelic activities were apparently in synchrony.

Table 8. The pattern of Amy-1 amylase in germinating F_1 maize seedlings.

Crosses & tissues	Days of germination					
	2	4	6	8	10	12
W64A x 38-11						
Endosperm	A	A	A	A	A	A*
Scutellum	A	A	A	A	A	A*
Shoot	AB	AB	AB	AB	AB	AB*
Root			AB	AB	AB	AB*
38-11 x W64A						
Endosperm	AB	AB	AB	AB	AB	AB*
Scutellum	A	A	A	A	AB	AB*
Shoot			AB	AB	AB	AB*
Root			AB	AB	AB	AB*
58-3-6 x 38-11						
Endosperm		A	A	A*		
Scutellum		A	A	A*		
Shoot		AB	AB	AB*		
Root			AB	AB*		
38-11 x 58-3-6						
Endosperm		AB	AB	AB*		
Scutellum		AB	AB	AB*		
Shoot		AB	AB	AB*		
Root		AB	AB	AB*		

*Extracts were made from pooled tissues of 5 seedlings.

The differential allelic expression of the *Amy-1* locus is also extended into F_2 progeny of A^1A^1 x B^1B^1 and the reciprocal. The data were presented in the section on genetic studies. In short, the latent expression of one allelic product in endosperm tissues had caused the segregation of the F_2 progeny to deviate significantly from the expected ratio for single gene inheritance which was subsequently proved by the leaf assays. Furthermore, there are some peculiarities to the Amy-1 pattern in stem tissues of the F_2 progeny (W64A x 38-11). Among the thirty F_2 seedlings (8-14 days old) assayed, those typed as A^1A^1, A^1B^1, and B^1A^1 by both endosperm and leaf showed only Amy-1A isozyme in their stem extracts, and Amy-1B in those otherwise identified as B^1B^1. Consequently, only two phenotypic classes A^1A^1 and B^1B^1 were detected when F_2 stems were used in the assays. The segregation ratio is nearly (24 A^1A^1 and 6 B^1B^1) 3:1 for F_2 stem tissues as it is compared to 13 A^1A^1, 10 A^1B^1 and B^1A^1 and 7 B^1B^1 to 1:2:1 ratio for F_2 leaf tissues. It seems as though the activity of Amy-1B is repressed in stem tissues of heterozygous origin *(Amy-1^A/Amy-1^B)*.

Discussion

The observation of the rise of amylase specific activity (amylase units/mg protein) in endosperm tissues and a concomitant fall of the specific activity in scutellar tissues poses the question whether amylase is

secreted from scutellum to endosperm, and if so, whether the amount secreted can account for the total increase of amylase in endosperm tissue during seed germination. A critical examination of the data with some calculations are presented in Table 9 and Table 10, which may shed some light on this issue. Table 9 gives the following values of each one of the three strains: (1) the amount of amylase in endosperm and scutelleum of germinating seedlings, (2) the percentages they represented in the pooled tissue extract, and (3) the ratio between the amylase content in endosperm and scutellum. Since the three strains essentially yielded the same information in this respect, only the results on 38-11 strain will be discussed in the following:

(1) The amylase was low in seeds soaked for 2 days. At this stage, scutellum amylase was about double the amount of amylase in endosperm. This relation was upset during the subsequent days of germination. The ratio of the amylase in endosperm to that in scutellum varies from 3.0 to 4.0 for seedlings grown under light and 2.1 to 5.8 for those grown in dark.

(2) Throughout 12 days of germination, the pooled amount of amylase in endosperm and scutellum was about 90% of the total amylase in the whole seedling.

(3) If the endosperm amylase originated from the scutellum, two possible relationships of the endosperm-scutellum amylase ratio might be observed. The ratio

Table 9. The quantitative distribution of amylase activity in endosperm and scutellum in the strains W64A, 38-11 and 58-3-6.

	Days of germination	Light			Dark		
		Amylase in ED + SC units/10 seedlings	% total amylase in 10 seedlings	Ratio of amylase ED/SC	Amylase in ED + SC units/10 seedlings	% total amylase in 10 seedlings	Ratio of amylase ED/SC
W64A	2	15.12	100	0.7	15.12	100	0.7
	4	29.40	86	1.7	39.43	90	1.2
	6	40.30	89	5.2	43.28	89	1.7
	8	62.30	86	1.6	57.60	74	2.0
	10	25.16	43	0.9	29.15	52	0.9
	12	24.00	63	1.3	43.93	84	1.6
38-11	2	18.95	100	0.5	18.95	100	0.5
	4	104.88	92	3.0	76.13	91	3.3
	6	170.10	95	3.9	195.85	96	5.8
	8	222.90	79	3.1	138.20	93	3.8
	10	104.79	89	3.8	85.70	95	4.0
	12	158.35	89	3.3	101.15	81	2.1
58-3-6	2	7.68	100	0.8	7.68	100	0.8
	4	24.38	86	0.9	23.43	91	1.0
	6	39.95	90	2.9	42.43	95	2.6
	8	54.65	88	5.6	48.40	93	3.9
	10	24.49	71	0.9	31.45	74	3.8

N.B. 1 Amylase unit = $\frac{OD_{620}\,m\mu}{\text{ml of enzyme X reaction time in minutes}}$

Table 10. Hypothetical conversion of endosperm amylase in germinating maize seedlings to an equivalent amount of protein that might have originated in the scutellum.

Strains	W64A		38-11		58-3-6	
	Light 8-day	Dark 8-day	Light 8-day	Dark 6-day	Light 8-day	Dark 8-day
Amount of amylase in endosperm (units)	38.00	38.40	169.00	167.00	46.40	38.50
Amylase specific activity in Scutellum (units/mg prot.)	2.52	1.98	1.86	2.05	0.81	1.10
Amy. eq. prot. mg	15.10	19.40	92.00	83.00	57.30	35.00
Total soluble protein (mg) in scutellum	9.63	9.69	29.00	13.83	10.19	9.00

might be decreasing in the case that the rate of secretion was constant and independent of the rate of amylase synthesis in the scutellum. Alternatively, the ratio might reflect the rate of amylase secretion from scutellum to endosperm which might have increased with successive days of germination to the 8th day and then dropped. Neither of these two phenomena was actually observed as shown in Table 9. Hence, it can be deduced that the increase of amylase in endosperm of germinating maize seeds could not be accounted exclusively by the secretion of amylase from scutellum.

(4) Table 10 shows the calculation indicating that at the peak stage the amylase in the endosperm of germinating seedlings was equivalent to an amount of soluble protein several fold of the soluble protein in scutellum of the same seedlings. If the total amylase found in the endosperm had truly originated from the scutellum, then one would have to assume the secretion of amylase from the scutellum to the endosperm was in such a rate that after 8 days of germination, the cumulative amount of secreted amylase exceeded the total amount of soluble protein in the scutellum of the same seedling.

In view of the gene-dosage effect, the differential allelic expression and the quantitative changes of amylase content in germinating seedlings, it may be concluded that the scutellum and endosperm of maize seeds synthesize their

own amylase independently though the rates may be different. Thus, Amy-1 amylase is synthesized in both endosperm and scutellum and Amy-2 amylase is synthesized in scutellar tissue on the basis of gene dosage effects. The present evidence is contradictory to what has been suggested by Dure (1960) that alpha-amylase has its exclusive tissue origin in the scutellum, but does not necessarily exclude the possibility that amylases (Amy-1 and Amy-2) synthesized in scutellum might be secreted into the surrounding starchy endosperm at the initiation of seed germination.

In developing maize kernels, three tissues are possible sources of amylase in liquid endosperm. They are the aleurone layer, scutellum, and endosperm. The aleurone of maize consists of a single layer of endosperm cells lying beneath the two integument layers. Duvick (1961) paid special attention to the development of this cell layer in developing kernels. The protein granules in aleurone cells were made visible by histochemical staining under the light microscope. He found that the deposition of these granules begins 15-20 days after pollination of dent corns. In the mature seeds, the aleurone cells contain numerous protein granules which make up 36% of the whole seed fresh weight in contrast to the 10% fresh weight of the protein content in endosperm tissue.

Detailed anatomical studies of maize kernel development have shown that the diploid embryo tissue differentiates

into embryo axis and scutellum 12-14 days post-pollination (Randolph, 1936). In a fully mature kernel (about 45 days post-pollination) the embryo axis and the scutellum are connected by vascular bundles extended from the first internode of the embryo axis through the scutellar nodal region and distributed into the scutellar proper with numerous branches (Wolf, et al., 1951 a, b). The outer epidermal single cell layer of the scutellum comes into close contact with the mature endosperm tissues. These epidermal cells differentiate into club-like cells with the swollen ends intruding into the intercellular gap between the scutellum proper and the endosperm tissue. The morphology of these epidermal cells resembles those of typical glandular cells. This epidermal layer is not a smooth cover of the scutellum, instead, it has numerous furrows or crevices which may be as deep as 10-15 cells in depth. The scutellar proper consists of isodiametric parenchyma cells with distinct pits on the cell wall. The neighboring cells are connected by protoplasmic strands across the pit membrane (Wolf et al., 1951c).

The starch content in developing endosperm increases rapidly 12-20 days post-pollination (Wolf et al., 1948). The starch is deposited in amyloplasts and becomes conspicuous under the electron-microscope 20 days post-pollination (Creech, 1968). It is likely that starch-synthesizing enzymes (phosphorylases and pyrophosphorylases)

and starch-degrading enzymes are either situated in amyloplasts or else the amyloplast membrane is permeable to all these enzymes.

The physiological significance of the scutellum is suggested from its anatomical structure. It may serve as a secretory tissue furnishing starch-degrading enzymes to initiate starch degradation in the surrounding starch endosperm during seed germination, and meanwhile serve as an absorbing tissue that takes up nutrients from endosperm and translocates them through the vascular bundles to the embryo axis.

Edelman et al. (1959) found that scutellar tissue has all the necessary enzymes for converting hexose to sucrose. The sugar conversion was demonstrated in both isolated and attached scutella. In germinating maize seeds, the proportion of sucrose and free hexose is high in scutellum but low in endosperm. The vectorial secretion and absorption is apparently an energy dependent process in the scutellum, and is probably regulated by hormones as in the case of barley (Paleg, 1960; Chrispeels and Varner, 1967b), and oats (Simpson and Naylor, 1962; Naylor, 1966).

The observation of differential allelic expression of the *Amy-1* locus of maize is, to my knowledge, the first case that has ever been reported in plants. Similar cases had been reported in animals. For simplicity, these cases

are presented in Table 11. They all have the following features in common: (1) They are interspecific hybrids with the exception of carp, and the viability of all these hybrid embryos is low. (2) Two or more autosomal allelic isozymes are used as molecular markers for distinguishing specifically the maternal and paternal genomes involved. (3) In all cases, the maternal allelic isozyme was detected during early embryo development. The paternal allelic isozyme was detected together with the maternal allelic isozyme at a relatively late but specific stage during morphogenesis. (4) The early expression of the maternal allelic isozyme in hybrid embryonic tissue could be distinguished from the mere transmission of stable material allelic isozymes. These phenomena were not observed in the intraspecific hybrids. In fact, Ohno et al. (1968, 1969) demonstrated that allelic expression of alcohol dehydrogenase and 6-phosphogluconate dehydrogenase were asynchronous in chicken-quail hybrids and yet they are synchronous in quail-quail hybrids.

The differential allelic expression of maize Amy-1 in diploid embryo and triploid endosperm may be independent from each other. In immature kernels heterozygous with respect to the *Amy-1* locus, the expression of *Amy-1* in triploid tissue seems to be strictly maternal. The maternal Amy-1 alone was detected after germination in endosperm of the genotype $Amy\text{-}1^{A}/Amy\text{-}1^{A}/Amy\text{-}1^{B}$. In reciprocal F_1 seeds,

Table 11. Examples of preferential expression of maternal allelic isozymes in heterozygous individuals.

Organisms*	Isozymes**	References
Drosophila	Aldehyde oxidase	Courtright (1967)
Frog	Lactic dehydrogenase	Wright and Moyer, (1966)
Trout	Alcohol dehydrogenase	Hitzeroth et al., (1968)
	Lactic dehydrogenase	Goldberg et al., (1969)
	Lactic dehydrogenase (retina)	Hitzeroth et al., (ibid)
Chicken-Quail	6-phosphogluconate dehydrogenase	Castro-Sierra and Ohno, (1968)
	Alcohol dehydrogenase (liver)	Castro-Sierra and Ohno, (ibid)
Carp	6-phosphogluconate dehydrogenase	Klose and Wolf, (1970)

*With the exception of the Carp, all others are interspecific hybrids.

**Whole embryonic tissue extracts were used unless indicated otherwise.

the expression of both maternal and paternal allelic products were in synchrony in endosperm tissue with the genotype *Amy-1*^{B}/*Amy-1*^{B}/*Amy-1*^{A}. The same phenomenon is observed in endosperm tissues of the F_2 progeny. The expression of *Amy-1* in embryo tissue was preferential for *Amy-1*^{A} regardless of its parental origin and whether it was in developing kernels or in germinating seeds. But the onset of the paternal allelic expression in diploid tissues was not at a specific stage during the morphogenesis of the young F_1 seedlings.

The major difference of differential allelic expression in maize Amy-1 and the aformentioned cases in animals is that the former is specific for one allele (*Amy-1*^{B}) irrespective of its parental origin, while the latter is specific for maternal alleles. It thus seems reasonable to deduce that differential allelic expression in interspecific hybrids of animals involves the whole maternal genome in contrast to maize amylase in which a single gene is involved.

The differential allelic expression in individuals heterozygotes for Amy-1 can be explained by three possible mechanisms:

(1) Differential allelic activation: Two allelic genes have the same potential to be transcribed and translated. However, only one of the two allelic geneswas translated in early stages of seedling development.

(2) Allelic exclusion: Two cell types evolve during seedling development. The cell type carrying the *Amy-1*^{A} allele evolves earlier than does the cell type carrying *Amy-1*^{B}. When both cell types come to exist during morphogenesis, one would detect both *Amy-1*^{A} and *Amy-1*^{B} in the tissue extract as they were synthesized in one homogeneous population of cells.

(3) Repression of one gene product by the product of its allelic gene: *Amy-1*^{B} may be repressed by in heterozygous conditions. Should this be the case, we would expect to observe a reduced number of heterozygotes (A^1B^1 and B^1A^1) in the F_2 progeny of *Amy-1*^{A}/*Amy-1*^{B} and its reciprocal. However, the experimental finding was that there were excess numbers of A^1A^1 whose existence was apparently at the expense of A^1B^1. In addition to the early and the latent expression of *Amy-1*^{A} and *Amy-1*^{B} respectively there is also a difference in their expression from tissue to tissue (see Table 8). Thus, if the repression mechanism prevails one would have to explain why *Amy-1*^{B} is "repressed" in endosperm and stem tissues and is "derepressed" in leaf tissues. This hypothesis cannot be established since there was no evidence of *Amy-1*^{B} being repressed when the two allelic isozymes were mixed *in vitro*.

While the third possibility is rejected, there is no basis for me to evaluate the first and the second possible mechanisms, except by citing the case of autosomal inherited rabbit gamma-globulin allotypes. Dray (1962) has shown that the maternal gamma-globulin allotype A4 appeared earlier, and was in relatively larger amount than its allelic paternal gamma-globulin allotype A5 in the heterozygous offspring (A_b^4 / A_b^5). By immunizing the maternal parent against A5 gamma-globulin prior to the mating A_b^4 A_b^4 x A_b^5 A_b^5, the paternal A_b^5 in the newborn was repressed while the maternal A_b^4 allele was fully functioning. The concentration of A4 was 1.8 times the amount of the controls while A5 was only 5% of the controls. Pernis et al. (1965) had successfully employed rhodamine and fluorescein in their double immuno-fluorescence straining technique to show there were two populations of plasma cells in the germinal centers of lymphoid follicles of heterozyote rabbits as characterized by two mutually exclusive gamma-globulin allotypes, (A4 and A5), controlled by allelic genes A_b^4 and A_b^5 respectively.

CHAPTER VI

CHEMICAL IDENTIFICATION AND CHARACTERIZATION OF STARCH-DEGRADING ENZYMES IN MAIZE

Introduction

The starch-degrading enzymes in maize liquid endosperm can be separated electrophoretically into three zones. Genetic and developmental studies have indicated that these three starch-degrading enzymes are inherited individually and are regulated independently in various tissues during the course of seed germination. The two immediate questions concerning these starch-degrading enzymes are (1) whether these enzymes can be identified individually as alpha-amylase, or beta-amylase, or phosphorylase: (2) how these amylases compare with other amylases in terms of their physico-chemical properties. The studies reported in this chapter entail the identification of Zone-3 as phosphorylase, Amy-1 of Zone-1 and Amy-2 of Zone-2 as amylases and some characteristic properties of Amy-1 amylase. The latter was studied more extensively than the others because Amy-1 has been shown to be a stable amylase at various developmental stages of maize.

Literature Review

Detection of Amylase Activity

Amylase degrades starch to dextrins and oligosaccharides. The amylolytic activity of the enzyme can be measured either by the decreased degree of polymerization, or the liberation of reducing sugars. The degree of polymerization of starch can be measured by three possible ways:

Turbidimetric method.--This technique is based on measurements of the reduction in turbidity of starch solutions after incubating with amylase-containing material. This method was adapted to clinical diagnosis of amylase activity in body fluids; serum, saliva, pancreatic juice, and urine which has high amylase content. Ware et al., (1963) found the reduction of turbidity was linearly proportional to the units of maltose liberated from starch substrates.

Starch-iodine method.--The intensity of starch-iodine color depends on the average size of starch or dextrin molecules (Bailey and Whelan, 1961). The reduction of starch-iodine color as a result of amylolysis can be monitored spectrophotometrically at a wave-length of maximal absorbance (Smith and Roe, 1949).

