HYDROLYSIS OF MAIZE ENDOSPERM

By

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ABSTRACT

The hydrolyses of endosperm storage reserves in maize caryopses was investigated. The following points were examined in detail: 1) The enzymes responsible for hydrolysis of the reserve starch and proteins during early germination; 2) The regulation of enzyme formation and starch hydrolysis in the endosperm by gibberellic acid and 3) Isolation of protein bodies and associated protease activity.

The principle protease extracted from maize endosperm after germination had an acid pH optimum, and a high temperature optimum with the substrate haemoglobin. Alpha-amylase was also present in this tissue. Electrophoretic gels were used in identifying the development of two distinct amylases during early seedling growth. The acid protease and the amylases are absent from quiescent caryopses. These enzymes appear 2-3 days after imbibition of intact caryopses, and increase in activity throughout the course of protein and starch breakdown.

In contrast to other reported results (76) neither the embryo nor exogenous factors were necessary for

initiation or continuation of amylase formation in de-embryonated endosperms. Changes in enzyme activity in excised endosperms following similar trends were compared to intact caryopses germinated at the same temperature. Neither enzyme production nor starch hydrolysis was stimulated by treating de-embryonated maize endosperms with gibberellic acid. The response to gibberellic acid of de-embryonated endosperms of barley was tested as a control. De-embryonated endosperms of this cereal had low α-amylase activities when incubated in buffer. Inclusion of gibberellic acid in the incubation medium stimulated enzyme production eight-fold.

Protein bodies were isolated from germinating maize endosperm using sucrose density gradients and were identified by electron microscopy. Acid protease activity was not associated with this fraction, and in fact over 90% of the original activity was recovered in the soluble fraction.

A second protease extracted from developing maize endosperm had a broad neutral-basic pH optimum, and a temperature optimum of 55 C. It actively degraded both α-N-benzoyl DL-arginine p-nitroanilide (BAPNA) and haemoglobin. This protease was present in all stages of

developing endosperm tested with activity peaks at 25 and 57 days post fertilization. This protease was also present in quiescent and early germinating caryopses. After imbibition, activity decreased rapidly and disappeared in approximately four days. Preliminary results indicate no association of this protease with the protein body fraction.

PREFACE

The experiments described in this thesis were carried our in the Department of Biology, McMaster University, from September 1975 to December 1977. Except where others are specifically mentioned, this thesis consists entirely of my own work.

I would like to thank my supervisor, Dr. B.A. Oaks, for her guidance and patience throughout this study. Special thanks to all other members of the Faculty and staff of the biology department, who offered valued suggestions, experience and technical assistance. I would also like to take this opportunity to express my gratitude to my wife, Clare, Mom and Dad, family and friends, who were always ready to listen and lend moral support. I would also like to thank Mrs. Kathy Howard and Ms. Ruth Hall for their excellent typing.

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ABBREVIATIONS

A	absorbance
ABA	abscisic acid
BAPNA	α-benzoyl-L-arginine-p-nitroanilide
C	degree Celsius
DCM	dichloromethane
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
GA_3	gibberellic acid
g	gram
g	gravity
hr	hour(s)
IAA	indoleacetic acid
mg	milligram(s)
ml	milliliter(s)
M	molar
mRNA	messenger RNA
TCA	trichloroacetic acid

INTRODUCTION

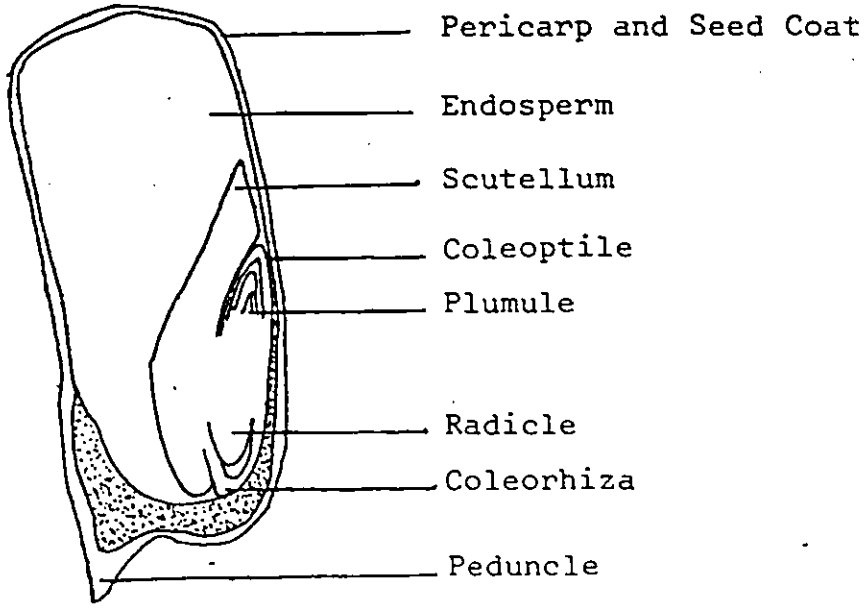
Structure and Germination of Grain

Maize grain consists of an embryo as a quantitatively minor component. In the mature kernel it represents only 10 percent of the total dry weight. The embryo consists of an axis with a lateral outgrowth, the scutellum, which is usually interpreted as a single modified cotyledon or seed leaf. In the ripe grain the root system and the shoot are well developed and both are enclosed in sheathing tissue; the coleorhiza in the case of the root and the coleoptile surrounds the first foliage leaves of the shoot (Fig. 1).

Abutting on the scutellum is the relatively massive endosperm. This tissue originates from the successive divisions of the triple fusion nucleus, that is, the fusion of the two polar nuclei and one nucleus from the male gamete (21). During grain ripening the cells of the central endosperm accumulate starch granules and protein bodies. The protein bodies contain the alcohol soluble prolamine fraction. Both the starch granules and protein bodies are embedded in a matrix of protein composed of the alkali soluble glutelin fraction.

Figure 1: Structure of the Maize Caryopsis

(Mayer, A.M. and A. Poljakoff-Mayber, 1975. The Germination of Seeds. Second Edition, Pergamon Press, Toronto).


Pericarp and Seed Coat
Endosperm
Scutellum
Coleoptile
Plumule
Radicle
Coleorhiza
Peduncle

As starch accumulates, the nuclei of the cells of the endosperm become disorganized, and by the time the grain is ripe, they cannot be detected by normal staining methods. At this stage the central endosperm is incapable of respiration and is exclusively a reserve tissue (110).

Around the periphery of the endosperm there is a layer of cells which differ structurally and physiologically from the rest of the endosperm. This is the aleurone layer which contains typical proteinaceous protein granules (99) called aleurone grains. Cells of the aleurone layer can respire actively and thus each contain a complement of functioning mitochondria proplastids, endoplasmic reticulum, dictyosomes and a nucleus.

Surrounding the embryo and the endosperm there is a thin envelope formed from the fused and modified pericarp plus testa: the whole structure--pericarp, testa, embryo and endosperm--constitutes the grass caryopsis (110).

Proteins, carbohydrates and lipids which accumulate in grain endosperms during development are hydrolysed during early seedling growth. Seedling development depends on the breakdown of the seed reserved and their utilization by the growing root and shoot. In maize the stored protein represents some 10-20 percent of the dry weight of the

endosperm, so that stored starch is the chief material used for energy production and cell wall synthesis in the developing tissues (12). The chief storage proteins, zein and glutelin, differ considerably in amino acid composition from the proteins which are to be synthesized by the developing embryo. Hence, extensive degradation of storage protein and interconversions of their amino acids accompany the utilization of this reserve (7). The hydrolysis products are transported via the scutellum to the embryo to support its early growth. Not until approximately 70 percent of the endosperm reserves have been used up is the first leaf functioning sufficiently to reverse the fall in total dry weight of the seedling or have the roots started actively assimilating nitrogen from the soil (7).