Reducing-sugar method.--The liberation of reducing sugars, such as glucose and maltose, can be measured spectrophotometrically on the basis of reduction-oxidation of

selected chromophores. This is generally achieved by the following four methods: Copper reduction (Somogyi, 1938), ferricyanide reduction (Fingerhut et al., 1965), dinitrosalicylic acid reduction (Miller, 1959) and neocupronic method (Strumeyer, 1967).

All the forementioned methods are either based on the decreasing degree of polymerization of starch or the release of reducing groups for measuring amylase activities. They are not discriminating toward alpha-amylase and beta-amylase since both enzymes cleave alpha 1,4-glucosidic bonds at C_1-0 linkages (Mayer and Larner, 1959), and release oligosaccharides and maltodextrins. However, the two amylases have different modes of action on starch. Alpha-amylase attacks starch molecules randomly from ends with reducing groups. Hence, the resulting decrease in the degree of polymerization of starch is proportional to the concentration of alpha-amylase and the time of the enzymatic action. Beta-amylase attacks starch molecules from non-reducing ends only, and breaks off maltose units sequentially along the polysaccharide chains. Hence, the degree of polymerization of starch is not affected initially while the release of maltose is proportional to the enzymatic action. For this reason, assaying reducing groups rather than measuring decreases in viscosity of starch (by viscometric method) are used in detecting beta-amylase activities. For measuring alpha-amylase, both the

viscometric method and the starch-iodine method can be used, although the latter is more sensitive to low alpha-amylase concentrations and is the method commonly used.

Baily and Whelan (1961) found that the maximal absorbance wave-length (λmax) of amylose--iodine complex is in the range of 600-620 mμ for amylose with a chain length longer than 135 glucose units. The λmax shifts toward a shorter wave-length when the chain length of amylose in the complex becomes shorter than 135 glucose units. The shift is however, not appreciable until the chain is less than 80 glucose units long. The λmax is 550 mμ for amylose molecules with a chain length of 36 glucose units long. Since the degree of polymerization of potato starch is in the order of several thousands, the wave-length at 620 mμ may be used for measuring amylolytic degradation of potato starch. Chrispeels and Varner (1967a) had devised a starch-iodine method by employing potato starch and measuring the change of absorbance at 620 mμ. They found that the change of starch-iodine absorbance at 620 mμ in the range of 15-80% of the initial absorbance caused by a purified barley malt alpha-amylase is linearly proportional to the enzymatic action.

In my opinion, the reducing sugar methods for measuring amylase activity has two disadvantages: (1) The reducing groups resulting from amylolysis of starch are heterogeneous in nature: including glucose, maltose,

various maltodextrins as well as newly exposed reducing ends of residual dextrins. (2) The amylase activity is usually expressed in units of maltose released from starch per minute per unit volume of the enzyme despite the fact that maltose may or may not be the predominant fraction of the reducing sugars released from starch molecules (Greenwood and Milne, 1968). Besides, the frequency distribution of oligosaccharides and maltodextrins resulting from initial amylolysis of starch varies with enzyme concentration (Banks, et al., 1970).

Detection of Phosphorylase Activity

Plant phosphorylase catalyzes the degradation of starch (G_n) in the presence of inorganic phosphate (P_i) and liberates glucose-1-phosphate (G-1-P).

$$G_n + P_i \xrightleftharpoons{\text{phospho-rylase}} G_{n-1} + \text{G-1-P}$$

The reaction is readily reversible, but the reaction equilibrium constant is pH dependent. The equilibrium constant is determined by the concentration of inorganic phosphate and glucose-1-phosphate. At pH 7.0, the equilibrium constant is about three times as much as it is at pH 5.0 (Trevelyan et al., 1952). The pH optimum of purified potato tuber phosphorylase for degrading starch is 6.5 (Lee, 1960). Plant phosphorylase also can synthesize straight chains of amylose *in vitro* by

transferring glucose from glucose-1-phosphate and adding it to non-reducing ends of maltodextrins. The maltodextrins serve as primers for enzymatic propagation of amylose chains. Parrish et al. (1970) used purified maltodextrins of various chain lengths (G_2 - G_7) to test K_m values of potato phosphorylase. They found the Km values for G_3 is 1.3×10^{-3} moles, 1×10^{-4}M for G_4, and 6.6×10^{-5}M for G_5. Lee (1960) reported a Km value of 5×10^{-3}M at pH 6.3 when amylopectin was used as the primer for potato phosphorylase.

Plant phosphorylase isozymes can be demonstrated by acrylamide gel electrophoresis. Siepmann and Stegemann (1967) devised a method for the detection of potato phosphorylases on the basis of the starch-iodine staining reaction. They incorporated glycogen into acrylamide gels on which phosphorylases were to be separated by electrophoresis. Upon the completion of electrophoresis, they incubated the gels in glucose-1-phosphate solution in acetate buffer, pH 5.6. The gels were then stained with potassium iodide-iodine solution. The phosphorylases were located on the gel-matrix as the synthesized polymer gave a positive starch-iodine staining reaction.

Distinctive Differences Among Various Plant Starch-degrading Enzymes

Phosphorylase is readily discernible from amylases since starch-degrading activity of this enzyme is dependent on the presence of inorganic phosphate (Pi), whereas the

amylolytic activity of amylase is Pi independent. In the presence of malto-dextrin primer and glucose-1-phosphate, phosphorylase but not amylase can catalyze starch synthesis (Hanes, 1940).

Among plant amylases, there are three known distinct types existing in nature. Their differences are primarily in the mode of action on the same substrate, starch. Alpha-amylase attacks alpha-1, 4-glucosidic bonds from the reducing ends of starch molecules, liberating glucose (G_1), maltose (G_2), and maltodextrins of various lengths (G_3 - G_7), but by-pass alpha-1, 6-glucosidic branching points along the polysaccharide chains. The alpha-amylase fragmented oligosaccharides all possess C_1 anomeric carbon in the alpha-configuration as defined by its direction of optical rotation. Beta-amylase cleaves maltose units sequentially from non-reducing ends of starch molecules, and unlike alpha-amylase it is not able to by-pass alpha-1, 6-glucosidic branching points but stops at two to three glucose units from the branching points, thus leaves the portion of glucan between the branching points untouched (Hopkins, 1946). The liberated maltose units are in the beta-configurations (Freeman and Hopkins, 1936, Mayer and Larner, 1959). Gluco-amylase is only found in fungi. This amylase, upon attacking starch yields only glucose, and it works from both reducing and non-reducing ends of starch amylose molecules (Pazur and Ando, 1959).

Besides the distinction in the mode of action of various amylases mentioned, they also differ in their pH sensitivity and thermal stability (Hopkins, 1946). Alpha-amylases are more labile to low pH, and more stable at 70C than beta-amylases.

Experimentally, alpha and beta-amylases are distinguished from each other on the basis of the chemical and physical properties of two well characterized amylases, hog pancreatic alpha-amylases and sweet potato beta-amylases. The methods which have been applied to identify alpha- and beta-amylases of various biological sources are summarized in the following table (Table 12).

Materials and Methods

Purification of Maize Amylases

The aim was to purify the two maize amylases, Amy-1 and Amy-2, which had been elucidated genetically and developmentally (Chapters IV and V). Special attention was given to the starting maize material for purifying Amy-1 and Amy-2 amylases since developmental changes of amylase patterns were observed, particularly the appearance of new bands in Zone-1 and the apparent isozyme pattern shifts in Zone-2 of germinating seedlings (see Chapter V). The amylase patterns in developing kernels of inbred strains were, however, consistent from 6 days to 30 days after pollination. During this period of kernel

Table 12. Criteria used in identifying alpha-amylases and beta-amylases.*

Criteria	Methods	Alpha-amylase	Beta-amylase
Action pattern**	Chromatography of enzyme digest of starch	$\underline{G_1} + \underline{G_2} + \underline{G_3}$ ∓ malto-dextrins	$\underline{G_1} + \underline{G_2}$ + Beta-limit dextrins
Opticat rotation of end-products	Polarimetric technique	decrease of OR toward EQ	increase of OR toward EQ
Limits of enzymic action	1. Beta-limit dextrin prepared from starch digested by sweet-potato beta-amylase.	further degradation	no further degradation
	2. λmax of limit-dextrin-iodine complex absorbance	620-500 mμ	620 mμ (blue)
pH sensitivity	Enzymic activity at pH 3.5 vs. that at pH 6.8	loss of activity up to 90% in 30 minutes	loss of activity up to 15% in 30 minutes
Thermal stability	Enzyme incubated at 70 C vs. at 23 C	slight loss of activity up to 20% of initial activity	loss of activity up to 75% of initial activity

*See Hopkins, 1964; Pazur, 1965; and Greenwood and Milne, 1968.

**Predominant end-products are underlined.

development, peak protein synthesis was observed about 16-20 days post-pollination (Duvick, 1961). Hence, immature kernels of 16-20 days old from strains homozygous for *Amy-1* or *Amy-2* were used as starting materials to purify the two amylases.

Initially, glycogen specific absorption of alpha-amylase (Schram and Loyter, 1966), and acetone precipitation were tried without any success in selective isolation of Amy-1 and Amy-2. Ammonium sulfate precipitation, and ethanol fractionation were used. Results presented later will show that ethanol fractionation is more desirable than ammonium sulfate precipitation. Amy-1 alone was found to be soluble in the 60% ethanol soluble fraction. Since Amy-1 and Amy-2 can be physically separated more readily by electrophoresis than by their differential solubility in organic solvents or ammonium sulfate, the purification of Amy-1 was carried further by a step of electrofocusing whereby Amy-1 may be isolated at its specific isoelectric pH. The following three steps were used as a general procedure to isolate Amy-1 amylase.

(1) 200-300 g of immature kernels, stripped from freshly thawed ears, were homogenized in a Waring Blender for 15 minutes without adding any buffer. The homogenized slurry was poured into a pair of 500 ml Sorvall centrifuge bottles and spun at 3,000 g for 1 hour in a Sorvall RC-3

refrigerated centrifuge (10 C). The supernatant was collected and chilled to 5 C before it was fractionated with ethanol.

(2) This step was carried out at 5 C. The volume of crude supernatant was measured and a total of 1.5 times that volume of chilled absolute ethanol was added slowly to the crude supernatant in a 500 ml volumetric flask which was half buried in an ice bucket and swirled while adding ethanol. The final concentration of ethanol was brought up to 60%, in 10 minutes. The cloudy solution was allowed to settle for another 5 minutes. The precipitated protein was removed by centrifugation at 12,000 g for 30 minutes in a Sorvall RC-2B centrifuge. A lipid layer floating at the top of the tube was removed and the clear ethanol soluble fraction was then transferred into several dialysis tubing (2 inches width) and dialysed against 20 volumes of Tris-HCl buffer (0.05M, pH 7.5) with 1 mM $CaCl_2$ for 36 hours with two changes of the buffer during the process. After the dialysis, the enzyme solution was again centrifuged at 12,000 g for 30 minutes to remove precipitated proteins. The amylase isozyme present in this fraction was examined by gel electrophoresis to make certain the presence of Amy-1 and the absence of Amy-2. This fraction is hence referred to as the ethanol fraction of Amy-1. Enzyme solutions were found to retain their specific activity (ca. 0.2 units/mg protein) at 5 C for several weeks.

(3) The ethanol fraction of Amy-1 was used for isoelectric focusing to separate Amy-1 from other proteins. An electrofocusing column with a volume of 110 ml (LKB 810 LKB Lab Instruments) was used. To establish a pH gradient along with a density gradient, Ampholyte carrier solution (40% Ampholine, LKB) and glycerol were used. Electrofocusing was done according to the method described in the LKB manual. I used glycerol instead of sucrose because the latter was found to interfere with amylase assays in the starch-iodine method. Sucrose concentration in fractions collected after electrofocusing can be as high as 30%. In the amylase assay, 15-20% sucrose concentration will cause a decrease of 0.3 units of OD_{620} in the standard assay. The compositions of gradient solutions and eletrolyte solutions for electrofocusing in the range of pH 3-6 are given in the following:

1. Light gradient solution:
   - Ampholine (10%) 2.5ml
   - Ethanol frac. of Amy-1 52.5ml

2. Heavy gradient solution:
   - Ampholine (10%) 7.5ml
   - Glycerol 36.0ml
   - Distilled water 11.5ml

3. Cathode solution:
   - 1% NaOH 10 ml

4. Anode solution:
   - H_2SO_4 0.2ml
   - 80% glycerol 21.5ml

The electrofocusing was carried out with a constant voltage power supply (LKB 3371D). The initial voltage was set at 420 volts with an initial current in the range of 4-5 ma depending on the amount of protein present in the electric field. The temperature of the electrofocusing column was maintained at 10 C by circulation throughout the external and internal jackets with water from a thermostat water cooler (LAUDA Model WB-20/R Brinkman Instruments). The electric current of the electrofocusing unit gradually drops as proteins settle at their isoelectric points along the linear pH gradient. Eventually, the current stabilizes at 0.8-1.0 ma indicating nearly all the proteins and peptides (Ampholyte carrier) in the electric field have been neutralized at their isoelectric points. The whole process of electrofocusing generally takes 60 hours. Constant volume fraction was collected by draining the gradient solution from the bottom of the column at a flow-rate of about 1 ml/minute with the aid of an LKB fraction collector (LKB Ultrorac 7000). Amylase activity was measured within 24 hours after the completion of electro-focusing to avoid possible decay of the enzyme at the pH of its isoelectric point. The amount of protein of each fraction was estimated by UV absorbance at 280 mμ, or by Lowry's method on properly diluted fraction samples. The pH value of each fraction was measured with a microelectrode and an Orion Model 801 digital pH meter (Orion Research

Inc., Mass.). The pH meter was standardized before use with pH 4.0 and pH 7.0 standard buffers. The errors of repetitive readings are in the range of 0.05 units. Amylase peak fractions were sampled and further assayed by gel electrophoresis to verify the location of the Amy-1 peak along the linear pH gradient.

End-product Analysis of Amy-1 and Amy-2

Chromatography.--Amy-1 and Amy-2 were eluted from four (20 x 20 cm) poly-acrylamide gels after electrophoresis. The two amylases were located on the unstained gels by staining a longitudinal strip cut out from each one of the (20 x 20 cm) gels. The poly-acrylamide gel strips containing Amy-1 and Amy-2 were macerated separately in a procelain mortar with a pestle. Two ml of Tris-HCl buffer (0.05 M pH 7.5, and 1mM $CaCl_2$) was added to the minced gel preparations. The mixture was centrifuged for one hour at 30,000 g in centrifuge tubes specially designed for this purpose. The centrifuge tube set consists of 2 plastic Nalgene centrifuge tubes; one 10 ml and one 5 ml with the small one fitting easily into the large one but the rim of the small tube keeps it situated at the top of the large tube. A pin-hole is punctured at the bottom of the small tube, and is covered with a layer of wet glass wool. On top of the glass wool, 0.5 gm of Sephadex G-25 powder was layered. The acrylamide gel was minced in the buffer and was loaded in this small tube which in

turn fitted into the large tube. After centrifugation, 0.3 ml of each eluate containing about 6 µg protein were obtained, and were incubated with 1 µc ^{14}C-starch (4 µg of tobacco leaf ^{14}C-U-starch from Calbiochem) for 12 hours at 23 C. At the end of incubation, undegraded starch was precipitated out by ethanol (70%). The supernatants were brought to dryness in a lyophilyzer, and then dissolved in 0.15 ml of water just before paper chromatography. The descending chromatography was done on Whatman No. 1 paper with a solvent system containing ethyl acetate-pyridine-water mixture (8:2:1, w/v). The autoradiogram of their products was made on Kodak No-Screen X-ray film (NS 54T) by pressing it against the paper chromatogram for three weeks. The amount of radioactivity in each spot was then measured by a liquid-scintillation counter (Beckman Model CPM-100) with 90% efficiency for carbon-14. Non-radioactive glucose and maltose were co-chromatographed with the radio-active end-products and located by the aniline phthalate reagent.

The ^{14}C-end-products were also chromatographed on Kieselguhr TLC plates (0.25 mm thickness) using a solvent system consisting of butanol-2, 6-lutidine-water mixed in 6:3:1 ratio (Weill and Hanke, 1962). The autoradiograms were made similarly as mentioned above.

The action pattern of Amy-1 amylase on soluble starch was studied by using the ethanol fraction of Amy-1 and a

1% potato starch solution. The end-products were separated on Whatman No. 1 paper with a solvent system containing nitromethane-ethanol-water (40:41:23, v/v). The reducing sugar spots were located by the silver nitrate reagent.

Detection of glucose in the enzymic digest with Glucostat.--The Glucostat was purchased from Worthington Biochemical Corporation. The principle of glucose detection is outlined below:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \underset{\text{(colorless)}}{\text{o-dianisidine}} \xrightarrow{\text{peroxidase}} \underset{\text{(brown)}}{\text{oxidized o-dianisidine}}$$

The color of oxidized chromogen is directly proportional to the amount of glucose present in the reaction mixture. The glucose oxidase used in the Worthington Glucostat was purified from Aspergillus niger. There are two special features of this commercially available glucose oxidase: (1) Although the enzyme is specific for beta-glucose, the enzyme preparation contains sufficient amounts of mutarotase which ensures the enzymic oxidation of alpha-glucose as well as beta-glucose (Dahlqvist, 1961). (2) This glucose oxidase preparation contains a low level of maltase which can be inhibited by high concentration of Tris (White and Subers, 1961). Dahlqvist (1961) found that 80% of the maltase activity was inhibited at pH 7.0 by 0.5M Tris, and the inhibition is competitive with maltose. High

maltose concentration can reverse the Tris inhibition on the maltase.