Ingle *et al.* (74) demonstrated that the two major constituents of the maize endosperm, insoluble protein and carbohydrate decreased drastically as early seedling growth proceeded. The solubilization of the protein began during the initial hours after grain imbibition, but during the first three days most of the soluble fraction is retained by the endosperm. Only later (5 days) did the soluble protein content of the endosperm decrease. At the same

time the soluble protein fraction of the embryo increased at a rate parallel with its growth. It is commonly accepted that the amino acids released by storage protein hydrolysis are incorporated into embryo protein (9,55,74,139,173) and appear to be the preferred nitrogen source for embryo protein synthesis during early growth. Experimentation shows that supplying exogenous nitrate does not increase the protein accumulation in the growing embryo until after the depletion of endosperm protein reserves (174).

Embryos can be cultured in the absence of the endosperm storage material. Dure (38) demonstrated that axial growth of excised maize embryos exceeded that of intact kernels for the first 8 days of growth if supplied with a nutrient medium containing sugars. Oaks and Beevers (136) found that although excised maize embryos showed normal increases in dry matter when cultured with glucose or sucrose, levels of amino acid pools fell, and subsequent increases in protein nitrogen was much less than observed in controls. Those amino acids which were most severely affected were those most abundantly supplied by the endosperm (ie. neutral and basic amino acids). An endosperm protein hydrolysate or synthetic mixture of amino acids of equivalent composition were most effective

in restoring protein synthesis in the excised embryos. These results help emphasize the importance of reserve protein hydrolysis for normal embryo development during early seedling growth. However excised embryos do have the capacity to synthesize their own amino acids after a lag period of about 3 days, and soon begin to accumulate both amino acids and proteins (135). This seems to suggest that the amino acid flow from the endosperm during early seedling growth may inhibit amino acid synthesis in the embryo.

Conversely Oaks (135) was able to demonstrate that the demands of the embryo for amino acids could regulate the degradation of storage proteins in maize endosperm. When an exogenous synthetic mixture of 15 L-amino acids known to be released by the endosperm was supplied to the seedling, loss of nitrogen from the endosperm was delayed. Thus there are clear interactions between the embryo and the storage tissues during seedling growth. These interactions appear to modulate both metabolic processes in the storage tissues, and embryo.

The Role of Gibberellins in Seed Germination

The aleurone layer, particularly those of barley, is frequently used to study hormone action. It is an easily

isolated tissue in which all cells respond to added gibberellins and abscisic acid. This response to added hormones can be studied by incubating half seeds or aleurone layers in a suitable buffer solution containing the hormone. Apparently the response of the isolated tissue to exogenously added gibberellins is similar to its response *in situ* to exogenously supplied gibberellins.

As early as 1890, Haberlandt recognized that diastatic activity responsible for starch digestion originated in the seed coat layers of cereals, and furthermore that a growing embryo was necessary for the production of this diastase (7).

In 1960 it was shown that the factor from the embryo necessary for activation of the secretory function of the endosperm was gibberellin-like (200). Paleg (141) and Yomo (200) demonstrated that GA_3 could substitute for the embryo in the initiation of events leading to subsequent substrate mobilization in the endosperm. Later Varner (202) showed that the GA effect and the production of hydrolases occured in the aleurone layer.

The barley aleurone layer synthesizes and secretes, in response to added gibberellins, α-amylase, protease (202), ribonuclease, β-glucanase (16), α-glucosidase, and limited

dextrinase (64). Part of the secreted ribonuclease, β-glucanase (16) and α-glucosidase (32) is present in the aleurone layers before addition of gibberellins. Although Clutterkuck and Briggs (32) found that high concentrations of helminthosporal or helminthosporic acid did induce a small proportion of α-amylase synthesis in isolated tissue, generally speaking all enzyme activities reported above are specifically evoked by gibberellins (32). The aleurone cells respond to GA_1, GA_3, GA_4 and GA_7, but not to GA_8 (32). Gibberellic acid (GA_3) has been used in most investigations.

Gibberellins similar to GA_3 and GA_1 are produced by the embryonic axis (61,108,109,111,159,160). In the intact germinating seed, only a few hours after grain imbibition (201) both the scutellum (159,160) and the nodal region of the barley embryo (109) synthesize the gibberellins that control the aleurone response. These gibberellins are then transported to the aleurone layer (108,109).

The response of isolated aleurone tissue to GA_3 has a lag period which varies from 0.5 to 20 hours after the addition of the hormone. Thirty minutes after GA addition the rate of labelling of soluble nucleotides changes (33). Four hours after GA addition an enzyme with

laminarase (β,1-3 glucanase) activity appears in the incubating medium (91). Synthesis and secretion of α-amylase and protease begins 8-10 hours after addition of the hormone (27,80). Ribonuclease does not appear in the incubation medium until 18-20 hours after GA addition (27). Because the production of the hydrolases is inhibited by dinitrophenol, p-fluorophenylalanine, and cycloheximide (185,186) it is clear that phosphorylative energy and protein synthesis are required for the appearance of hydrolase activity.

All alpha-amylase (α-amylase) becomes radioactively labeled when it is produced by aleurone layers incubated in ^{14}C-labelled amino acids. Tryptic digestion of this α-amylase demonstrates that the label is equally distributed throughout the enzyme (185). Alpha-amylase also becomes density labeled when the layers are incubated in the presence of $H_2{}^{18}O$ or D_2O (52). Results, like these, indicate that all α-amylase present in the aleurone layers of barley seedlings is synthesized *de novo*. Additional work with $H_2{}^{18}O$ and D_2O demonstrated that not only α-amylase but protease, ribonuclease and β,1-3 glucanase are synthesized *de novo* (16,52,80). Inhibition of hydrolase

production by inhibitors of protein synthesis is consistant with *de novo* synthesis.

A gibberellin-dependent increase in the rate of labelling of a poly(A)-rich mRNA-like fraction (71,81) is paralleled by a similar increase in translatable mRNA for α-amylase (70). Considering the fact that GA_3 does stimulate poly(A)-RNA synthesis (71,81), Higgins *et al.* postulate that the GA_3 stimulated increase in translatable mRNA may be due to promotion of α-amylase mRNA synthesis, but at the same time do not dismiss the possibility that this same increase could also be explained by a 1) decreased rate of degradation of existing mRNA; or 2) some kind of processing or activation of an inactive form of mRNA. Chrispeels and Varner (1967) have demonstrated that GA affects translation from mRNA rather than the synthesizes of that mRNA. Results show that upon addition of GA an 8 hour lag occurs before the appearance of α-amylase. Removal of GA from the incubation medium any time after the appearance of α-amylase activity results in a substantial reduction of α-amylase production. Replacing the GA back into the incubation medium results in an immediate resumption of α-amylase synthesis. The absence of a second lag period indicates that α-amylase mRNA is present and that

GA is affecting translation, or protein synthesis. The initial lag after GA additions may be that time needed for *de novo* synthesis of α-amylase specific messenger RNA (28).

Investigators have repeatedly sought the primary site of action of GA in the endosperm. The earliest observed responses of aleurone tissue to GA_3 are related to phospholipid metabolism and are not dependent on protein synthesis (17). These responses include changes within 30 minutes in the rate of labelling of soluble nucleotides (33) and increases within two hours in the activity of phosphorylcholine cytidyl transferase and phosphorylcholine glyceride transferase (17,84). Massive synthesis and secretion of hydrolases begins 8-10 hours after the cells of the isolated aleurone layers have been exposed to GA. The study of events during this 8-10 hour "lag" indicates that an entire change in metabolism is evoked by the hormone. Table I summarizes the principle hormone effects recorded to date.

A role of GA_3 in regulating the export of materials from aleurone cells has also been established (91,119,187). The release--without concomitant synthesis--of β,1-3 glucanase from aleurone cells is stimulated within 4 hours of GA_3 treatment (91). Similarly Melcher and Varner (119)

TABLE I

Early Effects of Gibberellic Acid on Imbibed Barley Aleurone Tissue

(hour) Time	Observation	Reference
0	GA added to isolated imbibed aleurones	-
½	changes in rate of labelling of soluble nucleotides	33
1	change in pattern of protein labelled by radioactive amino acids	53
2	increased activity of lecithin synthesizing enzymes	84
3	increase in the rate of labelling of Poly(A)-rich mRNA like fraction	71,81
	increase in translatable α-amylase mRNA	70
4	incorporation of ^{14}C-choline into ER stimulated	48
	increase in total number of ribosomes: increase in the number of ribosomes associated as polyribosomes: stimulation of ribosome aggregation	47,49

continued...