The presence of mutarotase and maltase in the Glucostat is not disadvantageous but makes the assay method more versatile since it allows the detection both of glucose and maltose from the mixture of the two sugars. The detection of glucose alone can be made by adding amylase and starch to the Glucostat in 0.13M Tris buffer. The detection of both glucose and maltose can be done in the same reaction mixture by replacing Tris buffer with phosphate buffer, thus lifting the inhibition of maltase which will convert maltose to glucose and add it to the total amount of glucose detected. Thus if an unidentified amylase degrades starch to glucose, this end-product alone can be detected by Glucostat while the maltase activity in the Glucostat is completely inhibited. Alternatively, if the end-product is mainly maltose, it will not be detected by Glucostat in Tris buffer, but it can be detected when maltase is active in the absence of Tris from the reaction mixture.

Beta-limit-dextrins

Beta-limit dextrin was made from a soluble starch (Lintner starch, Fisher Scientific Co.) solution which was exhaustively digested by sweet-potato beta-amylase (Robyt and French, 1963). To 200 ml of 1% starch solution which was made in acetate buffer (0.05M, pH 4.8), 60 µl of purified sweet-potato beta-amylase (Worthington

Biochemical Corp.) was added. The solution was incubated at 23 C with constant stirring by a magnetic stirrer. The starch digest was sampled periodically to check the starch-iodine color. The observation of no further decrease of starch-iodine color signifies the end-point of beta-amylolysis. To assure the complete beta-amylolysis, 1 ml of the enzyme digest was pipeted out to mix with 20 µl of sweet potato beta-amylase, and incubated for 1 minute at 23 C and the reaction was stopped by adding 1 ml of iodine reagent (5×10^{-3} M). No change of starch-iodine color was taken as evidence for complete beta-amylolysis. Another 40 µl of sweet potato beta-amylase was added to the bulk of the digested starch and the incubation was continued for 2 hours. The digested starch was then heated in a boiling water bath for 30 minutes to inactivate all the beta-amylase. The beta-limit dextrin solution thus prepared upon complexing with iodide became blue-purple, and was apparently susceptible to further amylolysis by hog pancreatic alpha-amylase. The beta-limit dextrin solution was stable at 5 C for several weeks.

The enzymic actions of Amy-1 and Amy-2 on beta-limit dextrin were examined in two ways: (1) Test-tube assay with isolated Amy-1. (2) Gel assay with Amy-1 and Amy-2 electrophoretic zymogram.

Test-tube assay with isolated Amy-1.--Both electrofocused Amy-1 and ethanol fractionated Amy-1 were used. The dextrin-iodine color changes were recorded.

Gel assay.--Crude amylases were subjected to acrylamide gel electrophoresis to separate Amy-1 and Amy-2 physically from each other in the gel matrix. Beta-limit dextrin was incorporated into a sheet of agar gel (1% Bacto-agar in distilled water) of the same size as the acrylamide gel on which Amy-1 and Amy-2 were separated. Likewise, an agar gel with 0.1% soluble starch was made. The acrylamide gel lifted out from the gel tray was sandwiched between the starch-agar gel and beta-limit-dextrin-agar gel. The three gels were laid in a moisture chamber and incubated at room temperature for 12 hours. The two agar gels were stained with 0.01 N KI-I_2 following the incubation. As a result, two prints of a single amylase band could be obtained: one on the starch-agar, and one on the dextrin-agar. The acrylamide gel served as a control. It was further incubated in a 1% starch solution for 6 hours and stained for amylase to make certain of the presence of and the separation of Amy-1 and Amy-2.

Polarimetric Technique

A perkin-Elmer model 141 polarimeter was employed. A starch solution (0.15% Nutritional Biochem potato starch in pH 4.8, 0.04M potassium phosphate buffer) was introduced into a 5 ml beaker cell with a 10 cm light path. The cell was aligned with its long axis to the sodium light beam (589 mμ). The cell containing the starch solution was left in the polarimeter to equilibrate with the internal

temperature for 30 minutes. The optical rotation reading was set to zero prior to the addition of amylase. To accomodate the added volume of enzyme solution in the filled beaker cell, 0.5 ml of starch was withdrawn and 0.5 ml of enzyme solution was added. The cell was inverted several times for thorough mixing and then placed back into the polarimeter. Optical rotation readings were taken every 2 minutes. After 30 minutes of amylase action on the starch, 0.5 ml of 10% Na_2C0_3 was added to the beaker cell to bring the pH up to 10. This alkaline condition accelerates the spontaneous mutarotation of oligosaccharides to reach the equilibrium point whereby the proportion of sugars in alpha and beta D-glucose are constant. For instance, the alpha-D-glucose and beta-D-glucose ratio in solution will be 1 : 2 at the equilibrium point. The direction of optical rotation in approaching that of equilibrium indicates what form of optically active oligosaccharides were present initially. Accordingly, alpha-amylase gives rise to oligosaccharides in the alpha-form which cause a decrease of optical rotation reading while approaching alpha and beta equilibrium by mutarotation. Conversely, beta-amylase gives rise to oligosaccharides in the beta-form which in turn cause an increase in optical rotation reading while the mutarotation is approaching the equilibrium. The critical optical rotation

readings during this process were taken at every 2 minute intervals for 30 minutes.

Molecular Weight Estimation of Amy-1 Amylase

Gel filtration on Sephadex columns: The elution volumes(Ve) of electrofocused Amy-1 amylase from a Sephadex G-75 column (84 cm packing length and 2 cm inner diameter) and a Sephadex G-200 (59 cm packing length and 1.8 cm inner diameter) were measured and compared to the Ve of lysozyme and hemoglobin (human hemoglobin with ferrous iron, Calbiochem Co.). The columns were calibrated with bromophenol blue (MW = 670, Fisher Scientific Co.) for the total bed volume (Vt) and blue dextran (MW = 200,000, Pharmacia Fine Chemicals, Inc.) for the void volume (Vo). The columns were eluted with Tris-HCl buffer (pH 7.5, 0.05M) containing 1 mM $CaCl_2$. Fractions of equal number of drops (70 drops/tube) were collected with an LKB fraction collector (Ultrorac 7000). The volume of each tube was measured by the average volume of ten tubes and it was estimated to be in the range of 4.2 - 4.3 ml per tube.

Protein was estimated spectrophotometrically by UV absorption at 280 mμ with either a Beckman DU or a Gilford 2400 spectrophotometer. Hemoglobin concentration in each fraction was measured by light absorbance at 410 mμ, the λmax for heme. Lysozyme activity was measured by the decrease of turbidity of Micrococcus lysodeikticus cell

(Sigma Chemical Co.) suspensions at 450 mμ. The reaction mixture of lysozyme assay consists of 1.5 ml of the cell suspension (0.3 mg/ml) in sodium phosphate buffer (0.06M, pH 6.2) and 0.05 ml of lysozyme solution. The reaction was carried out directly in a 2 ml Coleman round cuvette, and the decrease of OD_{450} in the first 2 minutes following the initial 15 seconds of mixing was recorded from Coleman Jr. II spectrophotometer (Model 6/35). Under this condition, 10 μg of lysozyme (Worthington Biochemical Co.) caused a drop of 0.008 OD units in two minutes. The change of OD is proportional to the lysozyme concentration ranging from 0.01 - 0.20 mg/ml.

Sucrose gradient centrifugation.--Molecular weight estimation of Amy-1 was done by comparing the apparent sedimentation coefficient of Amy-1 with that of lysozyme, according to Martin and Ames' method (1961). Sucrose gradients were made of 5 - 20% ultra pure sucrose (Mann Research Laboratory, New York) in a double chamber with a capacity of 4 ml in each chamber gradient apparatus (Brinkman Instruments Co.) and aided by a motor-driven stirrer (Buchler Instruments Co.). The procedure for making sucrose gradients described by Martin and Ames (1961) was followed in all experiments except one experiment in which convex exponential sucrose gradients were prepared according to Noll (1967). Sucrose gradients were made in 1/2" x 2" Beckman Polyallomer tubes. The amylase samples

were prepared from either electrofocused peak fractions or concentrated crude extract of germinating maize seedlings of known genetic make-up with respect to Amy-1 (homozygous for *Amy-1*^{A} or *Amy-1*^{B} or heterozygous for Amy-1 when mixtures of Amy-1A and Amy-1B were desired). A volume of 0.2 ml amylase samples were layered on the gradient by floating it on top of 5% sucrose solution. The layered protein samples were further stabilized with 0.5 ml parafin oil (Saybolt viscosity 125/135, Fisher Scientific Co.).

A swinging bucket rotor, SW 65 LTi (Beckman) was used in the ultra-centrifuge (Beckman, L2-65B). The experimental conditions for centrifugation are summarized in Table 16. At the end of each centrifugation, the rotor was allowed to coast to a halt without brake.

Fractions of centrifuged gradients were collected with the following two methods:--(1) Automatic fraction collection: The tubes were fixed in a metal ring and a hole was punctured from the bottom of the tube with the sharp end of a hypodermic needle. The efluant solution from the tube was forced out with an automatic syringe pump set at a constant flow rate of 0.325 ml/minute. The efluant was led by a tubing from the blunt end of the syringe needle to a quartz flow cell with 0.2 ml capacity and 0.4 cm path length (LKB, Lab. Instruments) placed in the light path of a spectrophotometer (Gilford 2400).

Protein was detected by UV absorbance at 280 mμ. The readings were directly recorded on a Gilford recorder connected to the spectrophotometer and operated at a constant chart speed, 1 in/minute. (2) Manual fraction collection: The tube was punctured with a hypodermic syringe needle (Gauge No. 22) with one blunt end and the other end filed to a wedge shape. Three-drop fractions were collected into a series of glass vials with 4 ml capacity. These fractions were stoppered and stored at 5 C until assays could be made.

Preparation of Rabbit Antiserum Against Amy-1A Amylase

Two New Zealand white rabbits, 4 months old, were immunized against Amy-1 amylase prepared from ethanol fractionation followed by electrofocusing. The initial immunization series consisted of four injections. The second and third injections were given on consecutive weeks, two weeks after the first injection. The fourth injection was 19 days after the third. Each injection consisted of Amy-1 amylase prepared from a fresh electrofocused fraction of Amy-1A amylase. The amount injected varied from preparation to preparation, and ranged from 1 - 2ml of focused Amy-1A fraction containing 0.5-2.5 mg protein with amylase specific activity being 0.3-0.5 units/mg protein. In the second injection, 1.0 ml of complete Freund adjuvant (Difco Laboratories) was injected

together with 1 ml amylase sample into the two rabbits. Blood samples were taken from the ears of each rabbit at the time of the second, third and fourth injections, and tested with Amy-1A amylase as the antigen by immunodiffusion methods, using the Ouchterlony double diffusion technique. Since no precipitin bands were detected in the test made at the end of the 4th injection, a "booster" injection was given 50 days after the initial immunizing series. Blood was obtained by heart puncture a week later. Clean serum was collected, divided into aliquotes and stored at -50 C.

Immunodiffusion was carried out with microslides (2.6 x 7.5cm) bearing a 1% agar (Difco Bacto agar) in 0.85% NaCl solution with precut 9 well patterns. The well pattern consisted of 1 center well and 8 peripheral wells. The distance from the peripheral wells to the center well was 0.8cm and that between neighboring wells was 0.5 cm. Antibody was placed in the center well.

Results

Identification of the Zone-3 Starch-Degrading Enzyme as Phosphorylase

Although three zones of maize starch-degrading enzyme activities are evident on zymorgrams, they could be either amylases or phosphorylases. The distinctions between amylase and phosphorylase were investigated directly on zymograms. There are two lines of evidence to show that

only Zone-3 has phosphorylase activity: (1) This zone is capable of synthesizing starch in polyacrylamide gels (Fig. 11) prepared and incubated according to the procedure devised by Siepmann and Stegemann (1967). (2) No starch-degrading activity could be detected in Zone-3 when the gel was incubated in 1% starch solution (pH 7.0) in the absence of orthophosphate. The genetics and chemistry of this phosphorylase was not pursued further.

Purification of Maize Amylases

Ethanol fractionation.--Amy-1 amylase of Zone-1 is more soluble in ethanol than both Zone-2 (including Amy-2) and Zone-3 (phosphorylase). Crude enzyme preparation from maize immature kernels was treated with ethanol at 5 C. The concentration of ethanol in the enzyme preparation was gradually brought to 60%. Stepwise ethanol soluble fractions and ethanol precipitated fractions were checked zymographically. Amy-1 alone was detected in the 60% ethanol soluble fraction. A gradual loss of Zone-2 and Zone-3 activity was observed during the stepwise increase of ethanol concentration. Fig. 12 shows that Zone-3 (phosphorylase) is eliminated from the 40% ethanol soluble fraction. Zone-2 amylase is present in the 45% ethanol soluble fraction, but is absent in the 50% ethanol soluble fraction. Amy-1 is the only soluble form of amylase in 60% ethanol. Quantitative data on total amylolytic activity

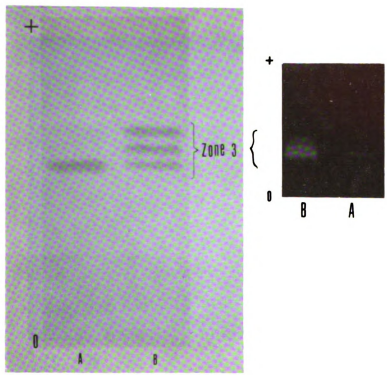


Fig. 11. Photographs of the zymogram showing the phosphorylase activity of Zone-3 of the starch degrading enzymes of maize liquid endosperm. A = one kernel of the inbred W64A; B = one kernel of the inbred 58-3-6. The polyacrylamide gel on the left was prepared as usual except 0.3% glycogen was dissolved and incorporated into the gel and was run for 24 hours. The gel was then incubated at 23 C in 0.2% glucose-1-phosphate dissolved in 0.1 N sodium acetate buffer, at pH 4.8 for 20 hours. After incubation, the gel was washed and stained with KI-I_2 for identifying phosphorylase isozymes. The result showed only Zone-3 with clear positive reactions. The polyacrylamide gel on the right was incubated in starch solution prepared in pH 7.0 phosphate buffer (0.04 M) for 5 hours and then stained with KI-I_2 for identifying the starch-degrading activity of the phosphorylase isozymes. 0 = point of sample insertion.

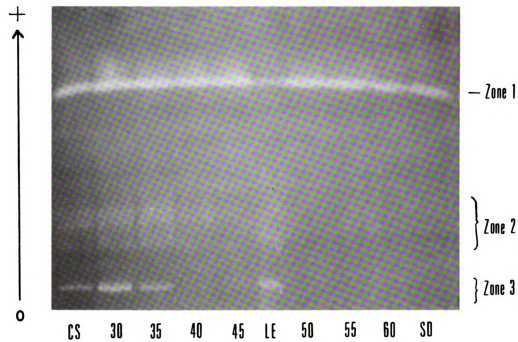


Fig. 12. Zymogram showing maize starch-degrading enzymes in ethanol soluble fractions. Numbers on the horizontal axis indicate the ethanol concentrations in each sample. CS = Crude supernatant, LE = Liquid endosperm, SD = 60% ethanol soluble fraction after dialysis. 0 = point of sample insertion.

in each fraction are presented in Table 13a and Table 13b. The specific activity of the amylase was increased 3-fold in the first experiment (Table 13a), and 15-fold in the second experiment (Table 13b). The extent of Amy-1 purification is probably underestimated since the starch-iodine assay method is non-discriminatory toward the two amylases and the phosphorylase. Hence, the initial amylase activity was a collective contribution of all three starch-degrading enzymes. The amylase activity in the final step (60% ethanol supernatant) however, is solely due to Amy-1 amylase.

The total amylase activity was notably increased during the process of ethanol fractionation. In fact, a 3-fold increase was found in the first experiment (Table 13a) and a 10-fold increase was found in the second experiment (Table 13b). The total amylolytic activity present in the 50% ethanol soluble fraction was nearly doubled at 60% ethanol fractionation in both experiments. One possible interpretation is that Amy-1 amylase was bound to membrane material initially and it was partially freed in 50% ethanol and to a greater extent in 60% ethanol. The solubilized Amy-1 is more active than the bound Amy-1 amylase.

Ammonium sulfate fractionation.--The supernatant of crude extract from immature kernels was prepared and a calculated amount of ammonium sulfate (Reagent grade, J. T.

Table 13a. Summary of ethanol fractionation of Amy-1 amylase in experiment I.

Fraction	Volume	Total amylolytic activity	Total protein	Specific activity
	ml	units	mg	units/mg
Crude supernatant	60.0	1,320	67.8	19.5
30% EtOH supernatant	87.0	1,392	80.9	17.2
35% EtOH supernatant	93.8	1,313	65.6	20.0
40% EtOH supernatant	102.0	1,122	67.3	16.7
45% EtOH supernatant	115.5	1,848	70.5	26.2
50% EtOH supernatant	120.0	1,560	81.6	19.1
55% EtOH supernatant	137.0	2,192	76.7	28.6
60% EtOH supernatant	154.5	3,399	81.9	41.5

Table 13b. Summary of ethanol fractionation of Amy-1 amylase in experiment II.

Fraction	Volume	Total amylolytic activity	Total protein	Specific activity
	ml	units	mg	units/mg
Crude supernatant	54	1,188	151.2	7.9
30% EtOH supernatant	77	1,340	194.0	6.9
40% EtOH supernatant	90	1,530	191.7	8.0
45% EtOH supernatant	99	2,080	159.4	13.1
50% EtOH supernatant	108	5,400	140.4	38.5
55% EtOH supernatant	120	9,850	109.2	90.1
60% EtOH supernatant	135	10,500	86.4	121.5

Baker Chemical Co.) was added to it to make the solution 40% saturated with the salt, and allowed to stand at 5 C for 5 minutes. The pellet was suspended in a minimal volume of the grinding buffer (0.025M glycyl-glycine pH 7.4). The supernatant was reused in the next step with increasing ammonium sulfate concentration. The quantitative data are summarized in Table 13c. The amylase activity was lost along with non-specific proteins as the ammonium sulfate concentration was raised to 90% of its saturation. Qualitatively, all three starch-degrading enzymes were present in salted-out fractions containing 40-80% saturated amount of ammonium sulfate. Amy-1 amylase alone was detected in the 90% saturated ammonium sulfate pellet. But the amylase recovery was only 1% of the input, and no apparent improvement on amylase specific activity was observed.