TABLE I (continued)

(hour) Time	Observation	Reference
	increased incorporation of ^{32}P into phospholipids	100
	decreased pentosan (cell wall precursor) synthesis	85
	proliferation of rough E.R.	88,90
	β,1-3 glucanase synthesis and release	91
8	selective incorporation of H^3-uridine into a polydisperse RNA fraction	179
	protease and α-amylase synthesis	52,80
	RNAse synthesis	30

have shown that the release of stored protein and peptides from aleurone cells is controlled by GA_3. Release of potassium and magnesium ions from aleurone cells only occurs after treatment of the tissue with GA_3 (92). The effects of GA_3 on the permeability of the aleurone plasmalemma to ions, storage proteins, and peptides suggests that this hormone could regulate storage events by controlling the permeability of membranes, including the distinctly membrane bound storage organelles of the aleurone cell (89).

There are two distinct GA effects described in the literature; one on the endosperm, and one on the embryo. Considerable evidence suggests that GA affects embryo metabolism. Intact caryopses can be stimulated to germinate with GA, and effects on embryo metabolism are apparent long before initiation of hydrolysis of the endosperm reserves (26). In barley embryos GA can stimulate α-amylase synthesis (204). Simpson (171) reported that embryos excised from dormant wild oats required GA in addition to sucrose and amino acids for growth.

GA is not the only hormone involved in regulation of embryo and endosperm metabolism in cereals. For example, Eastwood *et al.* (40) found that aleurones prepared from

quiescent wheat grains produced much more α-amylase when treated with GA and kinetin than when treated with either alone. Khan (97) proposed that kinins and inhibitors, such as abscisic acid, interact and their balance determines the ability of a seed to germinate. Even when this balance is favourable gibberellins are required to induce hydrolase activity in the imbibed seed. If this balance is unfavourable hydrolases cannot be induced by gibberellin addition (96,97).

Variability in Gibberellic Acid Effect

A series of papers by Paleg (141,142,143) have described the dependence of loss of dry weight, starch hydrolysis and protein release in excised barley endosperm on the presence of added GA. Work with barley, wheat and oats has confirmed the generality of this effect (144,164) but in each case there are varieties which do not respond to GA (79,130). In maize the regulation of hydrolysis of endosperm starch and protein reserves has been investigated, but conflicting reports appear in the literature. Dure (1960) (38) observed that starch hydrolysis or α-amylase production did not occur in excised endosperm and suggested

that the α-amylase was produced in the scutellum and released into the endosperm. Similarly Ingle and Hageman (1965) (76) found only minor hydrolysis of starch and protein reserves in excised endosperms, but established that a "stimulus" from the embryo necessary for activation of the hydrolases moved from the embryo about 36 hours after imbibition. In excised endosperms this "stimulus" could be replaced by exogenously supplied GA_3. These results indicate, as with barley, that GA normally produced by the embryo is required in the hydrolysis of the endosperm reserves. In contrast, Harvey and Oaks (69) demonstrated that excised maize endosperms incubated in buffer rapidly degrade their starch and protein reserves. These processes were not markedly stimulated by addition of gibberellic acid (GA_3), however protease and α-amylase activity were strongly inhibited by abscisic acid, and this inhibition could be overcome by the addition of exogenous GA_3 (69). Similarly, in barley (27) ABA inhibits the GA_3 induction of α-amylase synthesis and this inhibition can be overcome by increasing the concentration of GA_3 in the incubation medium. Thus in maize the presence of ABA counteracts the effect of endogenous GA and as a result the endosperms respond to added GA_3.