Other purification methods.--Glycogen absorption of alpha-amylase method (Schramm and Loyter, 1966; Jacobsen et al., 1970) and acetone fractionation method were tried. In each case, attempts to separate Amy-1 and Amy-2 by differential solubility failed.

Isoelectric Point of Amy-1 Amylase

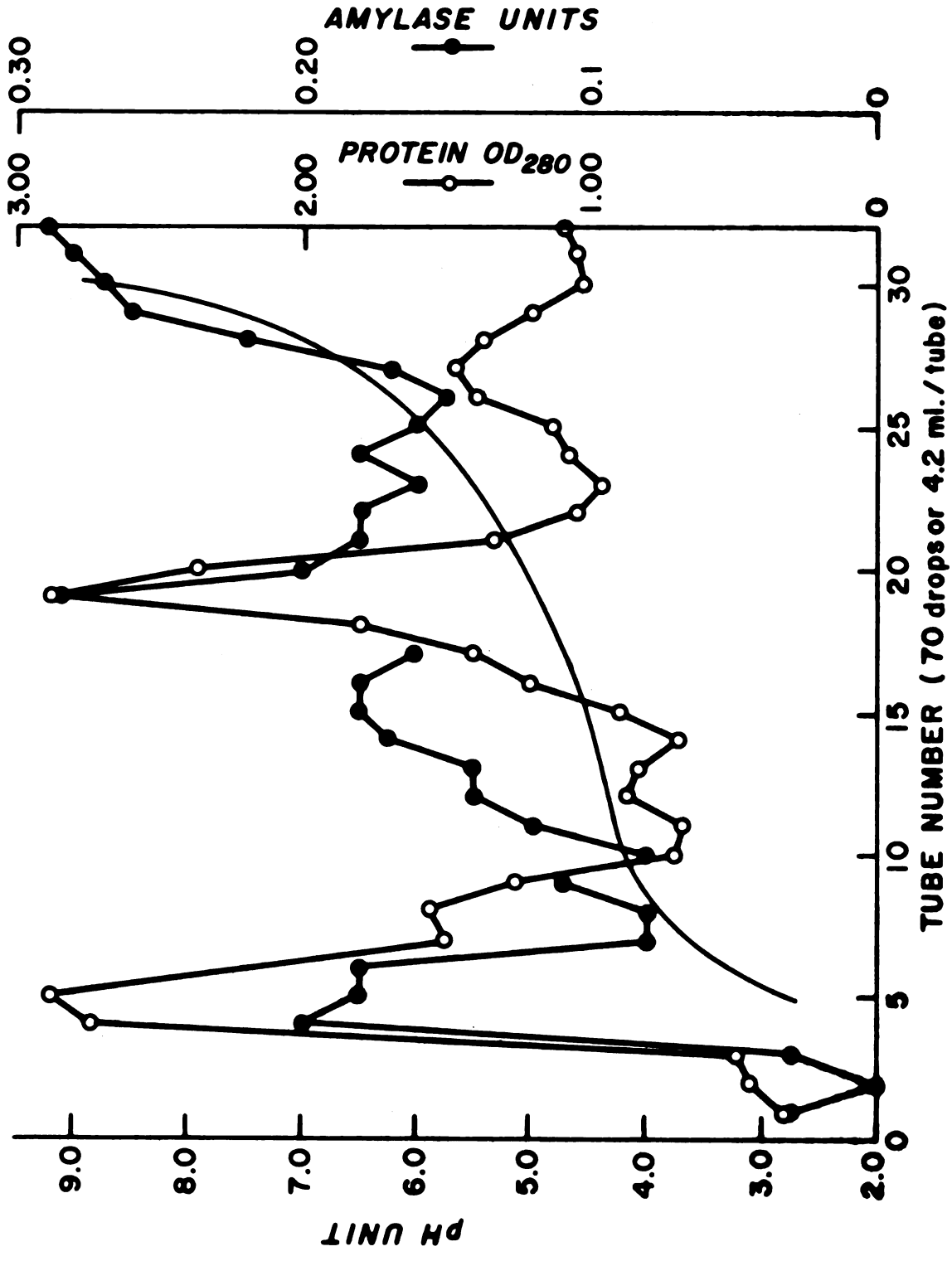
Amy-1 soluble fraction in 60% ethanol was electrofocused to determine its isoelectric point (pI). It was hoped that the electrofocusing method might prove to be a useful step to further the purification of Amy-1. Initially,

Table 13c. Summary of ammonium sulfate fractionation of Amy-1 amylase.

Fraction	Volume	Total amylase Activity	Total protein	Specific activity
	ml	units	mg	units/mg
Crude supernatant	320	624	1,084.8	0.575
Ammonium sulfate				
" 40% sat.	55	182	444.4	0.410
" 60% sat.	29	86	257.5	0.334
" 80% sat.	28	42	116.8	0.360
" 90% sat.	11	7	11.2	0.625

the pI of Amy-1 was estimated by experimenting with a pH 3 - 10 linear gradient to locate peaks of amylase activity. Two peaks were found with the major peak being at pH 4.9-5.2 and a minor peak at pH 7.0-7.5. Further electrofocusing experiments were done with pH 3-6 gradients in order to pinpoint the pI of Amy-1. The resulting pH gradients were linear from pH 4.0-6.0. The result of a typical electrofocusing experiment is shown in Fig. 13. In this experiment, 36 units of amylase (from W64A, and 58-3-6) in 50 mg protein was loaded on the 110 ml capacity electrofocusing column. After 60 hours of electrofocusing at 10 C, thirty fractions were collected with 4.0-4.2 ml per tube. A sharp protein peak was located in the middle of the gradient by UV_{280} absorbance with two other peaks located at two extreme ends of the pH gradient (pH 3.0 and pH 7.5). Amylase activity was found to coincide with the protein profile. Samples of each enzymic peak fraction was subjected to gel assay. The results of gel assays showed Amy-1 activity were in the tube numbered from 15 - 25 with the apparent highest activity at tube 19. The pH reading of this peak fraction was 4.95 and it was taken as the pI of Amy-1. Amylase activity recovered in the ten tubes was about 90% of the input. The electrofocusing experiments were repeated ten times. The average pI and the standard error were calculated to be pH 4.80 $\pm$ 0.15. Since Amy-1 was prepared from inbreds characterized by the fast

Fig. 13. The result of electrofocusing Amy-1A amylase. The smooth curve is a plot of pH values of eluted fractions. The curve with open circles (———o———) is the protein profile detected by UV absorbance. The curve with closed circles (———●———) is the amylase profile as measured by the decrease of starch-iodine absorbance at 620 mμ.


AMYLASE UNITS
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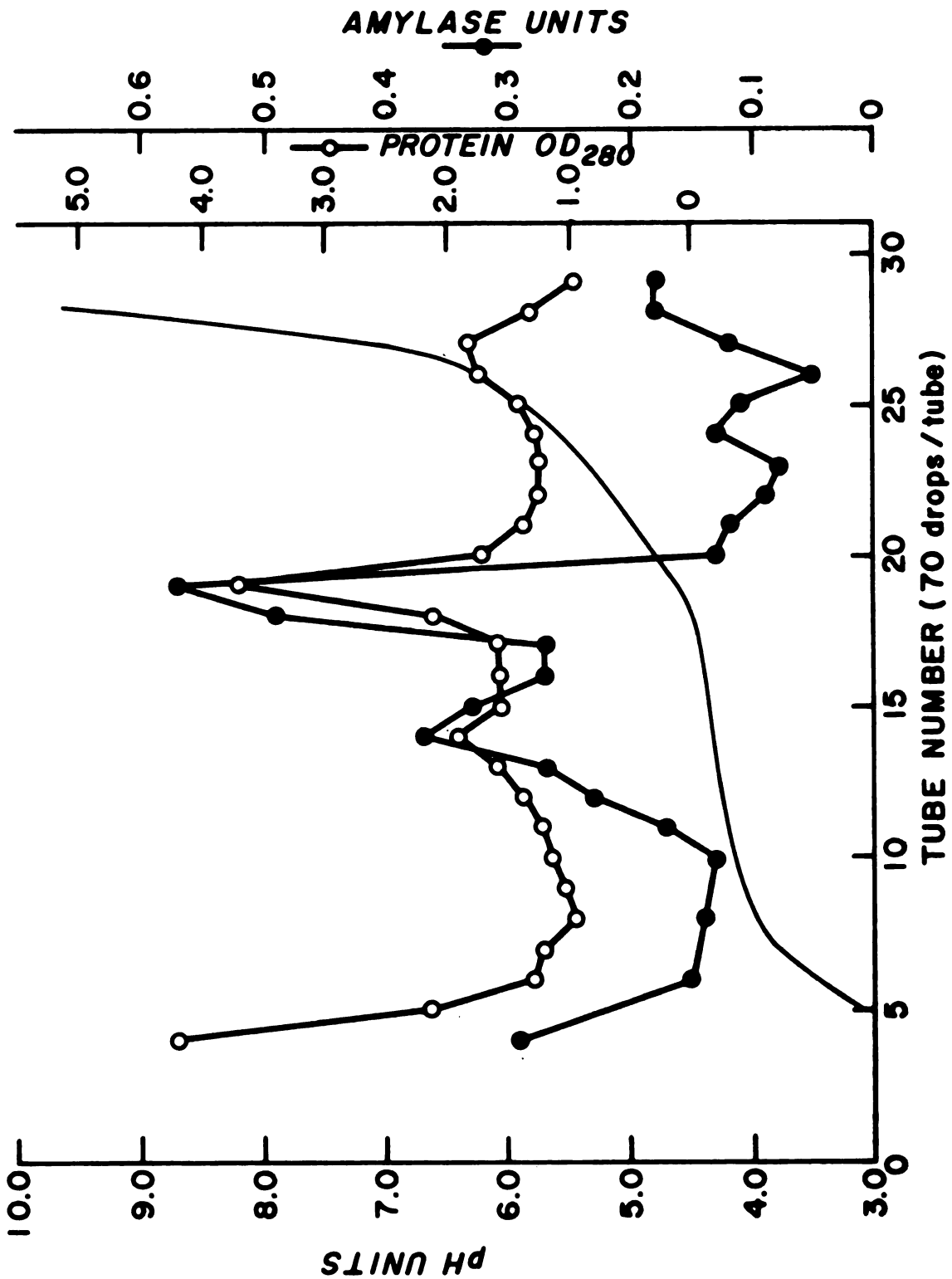
0.20
0.1
0
PROTEIN OD280
3.00
2.00
1.00
0
TUBE NUMBER (70 drops or 4.2 ml./tube)
0
5
10
15
20
25
30
pH UNIT
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0

electrophoretic variant of Amy-1, the pI so determined was the isoelectric point of Amy-1A, the gene-product of *Amy-1*^{A}/*Amy-1*^{A}.

The pI of Amy-1B, the slow electrophoretic variant of Amy-1 and produced by maize strain 38-11, was obtained in two experiments. Since an insufficient quantity of immature kernels of 38-11 was available, Amy-1B was obtained from two possible sources; 8-day old endosperm tissues of germinating 38-11 seedlings and also as a mixture with Amy-1A from immature kernels heterozygous for Amy-1 (*Amy-1*^{A}/*Amy-1*^{B}). Amy-1B from the first source, isolated by ethanol fractionation has a pI at pH 4.35. When Amy-1 from the second source was electrofocused, both Amy-1A and Amy-1B were recovered and demonstrated on gel assays. This experimental result is shown in Fig. 14. Two amylase peaks were found: one peak at pH 4.35 and the other peak at pH 4.65. Polyacrymide gel electrophoresis of these two fractions showed Amy-1A in the fraction with pH 4.65, and Amy-1B in the fraction with pH 4.35. The pI value for Amy-1B agrees with that prepared from endosperm tissues of germinating 38-11 seedlings.

Amy-1 can also be recovered in ethanol soluble fraction from tissues other than endosperm. Root extract of 8-day old W64A seedlings (*Amy-1*^{A}/*Amy-1*^{A}) was prepared and fractionated with 60% ethanol. A sample of the concentrated ethanol soluble fraction, containing 27 units

Fig. 14. The result of electrofocusing Amy-1A and Amy-1B amylases. The starting material was the mixture of Amy-1A and Amy-1B prepared from the extract of F_1 immature kernels of W64A and 38-11 which was fractionated with ethanol, dialysed and concentrated. The smooth curve is a plot of pH values of eluted fractions. The curve with open circles (——o——) is the protein profile detected by UV_{280} absorbance. The curve with closed circles (——●——) is the amylase profile as measured by the decrease of starch-iodine absorbance at 620 mμ.


AMYLASE UNITS
0.6
0.5
0.4
0.3
0.2
0.1
0
PROTEIN OD280
5.0
4.0
3.0
2.0
1.0
0
30
25
20
15
10
5
0
TUBE NUMBER (70 drops/tube)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
pH UNITS

of amylase in 73.5 mg protein was electrofocused. The Amy-1A peak was recovered at pH 4.90 which does not significantly deviate from the average pI 4.80 obtained by using immature kernels as the Amy-1 source material.

End-product Analyses

Enzymic action on ^{14}C starch.--Amy-1, Amy-2 and Zone-3 phosphorylase were eluted from gels after electrophoresis. One-tenth of a ml ^{14}C-starch containing about 1 μc was incubated with 0.1 ml of each one of the three eluted enzymes. Another set of the eluted enzymes were each incubated with 2 μc of ^{14}C-maltose. The reaction mixtures were incubated at 23 C for 12 hours. The end-products of amylolysis were separated on Kieselguhr G thinlayer chromatographic plates (TLC-plates) and autoradiographed. The results are shown in Fig. 15. Both hog pancreatic alpha-amylase and sweet potato beta-amylase upon digesting ^{14}C-starch gave rise to glucose and maltose, though with the proportions of the two sugars varying with the two amylases. Such a distinction was not observed with the three eluted maize starch-degrading enzymes. Actually, all three eluted enzymes gave rise to maltose and trace amounts of maltotriose. No maltase activity in the three eluted enzymes was detected since ^{14}C-maltose seemed to be left untouched. A similar experiment was repeated by separating the end-products by paper chromatography followed by autoradiography. The result is shown in

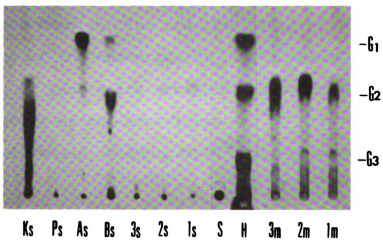


Fig. 15. Results of autoradiogram showing the detection of radioactive glucose (G_1), maltose (G_2), maltotriose (G_3) as the end-products of independent enzymatic hydrolysis of ^{14}C-starch and ^{14}C-maltose on TLC plates. The samples of hydrolysates resulting from various enzyme preparations are designated as the following:

K = crude extract of immature kernels.
M = ^{14}C-maltose. The subscript M indicates the substrate applied.
1 = eluted Amy-1 amylase from polyacrylamide gels.
2 = eluted Amy-2 amylase from polyacrylamide gels.
3 = eluted Zone-3 phosphorylase from polyacrylamide gels.
A = hog pancreatic alpha-amylase 10 microliter.
B = sweet potato beta-amylase 10 microliter.
P = rabbit muscle phosphorylase a 10 microliter.
S = ^{14}C-starch. The subscript S indicates the substrate applied.

Fig. 16. Again, I found that each one of the three eluted enzymes gave rise to glucose, maltose, maltotriose, and maltotetraose, in addition to what were detected by TLC. Sugar spots were outlined on the paper chromatogram by the aid of the autoradiogram. They were cut out and measured for the allocation of radioactivity with a Scintillation counter. The results are given in Table 14. The spectrum of the end-products of the three eluted enzymes are in fact quite strikingly similar despite the fact the extent of the amylolysis of ^{14}C-starch with the eluted Amy-1, Amy-2, and phosphorylase varied from 9.7% to 29.5%.

Action pattern of Amy-1 on soluble starch.--One ml of the ethanol fraction of Amy-1 (20 units of amylase in 0.5 mg protein) was incubated with 1% potato starch solutions in pH 4.8 sodium acetate 0.05 M buffer at 23 C. At various time intervals, 1 ml aliquots were withdrawn, heated to boiling, and centrifuged at 12,000 g for 10 minutes. The supernatants were directly spotted on Whatman No. 1 paper and chromatographed. The result is shown in Fig. 17. Maltose was released from the 3 minute sample and the accumulated amount appeared to increase with incubation time from 3 minutes to 2 hours. Similar results were obtained in repeated experiments. Maltose seemed to be the only detectable reducing sugar resulting from Amy-1 action on potato starch. I think it is unlikely

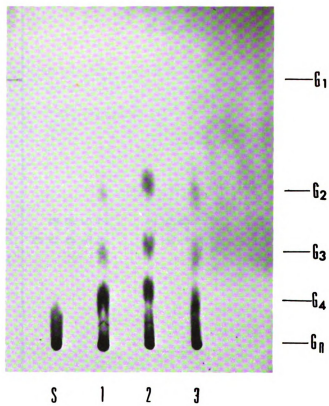


Fig. 16. The Autoradiogram of glucose and maltodextrins resulted from ^{14}C-starch digested by the eluted enzymes. S = ^{14}C-starch alone. 1 = Amy-1 amylase. 2 = Amy-2 amylase. 3 = phosphorylase. All three enzymes were eluted from polyacrylamide gel after electrophoresis.

Table 14. The distribution of radioactivity in end-products resulting from the ^{14}C-starch digested by the eluted starch-degrading enzymes. The end-products G_1, G_2, G_3, G_4 and Gn represent glucose, maltose, maltotriose, maltotetraose, and starch (at the origin) respectively.

Isozyme Zones	End Products	Counts per minute	I Counts of end-product / Total counts X 100	II Corrected % in I
Control	G_1	0	0	
	G_2	213	0.2	
	G_3	882	0.9	
	G_4	1,426	1.6	
	G_n	87,834	97.2	
		90,355		
1	G_1	86	0.1	0.1
	G_2	1,688	1.6	1.4
	G_3	2,012	1.9	1.0
	G_4	6,615	6.3	4.7
	G_n	95,492	90.3	92.8
		105,807		
2	G_1	316	0.4	0.4
	G_2	7,299	9.0	8.8
	G_3	5,655	6.7	5.8
	G_4	10,064	12.4	10.8
	G_n	57,837	71.5	74.2
		81,171		
3	G_1	277	0.3	0.3
	G_2	4,320	4.0	3.8
	G_3	5,084	4.7	3.8
	G_4	14,424	13.4	11.8
	G_n	83,891	77.7	80.3
		107,996		

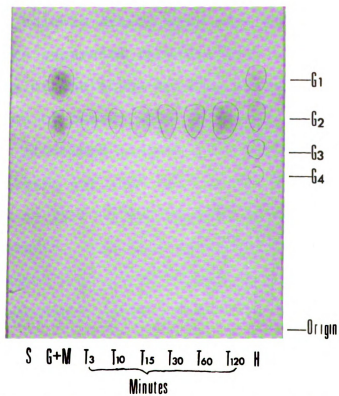


Fig. 17. The paper chromatogram of the end-products of starch digested by Amy-1 amylase. Fourty microliter of the hydrolysate sample were taken at minutes indicated at the origin. S = starch alone. H = starch acid hydrolysate. G = glucose marker. M = Maltose marker.

that glucose is present but not detected, since the reducing power of glucose is about 1.5 times that of maltose on equal weight bases.

Comparative time course studies of the action of Amy-1, hog pancreatic alpha-amylase, and sweet potato beta-amylase on soluble starch were also performed and the end-products were separated on Kieselguhr G TLC-plates. The end-products of the alpha-amylase following 3 - 15 minutes digestion of starch were primarily glucose and maltose. With prolonged incubation, maltotriose appeared at 30 minutes digest and this spot on TLC-plates became euqally stained as glucose and maltose spots in the 2 hours enzymic digest. The main end-products of the beta-amylase, throughout 2 hours of digestion of starch, was maltose; although trace amount of glucose was observed starting at 1 hour after incubation.

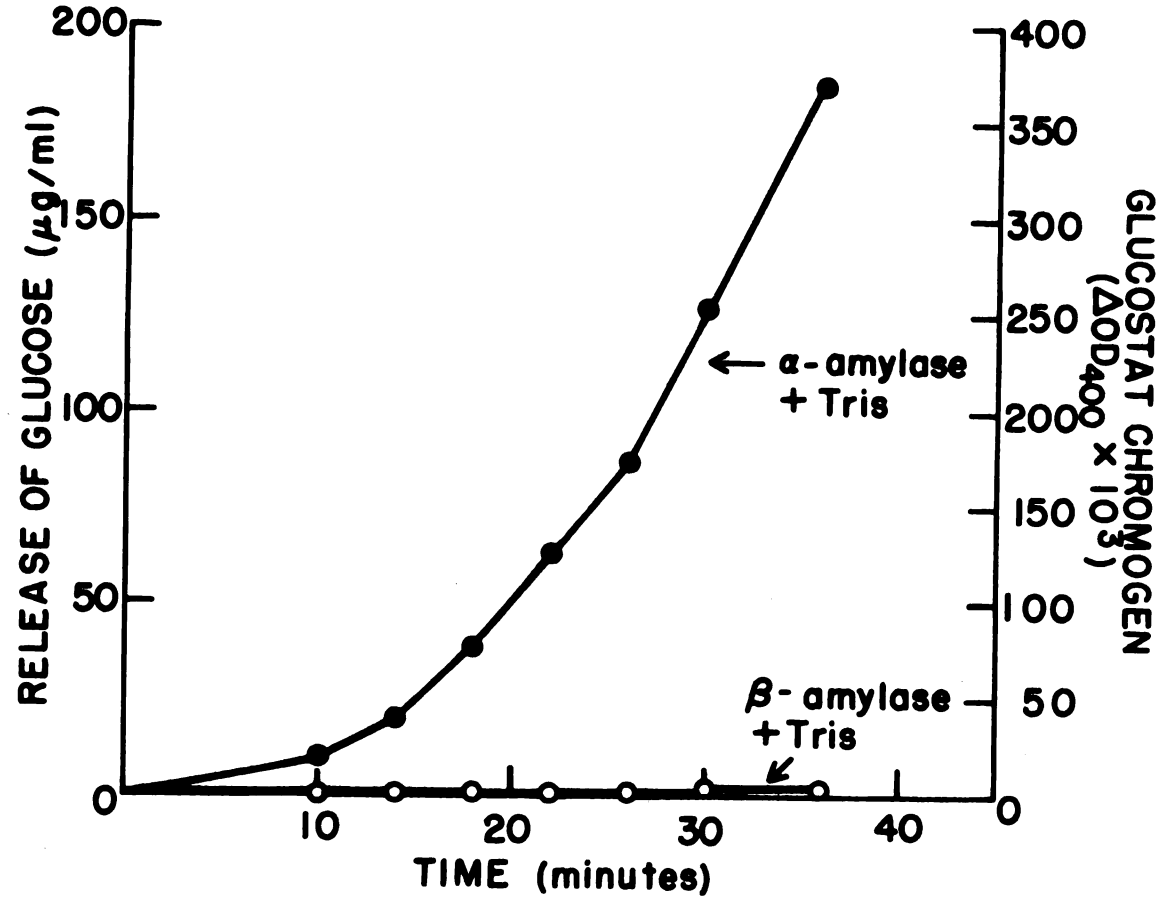
The experimental results on end-product analysis thus far indicated that the end-products of the alpha-amylase and the beta-amylase differ mainly in the initial period of amylolysis rather than in the exhaustive digestion of starch. To reiterate, glucose is released from starch by hog-pancreatic alpha-amylase, but not by sweet potato beta-amylase in the initial reactions.

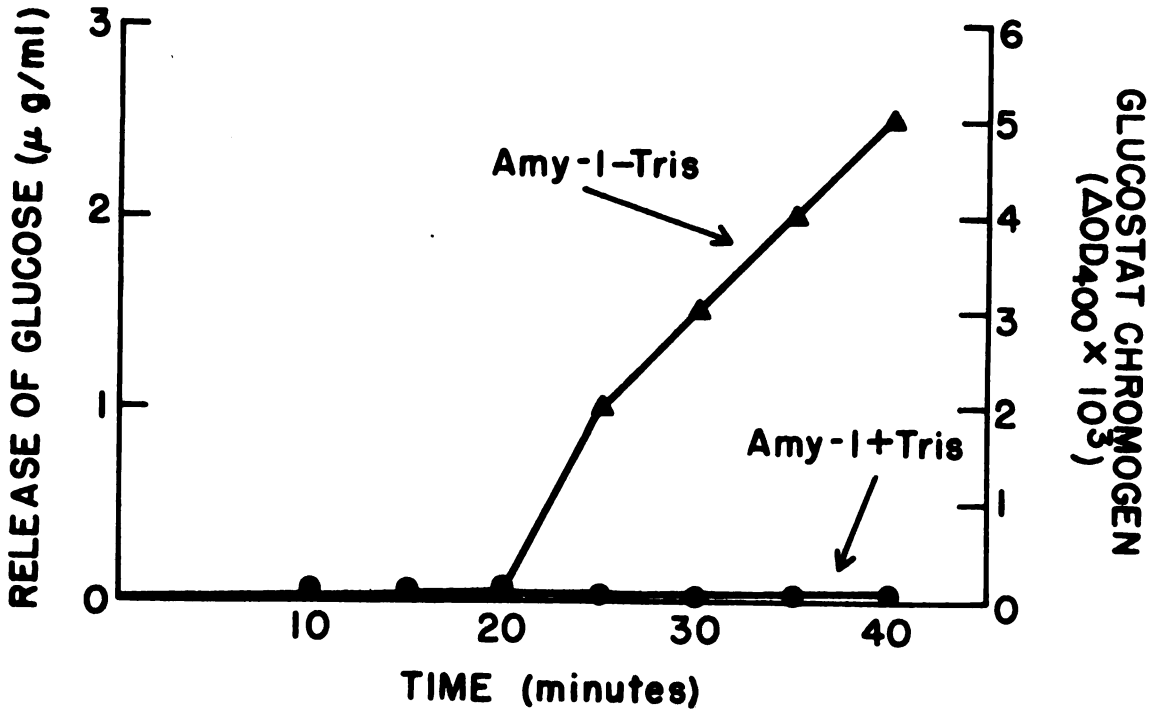
Detection of glucose with Glucostat.--Glucostat offers an assay method specifically for glucose with a high sensitivity (10 - 200 μg/ml), and the detection is

not prevented by other oligosaccharides. In order to follow glucose release by the action of hog pancreatic alpha-amylase, 20 µl of the enzyme were added to the 3 ml Glucostat-starch mixture in a 4 ml quartz cuvette. The time course of this enzymic action was traced on an automatic chart recorder. Fig. 18a shows such an experimental result. The release of glucose increases with time, and by 30 minutes 130 µg glucose was released from 5 mg soluble starch, thus representing 3.5-4.0% of the starch was converted to glucose by the alpha-amylase. In contrast to this, sweet potato beta-amylase, as much as 40 µl (2.2 mg with 500 units/mg) added to the reaction mixture did not give rise to any detectable amount of glucose even after 40 minutes of incubation. The distinctions of the alpha-amylase and the beta-amylase herein is noteworthy since it makes it possible to identify maize amylases with these two enzymes serving as reference standards. Scutellar extracts were prepared from 10-day-old seedlings of W64A and 38-11. The crude extracts were spun at 12,000 g for 30 minutes. The supernatants were collected and concentrated in collodion membrane bags (Schleicher and Schuell, Inc. New Hampshire) and effected by negative pressure concentrators (ibid) against Tris-HCl buffer (0.05 M, pH 7.5) and 1 mM $CaCl_2$. The concentrated supernatant after zymographic assay, showed strong Zone-1 activity (including Amy-1) and weak Zone-2 activity. Twenty µl of 38-11

Fig. 18a. The time-course curves of glucose released from starch by hog pancreatic alpha-amylase, and sweet potato beta-amylase.

Fig. 18b. The time-course curves of glucose released from starch by maize amylases in the presence and absence of maltase activity as effected by deleting or adding 0.13M Tris in the reaction mixtures.


RELEASE OF GLUCOSE (μg/ml)
GLUCOSTAT CHROMOGEN (ΔOD400 x 10³)
TIME (minutes)
α-amylase + Tris
β-amylase + Tris
0
50
100
150
200
250
300
350
400
10
20
30
40


RELEASE OF GLUCOSE (μ g/ml)
GLUCOSTAT CHROMOGEN (ΔOD400 x 10³)
TIME (minutes)
Amy-1-Tris
Amy-1+Tris
0
1
2
3
4
5
6
10
20
30
40

extract containing 0.23 amylase units, or twenty µl of W64A extract containing 0.20 amylase units were subjected to Glucostat-starch assays. No glucose release was observed despite the fact that starch was digested extensively because no starch-iodine color could be detected at the end of 40 minutes of the reaction. However, the above experiments were done with reaction mixtures containing Tris with a concentration 0.13 M per ml. At this concentration of Tris, maltase activity which is present in the Glucostat would be inhibited. By replacing Tris-HCl buffer with sodium-phosphate buffer (0.02 M, pH 7.0) in the reaction mixture containing maltose (0.2 mg/ml) the maltase activity was restored as it is shown in Fig. 18b. Maize amylase under this condition were expected to degrade starch to maltose, which would be converted by maltase to glucose. The results shown in Fig. 18b confirmed this supposition, although the glucose level detected was low. This experiment provides a qualitative evidence that maltose rather than glucose is predominant among the end-products resulting from amylolysis of starch by maize amylases.

Action of Maize Amylases on Beta-Limit Dextrin

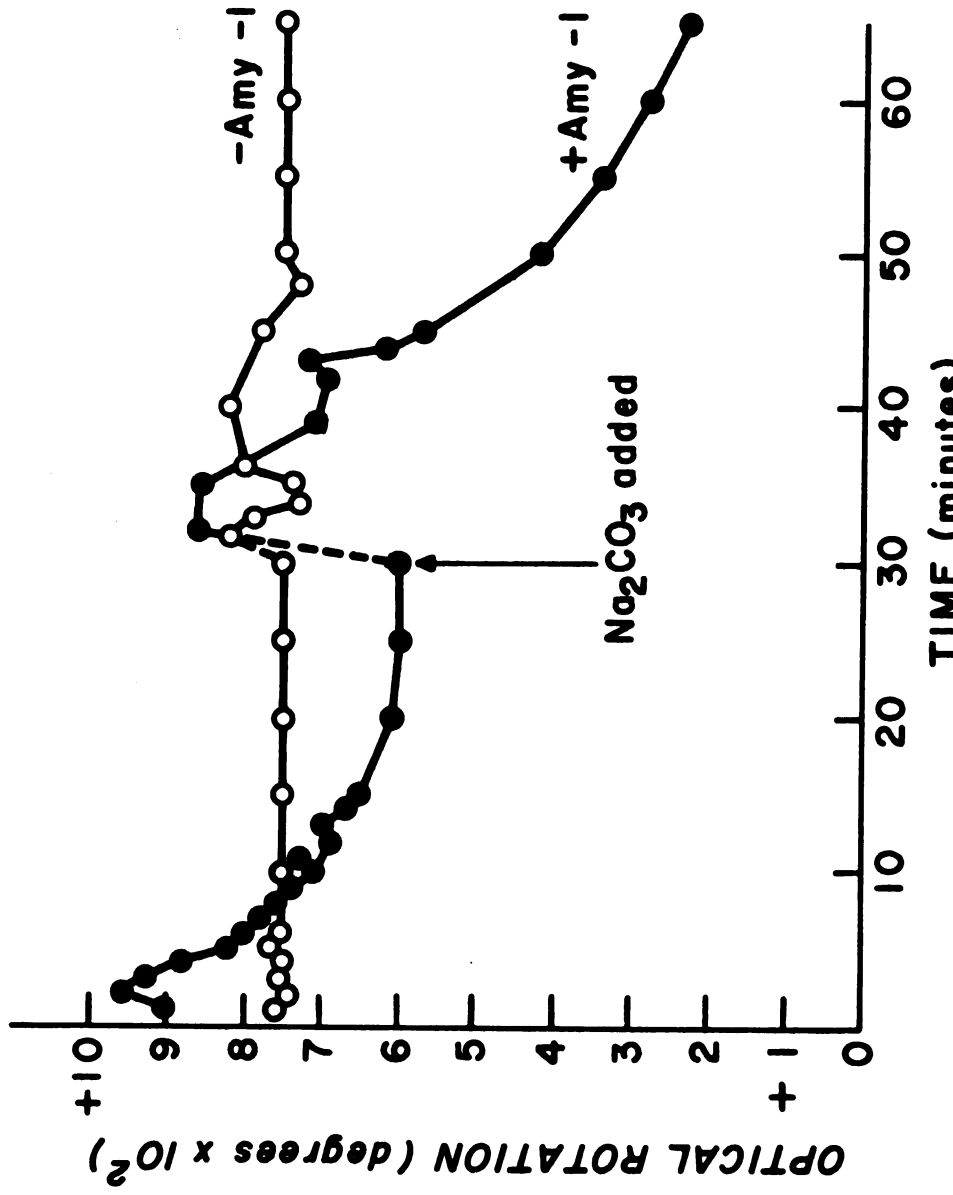
Beta-limit-dextrin can be degraded by hog pancreatic alpha-amylase but is immune to sweet potato beta-amylase. I found Amy-1, either as soluble ethanol fraction or in

electrofocused fraction, is capable of degrading beta-limit dextrin. For instance, an electrofocused Amy-1 with 0.25 amylolytic units/ml with respect to starch also showed 0.66 amylolytic units/ml toward beta-limit-dextrin, both in pH 4.8 sodium phosphate buffer (0.05 M). Since the isolation of Amy-2 was not attained, a similar test tube assay was not possible to achieve. Consequently, the beta-limit-dextrin assay was carried out for both Amy-1 and Amy-2 directly on a polyacrylamide gel after electrophoresis. The results clearly indicate that both Zone-1 (including Amy-1) and Zone-2 (including Amy-2) amylases can degrade the beta-limit-dextrin. The amylolytic zones detected on beta-limit-dextrin agar plate were identical to that on starch-agar plate with respect to both the dextrin-iodine color and the locations; i.e. clear at Zone-1 and pink at Zone-2. Furthermore, where Zone-2 amylase is no longer seen, Zone-4 is observed. This new band (Zone-4) is apparently capable of degrading beta-limit-dextrins based on gel assays.

Optical Rotation of End-products in Enzymic Digests of Starch

The experiment with the ethanol fraction of Amy-1 was conducted with the method described by Robyt and French (1961). The result is shown in Fig. 19. The decrease of optical rotation after adjusting the reaction

Fig. 19. Optical rotation study of the action of Amy-1 amylase on starch.


-Amy -I
+Amy -I
Na_2CO_3 added
TIME (minutes)
OPTICAL ROTATION (degrees x 10^2)
+10
9
8
7
6
5
4
3
2
1
0
+
10
20
30
40
50
60

mixture to pH 10, indicates the predominant optically active end-products are in alpha-configurations.