Harvey and Oaks (69) were also able to demonstrate that dwarf maize (d5) which is naturally deficient in GA (Phinney, 1959) showed a 3 to 5 fold increase of hydrolase production in response to added GA_3. This observation lends support to the idea that although GA is required in maize for the induction of amylase and protease as in barley and other small grain cereals, the reported variability in the response to exogenously added GA is probably related to various levels of endogenous GA or ABA in the different cultivars of maize. Results suggest that in many cases maize endosperms may contain sufficient gibberellin to stimulate maximal hydrolase production for starch and protein breakdown, with or without embryonic intervention.

To test this hypothesis it is necessary to:

1. develop a reliable technique to measure hydrolase activity in incubated excised endosperms and;
2. apply this technique to grain varieties with reportedly marked negative or positive hydrolase stimulation to endogenously added GA.

In this thesis a study has been made of the degradation of endosperm starch and protein reserves of

Zea mays during early seedling growth and the enzymes that may be responsible in that degradation. The regulation of enzyme activity due to GA has also been investigated.

Endosperm Protein Reserves

Two types of proteins are present in the mature seed. These are the metabolic proteins, both enzymatic and structural, involved in normal cellular activities, including the synthesis of the second type, the storage proteins. Storage proteins, along with reserves of carbohydrates and lipids are synthesized during seed development. Following seed germination and after hydrolytic breakdown they provide a source of nitrogen and carbon for the developing seedling. Storage proteins seemingly possess no overt biological activity and therefore have been classified by physiochemical means. The classification of major seed proteins on the basis of solubility as developed by Osborne (139) certainly has the advantage of simplicity and hence is still the most universally used. In cereals, albumins (proteins soluble in pure water) and globulins (soluble in salt solutions) are of relatively minor importance as nitrogen source proteins, but appear to contain most of the enzymatic proteins of maize caryopsis

(8,44,72). The albumin and globulin fractions of dry cereal seeds are mainly made up of proteins of the embryo and aleurone (50). The remainder of the protein left after salt extraction is almost entirely associated with the endosperm and can be extracted with organic solvents. One such protein fraction can be extracted in aqueous alcohol. This was termed prolamine due to its high proline and amide content. These are hydrophobic proteins found to reside in protein bodies (192). A fourth protein fraction, glutelin, can be extracted only by alkaline solvents. Although the origin of this fraction has never been conclusively established, it has been assumed that this is another group of endosperm storage proteins not contained in protein bodies (54). In maize, zein and glutelin are the principle storage proteins together comprising 60-80% of the total endosperm protein (199). Since the advent of the automated amino-acid analyser the proportions of the different amino acids in the Osborne fractions have been widely investigated (54,78,129,167,83). All workers to date have found that prolamines, for example zein, have high levels of glutamine and proline and low levels of lysine, methionine and tryptophan (Table II). In the salt-soluble fractions, the amino acids are present in more

TABLE II

Amino acid composition of Osborne fractions from a normal maize variety (180).

Amino Acid	Albumin	Globulin	Prolamine	Glutelin
	(percent of total protein fraction Nitrogen)			
Lysine	5.4	5.0	0.1	2.6
Histidine	1.9	2.0	0.7	2.7
Arginine	5.3	5.5	1.0	3.0
Aspartic Acid	8.2	8.6	5.4	5.7
Threonine	5.0	4.9	2.9	3.7
Serine	5.6	5.6	5.7	5.1
Glutamic Acid	12.3	11.6	21.0	16.4
Proline	6.9	7.4	10.2	10.9
Glycine	10.1	11.3	2.1	7.0
Alanine	11.0	10.4	14.2	10.2
Half-cystine	0.2	1.2	0.1	1.5
Valine	7.1	7.0	4.8	5.8
Methionine	1.3	1.2	0.3	3.4
Isoleucine	4.4	4.1	4.2	3.5
Leucine	9.3	8.1	19.5	11.6
Tyrosine	2.5	2.6	2.9	3.4
Phenylalanine	3.3	3.6	4.8	3.4
Percent of Grain Total Nitrogen	0.9	1.5	36.9	41.4

even proportions whereas the glutelins nearly always show an intermediate composition.

In maize the distinction between zein and glutelin has physiological significance. Microscopic examination of normal corn endosperm show that storage protein is deposited within the cells as two distinct components, globular bodies and an amorphorous matrix in which the bodies lie embedded.