Thermal Stability and pH Sensitivity of Amy-1

A electrofocused fraction of Amy-1 containing 0.20 units/ml in 4 mg protein was used to compare its stability at 70 C and its sensitivity at pH 3.5 with those of sweet-potato beta-amylase (0.40 units/ml in 0.1 mg protein). The results are shown in Fig. 20 for thermal stability; and in Fig. 21 for pH sensitivity. The percentage of initial activity of treated amylase was plotted against time. Amy-1 was found to retain 80% of its initial activity after 30 minutes in 70 C; contrasting to sweet potato beta-amylase only 20% of the initial activity was retained with the same treatment. At pH 3.5, sweet potato beta-amylase retained 95% of its initial activity while Amy-1 lost 90% of its initial activity during the first 20 minutes. The results clearly indicate that Amy-1 is distinctly different from sweet-potato beta-amylase with respect to its thermal stability and pH sensitivity.

Amy-1A amylase prepared from ethanol fractionation was used for determining the pH optimum of Amy-1. The pH of the reaction was controlled by the use of pH gradient fractions (pH 3-10) obtained from electrofocusing 1% Ampholyte in an LKB 8101 electrofocuser. These focused Ampholyte fractions were found to have buffer capacities

Fig. 20. Thermal stability study of Amy-1 amylase activity. One ml enzyme samples were incubated in 70 C water bath. At each time interval, two tubes were taken out, cooled at 10 C water bath, and stored at 5 C refrigerator until all samples were ready to be assayed. Each point represents the average of the duplicate samples.

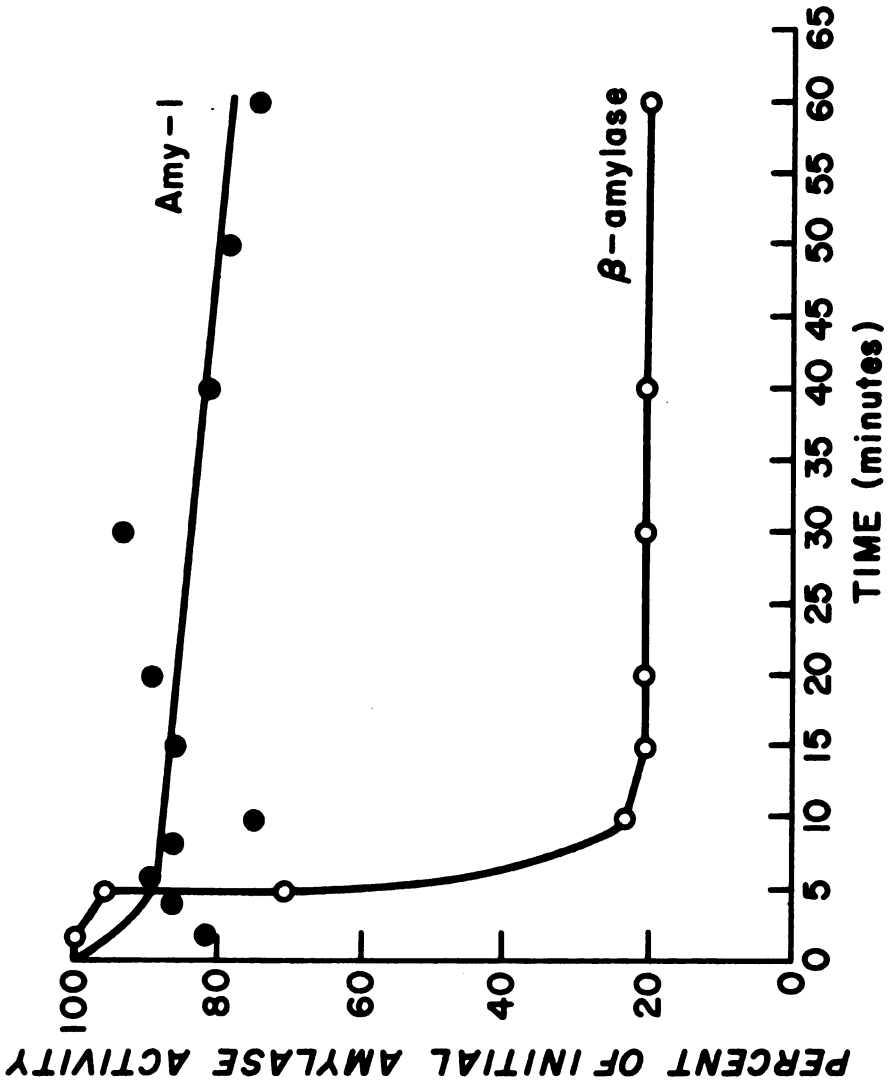
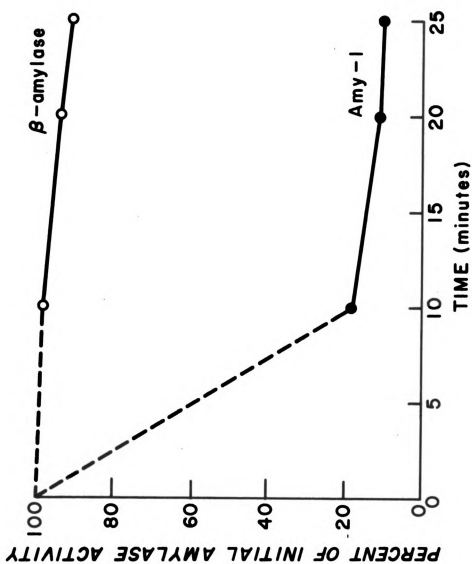

Amy-1
β-amylase
PERCENT OF INITIAL AMYLASE ACTIVITY
TIME (minutes)
0
20
40
60
80
100
0 5 10 15 20 25 30 35 40 45 50 55 60 65

Fig. 21. Results of pH sensitivity study of Amy-1 amylase activity. One ml enzyme samples were adjusted to pH 3.5 by adding 4N acetic acid. At the indicated time intervals, the acidified samples were neutralized with 4N NH_4OH. One-half of ml of each sample was assayed for amylase activity. Each point represent the average of two readings.

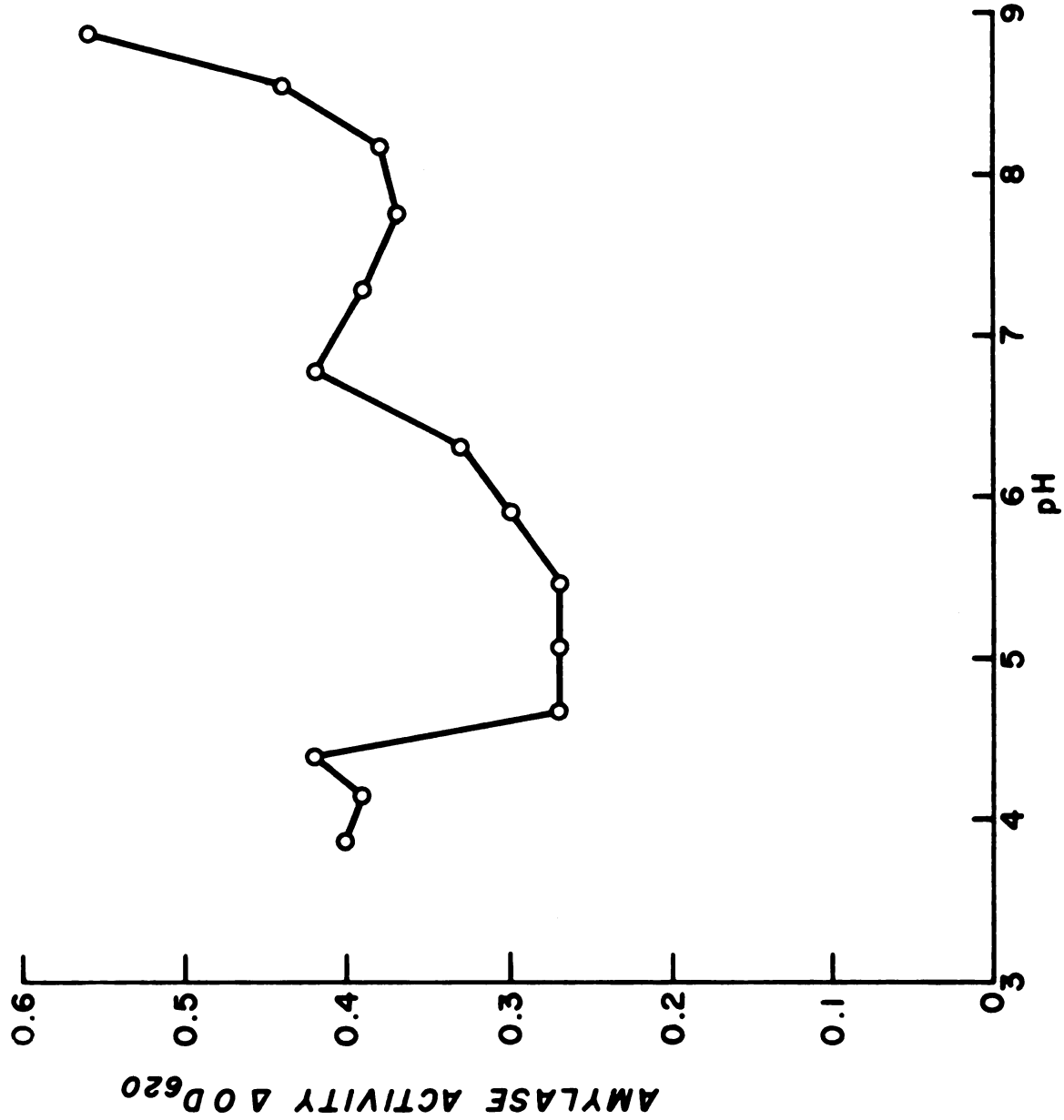

β-amylase
Amy-1
PERCENT OF INITIAL AMYLASE ACTIVITY
100
80
60
40
20
0
0
5
10
15
20
25
TIME (minutes)

sufficient to maintain the initial pH after a 10-fold of dilution. The reaction mixtures consisted of 0.5 ml of the amylase, 0.5 ml of a given pH gradient solution, 1.0 ml of starch reagent (0.15%, Nutritional Biochem. potato starch boiled to dissolve in distilled water). After 2 minutes of incubation, the reaction was stopped by 1 ml of iodide reagent prepared according to the assay used by Filner and Varner (1967) and diluted with a constant volume of water immediately before the OD_{620} reading was recorded. The optimal pH for Amy-1A amylase thus determined was 6.8.

Molecular Weight of Amy-1 Amylase

Gel filtration with Sephadex columns.--One and one-half ml of Amy-1 prepared from electrofocused fraction (Fig. 13) was passed through a Sepadex G-200 column (59 x 1.8 cm) and eluted with Tris-HCl buffer (0.05M, pH 7.5) and 1mM $CaCl_2$. Equal volume efluant fractions were collected. Amylase activity and protein concentrations were measured. The result is shown in Fig. 23. The only peak of Amy-1 was located in Tube 25 which gave a calculated value of partition coefficient Kav = 0.610. The experiment was repeated once again with one ml of the same Amy-1 preparation. Amy-1 peak was eluted out with exactly the same volume of the buffer as in the previous experiment. The column was standardized with two proteins, chicken egg

Fig. 22. The study of pH optimum of Amy-1 amylase. The amylase activity is plotted against pH of the reaction mixtures. The pH 3-10 gradient fractions resulting from the electrofocusing of 1% Ampholyte were used instead of using several buffers of proper pKs. The amylase peak activity was found at pH 6.8. The amylolytic activities detected at two extremes (below pH 4 and above pH 8.5) are probably due to non-enzymatic hydrolysis of the starch.


AMYLASE ACTIVITY Δ O D620
0.6
0.5
0.4
0.3
0.2
0.1
0
3
4
5
6
7
8
9
pH

white lysozyme (MW = 14,400, 2x crystallized, salt free, Worthington Biochem Corp.) and human hemoglobin (MW = 68,000, 2x crystallized, Calbiochem.). The profiles of lysozyme and hemoglobin on the Sephadex G-200 column are plotted on the same graph with Amy-1 and are shown in Fig. 23.

Since Kav is a coefficient measuring the partition of a macromolecule between the liquid phase and the gel phase, it is independent of the geometry of the column employed. Extensive gel filtration data of several proteins on a Sephadex G-200 column had been published by Andrews (1964). I took his data calculated the Kav values of proteins of various sizes and replotted them against the logarithm of their molecular weights as shown in Table 15. A straight line was obtained and is shown in Fig. 24. The Kav values of lysozyme, hemoglobin and Amy-1 obtained from my experiments were plotted on the same graph. Accordingly, lysozyme has an estimated molecular weight of 15,500; Amy-1, 14,500; and human hemoglobin, 35,000. Human hemoglobin was applied to the column as a 0.2% w/v solution, and during the runs this concentration decreased. The value obtained for its molecular weight indicates dissociation into half-molecules under the experimental condition, a result being consistent with Andrew's observations (Andrews, 1962) on agar

Table 15. Comparison of gel filtration data of Amy-1, lysozyme, hemoglobin on Sephadex G-75, and G-200 columns with some reference proteins. Gel filtration data of reference proteins were obtained from literature (Andrews, 1964).

Proteins	Reference Molecular weight	Partition coef. Kav	
		G-75	G-200
Pseudomonas cytochrome c 551	9,000	0.573	0.717
Cytochrome c	12,400	0.490	
Ribonuclease	13,700	0.483	0.632
α-Lactalbumin	15,500	0.434	-----
Myoglobin	17,800	0.399	0.572
Ovalbumin	45,000	0.133	0.316
Serum albumin	67,000	-----	0.197
Hemoglobin[1)]	68,000	0.186	0.382
Lysozyme[2)]	14,400	0.148 0.452	0.587
Amy-1A[3)]		0.445 -----	0.610
Amy-1B[4)]		0.753	-----

[1]Human hemoglobin with a concentration 0.2% (w/v) was used. The estimated molecular weight of this hemoglobin from the Sephadex G-75 column is 42,000, whereas that from the Sephadex G-200 is 35,000.

[2]Chicken egg white lysozyme with a concentration 4-8% (w/v) was used. The estimated molecular weight is 15,000 on Sephadex G-75 column and 15,500 on the Sephadex G-200 column.

[3]Amy-1A isolated by means of ethanol fractionation and electrofocusing. Duplicate experiments were done with samples of Amy-1A containing 6-10 mg protein in 1-1.5ml with 1.2-2.0 amylase units. The estimated molecular weight is 14,500 on Sephadex G-200.

[4]Amy-1B isolated by means of ethanol fractionation. The sample of Amy-1B contained 3.0 units of amylase activity in 15 mg protein, 4 mg lysozyme and 1 mg hemoglobin in 1.5 ml. The recovery of amylase activity in efluant was low about 12% of the input. The estimated molecular weight is about 6,000. The low recovery of activity makes this estimation doubtful.

Fig. 23. Gel filtration Amy-1 amylase, human hemoglobin, chicken egg white lysozyme, and two markers; bromophenol blue and blue dextran using pH 7.5 Tris-HCl buffer (0.05M) containing 1 mM $CaCl_2$.

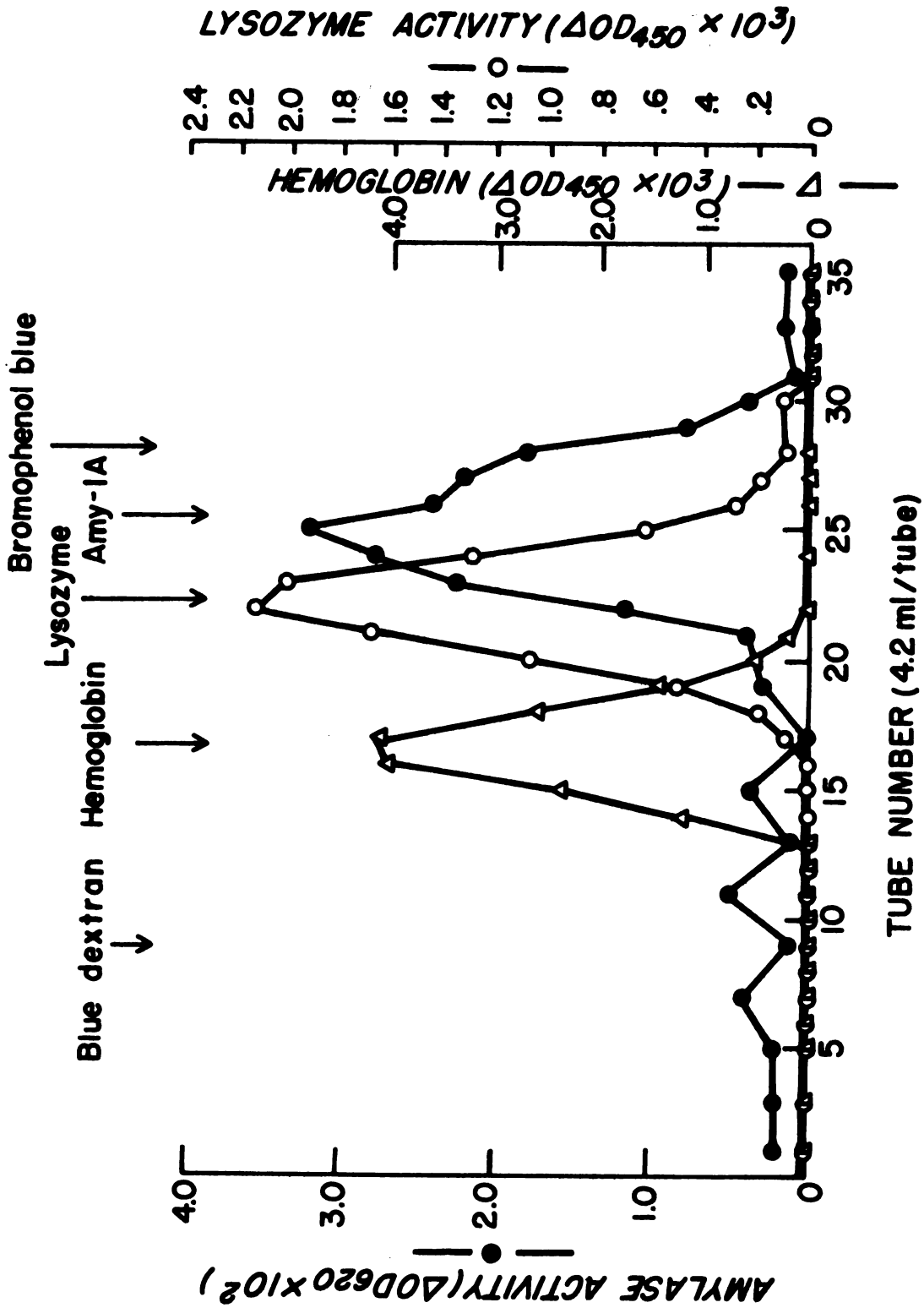
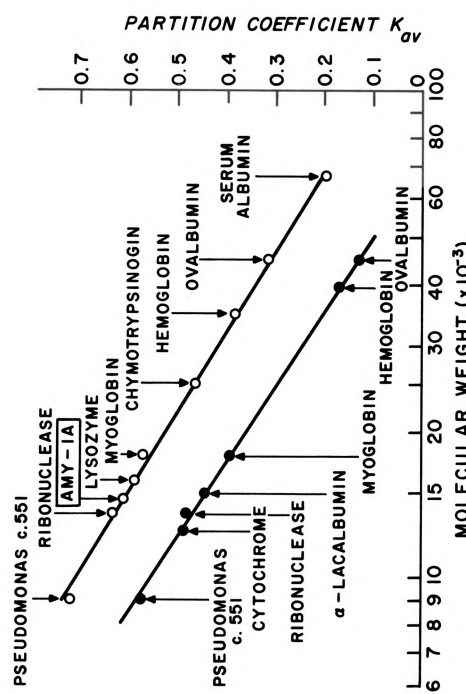

LYSOZYME ACTIVITY (ΔOD450 × 10³)
HEMOGLOBIN (ΔOD450 ×10³)
AMYLASE ACTIVITY (ΔOD620 ×10²)
TUBE NUMBER (4.2 ml/tube)
Bromophenol blue
Lysozyme
Amy-1A
Hemoglobin
Blue dextran

Fig. 24. Plots of partition coefficient, Kav, against log (molecular weight) for proteins on Sephadex G-75 (●) and G-200 (o) columns equiliburated with 0.05M Tris-HCl buffer, pH 7.5 containing 1 mM $CaCl_2$. The gel filtration data are taken from Table 15.