In a study of protein degradation in corn wet-milling Cox *et al.* (34) reported a globular component of protein which was resistant to dilute sulfurous acid. On the basis of histochemical tests, and positive correlation between appearance, number, and location of the protein granules with the appearance, amount, and location of zein during development of the endosperm, Duvick (39) suggested that these bodies were the major site of zein storage in maize. That these granules are rich in zein is demonstrated by their solubility in 80 percent ethanol (194,195). On the basis of analysis of other particulate structures, zein protein does not appear to be deposited in locations in the endosperm cell other than in membrane-bound protein bodies (30). The proteinaceous character of the protein bodies and cross-linked glutelin matrix was demonstrated by digestion with protease, pronase, and

the positive staining reactions indicative of protein using ninhydrin-Schiff or chloramine-T-Schiff reagent (194). Thus intracellular locations of the two classes of proteins differ as do their patterns of synthesis in developing grain. Glutelin accumulation begins 10 days after pollination in maize, continues at a steady rate until 42 days then ceases. In contrast, zein synthesis is slight until about 22 days and then becomes very rapid until 42 days. Between 22 and 42 days the rate of zein accumulation is about three-fold greater than that of glutelin (36).

Genetic evidence adds to the validity of dividing endosperm storage proteins into the two classes. Mertz *et al.* (121) was the first to demonstrate a radical change in the protein composition of maize endosperm caused by a single mutant gene, opaque-2. Preliminary tests showed that the endosperms of maize grain homozygous for the opaque-2 mutant gene had a lysine and tryptophan content which was double that of normal corn. This was accompanied by a dramatic reduction in the ratio of zein to glutelin. A second mutant (132), floury-2 displayed similar characteristics. Other mutants of similar phenotype; opaque-1, floury-1, and soft starch do not cause major changes in amino acid pattern. Although the enhanced

lysine content of opaque-2 stocks result in part from a higher lysine content of the zein (132), in general the increase in both high lysine single gene mutants is attributed to the change in the zein to glutelin ratio of the endosperm. Wolf (195) reported that the high-lysine mutants, opaque-2 and floury-2, were deficient in protein bodies. Since protein body compartmented zein contains little lysine in its amino acid make-up, whereas other endosperm protein fractions have normal amounts of lysine, Wolf's observations provide structural evidence for the altered amino acid composition of storage proteins originally reported in the high lysine maize mutants by Mertz *et al.* (121). The accumulation of zein in the endosperm is therefore under individual genetic control.

Environmental factors also influence the total amount of protein synthesized during seed development. The effect of nitrogen fertilizers have been investigated (63,95,157,193) and the results show that zein content increases preferentially when ample nitrogen is present, whereas glutelin content remains relatively constant. With this in mind, Harvey (67) studied the sequence and rates of degradation of zein and glutelin during germination. Results showed that high glutelin caryopses degraded

glutelin more rapidly than zein, whereas in high zein caryopses the reverse was true. Batches of caryopses with initially the same amount of zein and glutelin degraded equal amounts of both storage proteins. Harvey concluded that the more abundant protein was degraded most rapidly. Additional results indicated early zein and glutelin losses were not initially reflected in loss of total nitrogen in the endosperm due to increases in the salt and water soluble nitrogen. Increases in the water soluble fraction during the first days of germination were also observed by Ingle *et al.* (74).

Endosperm Proteases

Many workers have shown that proteases and peptidases are present in both ungerminated and germinating seeds. Proteolytic enzymes involved in the mobilization of protein reserves of seeds during early seedling growth may be classified as endopeptidases or exopeptidases depending on whether they hydrolyze internal or terminal peptide bonds of a polypeptide. Proteolytic enzymes that attack the terminal peptide bonds are called carboxy-peptidases or aminopeptidases depending upon which end of

the molecule they act on.