PARTITION COEFFICIENT K_{av}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100
80
60
40
30
20
15
10
9
8
7
6
MOLECULAR WEIGHT (x10^{-3})
PSEUDOMONAS c.551
RIBONUCLEASE
AMY-1A
LYSOZYME
MYOGLOBIN
CHYMOTRYPSINOGIN
HEMOGLOBIN
OVALBUMIN
SERUM ALBUMIN
PSEUDOMONAS c. 551
CYTOCHROME
RIBONUCLEASE
α-LACALBUMIN
MYOGLOBIN
HEMOGLOBIN
OVALBUMIN

gel-filtration of dilute bovine hemoglobin solutions (0.06%, w/v).

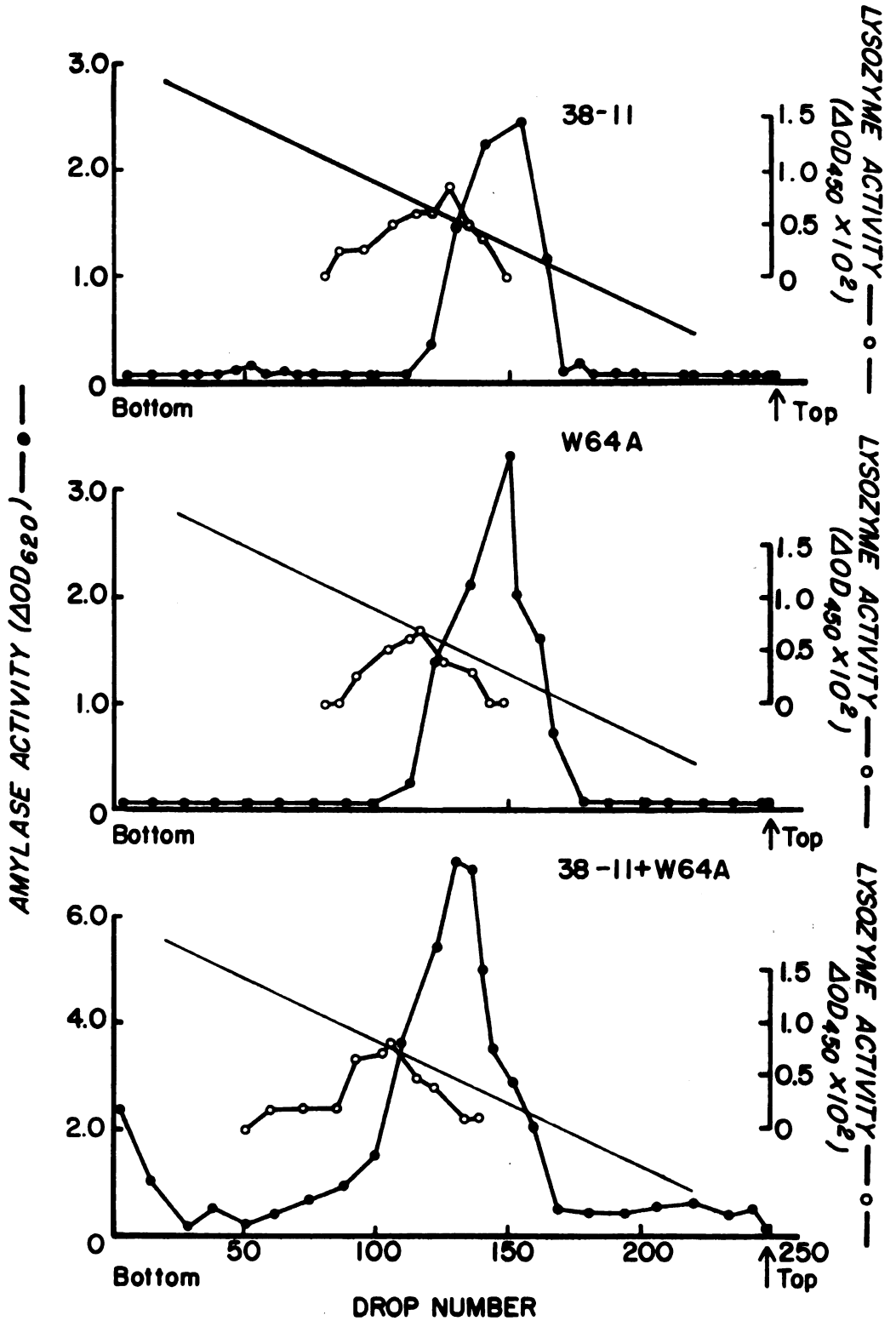
Sucrose gradient centrifugation.--Four independent experiments were done to estimate the molecular weight of Amy-1A and Amy-1B. A Beckman L2-65B ultracentrifuge and a SW 65 L Ti rotor were used in all these experiments. Electrofocused Amy-1 was used in the first experiment. Crude amylase obtained from either scutella or endosperm of 8 - 10 day old seedlings (W64A, 38-11, or mixture of the two depending on whether Amy-1A, Amy-1B, or Amy-1A & Amy-1B were desired) were concentrated in collodion membrane bags (Schleicher and Schuell, Inc. New Hampshire) and used in the subsequent three experiments. The experimental condition and the calculated apparent sedimentation coefficients are given in Table 16. Lysozyme was used as a marker in the same tube with Amy-1. After centrifugation, 3-drop fractions were collected. When crude amylase was used it was necessary to correlate the quantitative amylase profile with a qualitative zymogram. Ten µl samples of every fourth fraction along the sucrose gradient were subjected to polyacrylamide gel electrophoresis. Zymograms of amylase showed that Amy-1, the most active band and the pink band right beneath Amy-1 (together comprises Zone-1) always came in the same fraction to contribute the peak activity in quantitative assays. The result of the fourth experiment (Table 16) is shown in Fig. 25.

Table 16. Summary of sucrose gradient centrifugation experiments.

Exp.	Sample	Sucrose gradient	Conditions of centrifugation			Distance from miniscus cm	Apparent sedimentation coefficient 10^{-13} cm/sec	Calculated molecular weight dalton*
			Rotor speed in Krpm	Time hour	Temperature centigrade			
1	Amy-1A, focused	5-18.5% convex exponential	65	8	5		1.90	12,800
	Lysozyme						2.06	14,400
2	Amy-1A crude	5-20%, linear	40	15	0		1.37	
	Amy-1B crude	"	"	"	"		1.37	
	Amy-2A, crude	"	"	"	"		1.37	
3	Amy-1B crude	5-20% linear	55	16	0		1.82	9,300
	Lysozyme						2.43	14,400
4	Amy-1A crude	5-20% linear	55	16	0	1.50	1.83	10,588
	Lysozyme					1.90	2.26	14,400
	Amy-1B, crude	"	"	"	"	1.50	1.83	10,588
	Lysozyme					1.90	2.13	14,400
	Amy-1A & Amy-1B crude	"	"	"	"	1.70	2.03	11,707
	Lysozyme					2.00	2.40	14,400

*The molecular weight of Amy-1, are calculated in reference to that of lysozyme, 14,400 (Sophianopoulos, et al., 1962).

Fig. 25. Sucrose density gradient centrifugation of Amy-1A and Amy-1B. The detailed experimental conditions are shown in Experiment 4, Table 16. The lysozyme profile is indicated by (—— o ——). The amylase profile is indicated by (——●——). The fine straight line is the plot of refractive index of sucrose to the drop number although the scale of refractive index is not shown here.


38-11
W64A
38-11+W64A
AMYLASE ACTIVITY (ΔOD620) —●—
LYSOZYME ACTIVITY —○— (ΔOD450 ×10²)
Bottom
Top
DROP NUMBER
3.0
2.0
1.0
0
6.0
4.0
1.5
0.5
50
100
150
200
250

The following conclusions may be drawn from the data: (1) Amy-1 is slightly smaller than the lysozyme with MW = 14,400. (2) The molecular weights of Amy-1A and Amy-1B are apparently identical. The molecular weight of Amy-1 was calculated on the basis of the following relation and shown in Table 17.

$$(MW^L/MW^A)^{2/3} = S^L/S^A$$

$$= \frac{\ln r_2^L - \ln r_1^L}{\ln r_2^A - \ln r_1^A} \quad \text{since } S = \frac{1}{\omega^2} \cdot \frac{\ln(r_2/r_1)}{t_2 - t_1}$$

where MW = molecular weight
S = apparent sedimentation coefficient
L = lysozyme
A = Amy-1
ω = angular velocity

Reaction of Anti Amy-1A Rabbit Serum with the Two Allelic Amy-1 Amylases

Various preparations of Amy-1A and Amy-1B were used in the immunodiffusion experiments to test their antigenic specificities with anti Amy-1A rabbit serum. Two of the Amy-1A preparations were obtained from two independent electrofocusing experiments. Other Amy-1A samples were obtained from ethanol fractionations of endosperm, scutellum, or leaf of 8-day-old germinating W64A seedlings. Crude Amy-1B was obtained from scutellar extract of 8-day-old 38-11 seedlings. The crude Amy-1B was further concentrated by a negative pressure dialysis in a collodion membrane bag (Schleicher and Schuell Inc. New Hampshire).

Table 17. Calculations of Amy-1 molecular weight with reference to lysozyme using sucrose gradient centrifugation data.

Tube no.	Sample	Distance from rotation axis r_1	Distance from rotation axis r_2	$\frac{S^L/S^A}{\ln r_2^L - \ln r_1^L}{\ln r_2^A - \ln r_1^A}$	MW of Amy-1 = $MW^L/(S^L/S^A)^{3/2}$ dalton
1	Amy-1^A	5.0	6.5	1.228	10,588
	Lysozyme	5.0	6.9		
2	Amy-1^B	5.0	6.5	1.228	10,588
	Lysozyme	5.0	6.9		
3	Amy-1^A & Amy-1^B	5.0	6.7	1.150	11,707
	Lysozyme	5.0	7.0		

N.B. MW^L = 14,400.

Results of immunodiffusion indicate both Amy-1A and Amy-1B amylase possess identical antigenic specificities as shown in Fig. 26. The two electrofocused Amy-1A preparations formed a precipitin band with the anti serum but the ethanol fractionated Amy-1A did not. This could be due to the low titer of Amy-1A in the ethanol fraction. The concentrated crude scutellar extract containing Amy-1B formed a precipitin band with the antiserum readily.

Other purified amylases were also tested with anti Amy-1A rabbit serum. A precipitin line was formed with hog pancreatic alpha-amylase, but not with sweet potato beta-amylase, or barley alpha-amylase. This result suggests there are some structural dissimilarities existing between maize Amy-1 amylase (identified as alpha-amylase) and barley alpha-amylase or sweet potato beta-amylase.

Amy-1 amylases prepared in various ways were placed in the wells surrounding the center well. Slides were incubated at 23 C in a humidified chamber for 3-7 days. The slides were then soaked in 1% saline for 24 hours with 3 changes of the saline, and 1 hour in distilled water. The agar gels were allowed to be air-dried. For staining the protein precipitin band, slides were immersed in 0.5% Nigrosin (dissolved in methanol-water-acetic acid 4.5 : 4.5 : 1 mixture) for 5 minutes and washed in 7% acetic acid for several days until protein precipitin bands stood out clearly from the non-stained background.

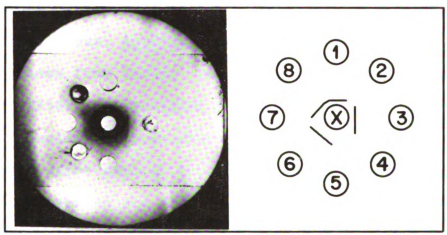


Fig. 26. Immunodiffusion of preparations of Amy-1A and Amy-1B amylases. A preparation of antibodies against Amy-1A was placed in the center well marked X. The number of the well into which each enzyme preparation was placed, and the material from which the samples were obtained is indicated as follows: 1 and 3 are the electrofocused Amy-1A; 2, 5 and 7 are the barley alpha-amylase obtained from extracts of the aleurone layers, 4 is sweet potato beta-amylase; 6 and 8 are Amy-1B present in the concentrated crude extracts of scutellar tissue of 8-day-old 38-11 seedlings.

Discussion

Identification of Amylases

The classification of amylases into alpha-amylase and beta-amylase has been based on the mutarotation of the end-products in the enzymic starch hydrolysates resulting from the actions of several purified amylases (alpha-amylases from hog pancreas, Aspergillus oryzae, and Bacillus subtilis; beta-amylases from barley, and sweet potato; (Freeman and Hopkins, 1936). Correlations of several alpha and beta forms of amylase with physico-chemical properties of hog pancreatic alpha-amylase and sweet potato beta-amylase were generally observed but not without exceptions. Table 18 lists thirteen sources of amylases that have been so identified. Bacillus polymyxa extracellular amylase degrades starch to maltose in beta-configurations but is also capable of attacking cyclic G_8 dextrins and beta-limit-dextrins (Robyt and French, 1964). Another class of fungal amylase, gluco amylase, degrades starch from both reducing ends and non-reducing ends by removing sequentially single glucose units, is unlike either the standard alpha- and beta-amylases (Pazur and Ando, 1959). Most workers have purified plant amylases from tissue extracts with a pretreatment of either heat or acid depending on whether alpha-amylase or beta-amylase was desired. Consequently, it is not surprising that no alpha-amylase

Table 18. Collective evidence for identifying amylases of plant and bacterial origin.

Origin	Amylases identified	Stability at pH 3.5	Stability at 70 C	Main end-products	Degradation of beta-limit dextrin	Degradation of cyclic G_8 dextrin	Optical Mutarotation	Calcium requirement	References
Barley, malt	alpha	labile	stable	$G_1 - G_6$	Yes	----	----	Yes	Greenwood & MacGregor '65
Sorghum, malt	alpha	labile	stable	$G_1 - G_9$	----	----	----	Yes	Dube & Nordin, 1961
	beta	stable	labile	G_2	----	----	----	No	
Wheat, flour	beta	stable	----	G_2	----	----	----	----	Rexova et al. 1967
Soy-bean	alpha	liable	stable	----	Yes	----	----	Yes	Greenwood et al. 1965 a,b
	beta	stable	----	----	No	----	----	No	
Pea, cotyledon embryo axis	alpha	----	stable	$G_1 - G_6$	----	----	minus	Yes	Swain & Dekker, 1966
	beta	----	labile	G_2	----	----	plus	No	
Maize, immature kernel	alpha	labile	stable	G_2	Yes	----	minus	No	These studies
Bacillus subtilis, extra- & intracellular	alpha	----	----	$G_1 - G_6$	Yes	Yes	minus	----	Robyt & French, 1963, 1964
Bacillus stearothermophilus, extra- & intracellular	alpha	labile	stable	----	----	----	----	Yes	Manning & Campbell, 1961 a
Bacillus polymyxa, extra- & intracellular	?	----	----	G_2	Yes	Yes	plus	----	Robyt & French, 1964
Streptococcus bovis, extra- & intracellular	alpha	----	----	$G_1 - G_4$	----	----	----	----	Walker, 1965

stable at low pHs, and no beta-amylase stable at 70 C could be found. Maize Amy-1 isolated by ethanol fractionation and electrofocusing was found to be similar to alpha-amylase in all respects. Its action pattern differs from hog pancreatic alpha-amylase perhaps only in the initial rate of glucose released from starch.

End-product Analysis

The spectra of end-products of alpha-amylases vary with the biological sources for the enzyme. There are two distinct action patterns of alpha-amylase with amylose; multiple-attack and random-attack. In a multiple-attack pattern the enzyme catalyzes the hydrolysis of several 1,4α-glycosidic bonds before the enzyme-substrate complex dissociates and forms a new complex with another substrate molecule. In a random-attack pattern the enzyme catalyzes the hydrolysis of one bond per active enzyme-substrate complex. The two action patterns can be distinguished by relating either the amount of residual polysaccharide to the amount of reducing sugar value (Robyt and French, 1967) or the viscosity to the number of glucose units in the residual polysaccharides resulting from the enzymatic hydrolysis (Banks et al., 1970). The ratio of either pair of parameters will be constant in the case of random attack, but increases with time in the case of multiple attack. The action patterns of three alpha-amylases from

hog pancreas, Aspergillus oryzae, and human saliva were examined at their own optimal pH and temperature. The action pattern of hog pancreatic alpha-amylase is typical for multiple attack and is distinctly different from those of the remaining two alpha-amylases which are characterized by random-attack (Banks et al., 1970). The action pattern at the initial stage of enzymic hydrolysis of starch is important inasmuch as the enzyme-substrate affinity varies with the size of the polysaccharide (Greenwood and Milne, 1968). The spectrum of end-products of a given alpha-amylase however is determined primarily at the initial stage of enzymic hydrolysis. Comparisons on action patterns of a wide range of alpha-amylases are still lacking. It is conceivable that various alpha-amylases may have different action patterns but are otherwise similar with respect to the eight criteria listed in Table 18.

The Molecular Weight of Amy-1

Molecular weights of alpha-amylases from diverse biological sources have been reported to be in the range of 45,000 to 50,000 (see the review by Greenwood and Milne, 1968). The only exception is Bacillus stearothermophillus exocellular alpha-amylase with a molecular weight of 15,600 as it was concluded from analytical centrifugation, osmotic pressure, and amino acid composition data (Manning and Campbell, 1961b). The present experimental data on

the molecular weight of maize Amy-1 estimated by gel-filtration and sucrose gradient centrifugation, indicate its size is about 12,000 and is smaller than lysozyme (14,400). Empirical means of estimating molecular weights of soluble proteins are based on three parameters, namely; Stokes radius, diffusion coefficient, and specific volume. The relevance of each parameter to the estimated molecular weight depends on the empirical method employed. Stokes radius is a predominant factor in determining the gel-filtration behavior of a protein and the sedimentation constant of a protein is determined mainly by both Stokes radius and diffusion constant and to a lesser extent by its specific volume. Since the results of the two methods employed to determine the size of Amy-1 are consistent with each other, it is reasonable to conclude that Amy-1 is a globular protein.

In nature, active enzymes with molecular sizes close to that of lysozyme are rare. A family of 6 proteases purified from Ficus glabrata latex was reported to have molecular weights ranging from 10,000 - 20,000 (Williams and Whitaker, 1969). They are probably structural conformers because the amino acid compositions are quite similar. The biological significance of these small active proteases is not known.

Enzyme Secretion

Neurospora crassa invertase exists in two active forms; plasma membrane bound invertase and extracellular invertase (Trevithick and Metzenberg, 1966). The $s_{20,\omega}$ values of the two forms are 10.3 and 5.3 respectively (Metzenberg, 1969). These two invertase are inter-convertible in vitro by manipulating salt concentration in the enzyme solution and the conversion was evident from the shift of electrophoretic mobility of one form to the other. Penicillinases of Bacillus licheniformis also exists in two forms (Sargent and Lampen 1970a). The extracellular penicillianse has a molecular weight of 24,000. The intracellular penicillinase in both plasma membrane and vesicle fractions can be solubilized by sodium deoxy-cholate, an ionic detergent, into a 45,000 molecular weight form. By removing and adding sodium deoxy-cholate, this form can be reversibly converted to a 24,000 molecular weight form with an electrophoretic mobility identical to that of the extracellular penicillinase. The latter, however, cannot be dimerized in the presence of sodium deoxy-cholate (Sargent and Lampen, 1970b). These authors suggested that the intra-cellular penicillinase undergoes a conformational change and is secreted as a stable form in aqueous media with a hydrophilic surface. It is conceivable that the enzyme to be secreted would have to be small in size and facilitated

with a hydrophobic surface enabling its passing through lipid-riched cell membrane.

Aspergillus oryzae alpha-amylase is also located in the periplasmic space (Tonomura, and Tanabe, 1964), and has monomer and dimer forms. The mechanism of its secretion is likely to be analogous to that of B. licheniformis. Cereal alpha-amylases are secreted from barley aleurone cells (Varner and Chandra, 1964), oat scutellum (Simpson and Naylor, 1962) and maize scutellum (Dure, 1960). The mechanism of their secretion may well be analogous to that proposed for Bacillus licheniformis penicillinase (Sargent and Lampen, 1970b).

Characterization of Amy-2

Amy-2 amylase of maize is slightly larger than Amy-1 amylase as judged by the appearance of Amy-2 in sucrose gradient fractions assayed by gel electrophoresis. The estimated molecular weight of Amy-2 is about 14,000. Despite the observation that on amylase zymograms Amy-1 and Amy-2 differ in the resulting starch-iodine color (Amy-1 is colorless and Amy-2 is pink) the fact remains that Amy-2 when eluted from gels after electrophoresis, is similar to Amy-1 with respect to the radioactive end-product analysis and the degradation of beta-limit dextrin, it may be concluded that Amy-2 is also analogous to alpha-amylase. The identification of Amy-2 cannot be made conclusively without further purification of this enzyme.

Comparison between Amy-1A and Amy-1B

The two allelic products $Amy\text{-}1^{A}$ and $Amy\text{-}1^{B}$ produced at the locus *Amy-1* are identical in molecular weight and antigenic specificity but differ in their electrophoretic mobility and isoelectric points (pI). The pIs of Amy-1A and Amy-1B are 4.80 and 4.35 respectively. Theoretically, it may be expected that of two proteins of the same size but different pI, the one with lower pI would migrate faster toward the anode at alkaline pH than the one with higher pI in an electric field. However, the reverse order of the electrophoretic mobility of Amy-1A and Amy-1B at pH 8.2 was observed. One possible interpretation is that Amy-1A and Amy-1B differ in their molecular conformations at regions of the polypeptides not involving their antigenic sites.

CHAPTER VII

GENERAL DISCUSSION

The significance of studying isozyme genetics need not be reiterated since this subject has been discussed extensively by Markert and Whitt (1968), and Scandalios (1969). It is evident from these studies that any attempt to elucidate the genetic control mechanisms of a given isozyme cannot be separated from developmental studies of this enzyme. The function of a gene can only be studied while the gene is active or when it is represented by its stable products, as it is in the case of Amy-2 in developing maize kernels. Neither can the genetic studies be confined to one developmental stage since the deduced mode of inheritance of this gene-product should be consistent in all phases of the life cycle of the organism, as it is in the case of Amy-1 in maize.

The concept of dominance and recessiveness in monogenic morphological traits of higher, diploid organisms has not been explained on the level of proteins, the immediate physiologically functional products of genes. Numerous isozymes have been shown to be controlled by codominant alleles. Heterozygous individuals generally possess both the parental allelic products. However, if

any isozymes, specific to any tissue or organ, have an ultimate effect on the differentiation and development of a distinct morphological trait, it may be possible that other mechanisms than simple dominance-recessiveness or codominance are operative. The significance of allelic hybrid isozymes as in the case of lactic dehydrogenase in many mammilian, avian and amphibian species, and in the case of maize catalase has not yet been understood. In other isozyme systems where allelic hybrid isozymes are apparently lacking, as in the case of maize Amy-1, the differential allelic expression may prove to have a significant effect on cellular differentiation in as much as the allelic expression is governed by a temporal and spatial controlling mechanisms. The allelic expression of a given gene may be remote from the action of that gene (transcription), but it can be defined more precisely by studies which enable us to discern the possibility of de novo synthesis versus activation of a preformed product (enzyme).

Apart from differential gene activation as a possible mechanism of cell differentiation, the steps leading from the genotypic input to the phenotypic output in a cell lineage are no less important in the contributions to cell differentiation. Particularly, the significance of protein-protein interactions has recently been recognized. In well established cases it has been shown that two

functionally related proteins encoded by two different loci are "hybridized" *in vivo* to form a multimeric protein with a distinct function, namely; the two components of E. coli tryptophan synthetase, and mammalian hemoglobin (see the review by Williamson, 1969). Numerous other multimeric enzymes resulting from protein-protein interaction are now known. The genetic basis for some hybrid isozymes is known but their physiological function(s) is not understood; examples are lactic dehydrogenase in mammals (Shaw and Barto, 1963; Markert, 1968), catalase in germinating maize seedlings (Scandalios, 1965 and 1970), and mitochondrial malate dehydrogenase in *Neurospora crassa* (Munkres, 1968). There are others with known *in vivo* functions but unfortunately have not been genetically defined, for instance; lactose synthetase of the guinea pig (Brew, 1969). Interactions between two structurally and functionally entirely unrelated proteins are possible *in vitro* while they are in unfolded states. Such an interaction was found between sweet potato beta-amylase and rabbit muscle aldosase (Cook and Koshland, 1969). It should be pointed out that in the developmental studies of maize amylase, Amy-2 gradually phased out and a new amylase band appeared 8 days post-germination with an intermediate electrophoretic mobility between Amy-1 and the original Amy-2. The question whether this new amylase band is the product of yet another gene (or gene product)

newly activated during seed germination, or is the result of an interaction between Amy-1 and Amy-2 will be a challenging problem in the future.

It was felt during the course of this studies that a more definitive classification of plant amylases based on such parameters as specificity of end-products, catalytic properties, or enzymatic mechanisms might help clarify the physiological function(s) of each amylase enzyme. In view of the fact that amylase, like many other enzymes, occurs in multiple molecular forms (isozymes), any attempts at characterization must account for the individual isozymes of the enzyme system. The characterization of isozymes can only be accomplished if we have good knowledge of the genetic origin(s) of a given enzyme; this in the long run is far more important than the gross chemical classification of any enzyme and should be the foundation for detailed biochemical and physiological studies.

Although amylases are probably some of the best studied enzymes in plants, the bulk of the earlier physiological studies were based on gross quantitative assays of the enzyme. The present findings show clearly that we are dealing with more than one molecular species of the enzyme, and that the isozyme pattern depands on the given stage in development. Both the genetic and developmental studies, as well as the chemical data suggests that interaction may be occurring between the different isozymes of amylase which affect both its structure and function.

The present work can be further expanded in several interesting directions. These are briefly listed below:

To investigate the possible interactions between Amy-1 and Amy-2 which may result in new forms of amylase. One approach is to test the amylase in question with rabbit anti-sera made specifically for Amy-1 and Amy-2 by an immunoelectrophoresis technique.

To study the intracellular localization of maize amylases. Preliminary results have shown that amylase activity is not associated with subcellular organelles (either mitochondria or glyoxysomes) which can be isolated by sucrose gradient centrifugations. However, the possibility that certain amylase isozymes are associated with some membrane fractions, such as plastid and cytoplasmic membranes, has not been examined. The fluorescent antibody technique may be useful in locating amylases in intact tissues. The commercially available fluorescein tagged goat antibody made against rabbit gamma-globulin may be used as an indirect histochemical method to study the intracellular localization of maize amylases.

To study the possible role of amylase isozyme by use of known carbohydrate mutants in maize. There are several carbohydrate mutants in maize. They differ significantly in the proportion of amylose and amylopectin in their starch. The enzymic digestion of the starch in vivo may have been programmed

in such a way that the combinations of various amylase isozymes may be starch-composition dependent.

To isolate and purify Amy-1A and Amy-1B and study their catalytic properties. Amino acid sequencing will be rewarding because the two amylases are small molecules consisting of roughly 100 amino acids. The elecidation of the primary structure of Amy-1 may lead to insight into its substrate binding sites.

To study hormonal effects on amylase activities from the genetic point of view. Emphasis would be on the hormonal regulation of the differential allelic expression of Amy-1 amylases in heterozygotes.

To analyze hormonal and environmental effects on amylase secretion from excised maize embryo deserves further investigations. Attention may be focused on the kinetics of amylase secretion in response to single external factors.

Studies of maize amylases have suffered from a major drawback in that there are no available analytical methods for quantitative determination of alpha-amylase, beta-amylase, and maltase independently from tissue extracts. As a result, no absolute correlation can be drawn from the quantitative assays to the qualitative zymograms of tissue samples from various developmental stages of seed germination; though indications are there. The present studies suggest the following scheme which may be applicable

for assaying alpha-amylase, beta-amylase, and maltase independently from their mixtures.

(1) Total reducing sugars released from starch and representing the overall effect of alpha-amylase, beta-amylase, and maltase can be most efficiently assayed by the neocupronic method (Dygert et al., 1965), in conjunction with starch pretreated with sodium borohydrade (Strumeyer, 1967). The effective range of this assay is 10 - 60 µg of glucose equivalents.

(2) Maltase activity can be sorted out by selectively inhibiting maltase with 0.1 - 0.4M Tris.

(3) Alpha-amylase activity can be sorted out by the use of the Glucostat-starch combination, and the complete conversion of maltose to glucose effected by the presence of fungal maltase generally found in Glucostat. The effective range of this assay is 50 - 200 µg glucose.

The activity of all three enzymes can thus be expressed on the common basis as units of glucose released per unit of reaction time. This procedure would be applicable in studying plant amylases assuming the effects of alpha-amylase and beta-amylase are additive in degrading starch.

CHAPTER VIII

SUMMARY

1. Starch-degrading enzymes in maize were separated by means of polyacrylamide gel electrophoresis into three zones; two amylase zones and one phosphorylase zone.
2. Genetic analyses were done for the major band of each of the two zones of amylase activities. The results showed Amy-1 of Zone-1 and Amy-2 of Zone-2 are controlled by two codominant alleles at two genetic loci.
3. Amy-1 amylase was isolated by ethanol fractionation and electrofocusing. It was identified as an alpha-amylase based on several criteria namely; thermal stability, pH sensitivity, optical mutarotation of end-products, distribution of end-products, enzymatic hydrolysis of beta-limit-dextrin.
4. The molecular weight of Amy-1 amylase was estimated by gel-filtration and sucrose gradient centrifugation. These methods led to the same conclusion, that Amy-1 amylase is a globular protein with a molecular weight of 12,000.
5. Amy-2 amylase is analogous to Amy-1 amylase with respect to its distribution of end-products and the capability of degrading beta-limit-dextrin. By

combining sucrose gradient centrifugation and gel electrophoresis the molecular weight of Amy-2 amylase was estimated to be about 14,000, slightly larger than Amy-1 amylase. However, Zone-2 is pink in color on the zymogram and Zone-1 is colorless; indicating the average size of residual limit dextrins in Zone-2 is larger than that in Zone-1.

6. The amylase zymograms of heterozygotes with respect to either Amy-1 or Amy-2 consist of two parental isozymes but no detectable allelic hybrid isozyme. This result is consistent with the findings in alpha-amylases of various sources that alpha-amylase is active as a monomer.
7. Gene-dosage effects were apparent with Amy-1 but not Amy-2 in zymograms of triploid endosperm tissues suggesting the former is synthesized in the endosperm tissue while the latter is synthesized in diploid tissues of developing kernels.
8. The amylase content increased during seed germination and reached its peak on the 8th day of germination. The amylase peak activity was 3-10 fold the activity in ungerminated seeds (depending on the strain). The change of amylase content in germinating seedlings was parallel with the change of amylase specific activity in endosperms but was not in phase with that in scutella. About 80% of the amylase content in

seedlings was found in endosperm and scutellum, and the remaining 20% in shoot and root tissues.

9. Amy-1 amylase is stable in all tissues from immature kernels to leaves of adult plants. Amy-2 is stable in developing kernels but disappears at about four days after germination and new bands with different electrophoretic mobility appear in Zone-2. Whether the instability of Amy-2 is due to a protein-protein interaction, non-protein conjugation in vivo, or conformational changes in vitro is yet to be determined.

10. The allelic expression of Amy 1 in heterozygotes ($Amy\text{-}1^{A}/Amy\text{-}2^{B}$) is under temporal and spatial control. The expression of $Amy\text{-}1^{B}$ is either absent or latent in endosperm and the scutellar tissues of germinating F_1 seedlings of ($A^1A^1 \times B^1B^1$), although it is in synchrony with $Amy\text{-}1^{A}$ in shoot and root tissues of the same seedlings. This differential allelic expression of Amy-1 is less pronounced in the F_1 seeds of the reciprocal cross ($B^1B^1 \times A^1A^1$). In liquid endosperm of developing F_1 kernels, only the maternal Amy-1 (either Amy-1A or Amy-1B) was present, while Amy-1A alone was invariably detected in embryo tissues of the same kernels. The differential allelic expression in maize seedlings could be accounted by one of the three possible mechanisms: differential gene

activation allelic exclusion, and repression of one gene-product by the product of its allelic gene. The differential allelic expression of Amy-1 in immature kernels is apparently independent to that in germinating seedlings but is consistent with the explanation of allelic exclusion.

11. The two allelic products, Amy-1A and Amy-1B, are identical in terms of molecular weight and the antigenic specificity, but differ in isoelectric point by 0.4 pH units.

12. Genetic linkage tests for *Amy-1* and *Amy-2* cannot be done directly because of the instability of Amy-2 in developmental stages suitable for assaying Amy-1. This test can be achieved indirectly by a linkage test for *Amy-1* and Ct (catalase) loci, since the latter is known to be linked with the *Amy-2* locus. This information may lend us an insight as to whether *Amy-1* and *Amy-2* might have evolved by means of gene duplication.

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