In germinating seeds the endopeptidases and carboxypeptidases are apparently involved with breaking down reserve proteins (165). Subsequently peptidases can complete the hydrolysis to amino acids and small peptides suitable for transport to the embryo (123). Proteolytic activity has been studied in a number of cereal crops (Table III) and common denominators now seem apparent. They usually have acidic pH optima, and in this respect they support a concept presented by Matile (113), Yatsu (197), and St. Angelo and Ory (176) that they are part of a lysosomal-like digestive process that takes place at acid pH.

Many specific peptidases have been identified in germinating barley (104,123,153,190), and their distribution and activity during germination investigated. However endopeptidases, which would appear to have the more important role in protein breakdown in the earlier stages of germination have not been as thoroughly described.

In most modern reports on protease activity, in either resting or germinating cereal grains it is unclear whether the assays used are measuring enzymes with either endo- or exopeptidase activity. It is also important to note that in most developmental studies enzyme activities

TABLE III

Characteristics of Proteases from Quiescent and Germinated Cereal Endosperm

Grain	Activity	pH Optimum	Assay	Ref.
Ungerminated Wheat	Protease	5.0 - 6.0	Tryrosine release from haemoglobin	172
Ungerminated Wheat	Protease	3.8	Tryrosine release from haemoglobin	116
Ungerminated Wheat	Endopeptidase	3.8	Peptide release in flour suspensions	59
	Exopeptidase	n.d.	Amino acid release in flour suspensions	59
Ungerminated Wheat	Protease	n.d.	Change in nitrosine staining of haemoglobin	94
Ungerminated Wheat	Protease	n.d.	Tryrosine release from haemoglobin	191
Ungerminated Barley Protein Bodies	Endopeptidase	n.d.	Gelatin viscosity change BAPNA hydrolysis	137
Ungerminated and Germinated Barley	Endopeptidase	n.d.	Gelatin viscosity change	44
Ungerminated and Germinated Barley	Endopeptidases(5)	n.d.	BAPNA hydrolysis	24
Germinated Barley	Protease	acid	α-amino nitrogen release from gliadin	80
Germinated Barley	Endopeptidases(5)	acid	Total nitrogen release from gliadin	23

Continued...

TABLE III (continued)

Grain	Activity	pH Optimum	Assay	Ref.
Germinated Barley	Endopeptidase	3.8,5.0-6.5 and 7.0	Gelatin viscosity change	77
Germinating Barley	Endopeptidase	8.6	BAPNA hydrolysis	18
	Protease	3.8	Release from haemoglobin of material absorbing at 280 nm	18
Germinating Barley	Carboxypeptidases (3)	4.8,5.2,5.7	Liberation of C terminal amino acid from Z-Phe-Ala, Z-Phe-Phe and Z-Pro-try	123
Germinating Barley	Aminopeptidase (3)	5.8-6.5,7.2	Measurement of liberated B-naphthylamine, p-nitroaniline or amino acids	101
Germinating Oats	Protease	n.d.	Release from haemoglobin of material absorbing at 276 nm	178
Germinating Sorgum	Endopeptidase	3.6	Tryosine release from Bovine Serum albumin	56
Ungerminated Maize	Endopeptidase	7.5	BAPNA hydrolysis	120
Ungerminated and Germinated Maize	Aminopeptidase	n.d.	Hydrolysis of L-leucine.para nitroanalide and N-carbobenzoxyl-L phenylalanine-L-alanine	51
	Carboxypeptidase	n.d.		
Germinated Maize	Endopeptidase (3)	3.8,5.4-7.5	Rate of trichloroacetic acid soluble amino groups produced from casein	51
Germinated Maize	Endopeptidase	3.8	Release from haemoglobin or gliadin at material absorbing at 280 nm	67,68

Continued...

TABLE III (continued)

Grain	Activity	pH Optimum	Assay	Ref.
Germinated Maize	Endopeptidase	3.0	Hydrolysis of denatured haemoglobin	3
Germinating Wheat	Carboxypeptidase	5.7	Rate of liberation of alanine from CBZ-Phe-ala	190
	Exopeptidase	4.0	Amount of glycyl-glycine produced from acid denatured haemoglobin	154
	Endopeptidase	6.0	Release from Azocasein of material absorbing at 440 nm	104
	Endopeptidase	8.6	BAPNA hydrolysis	46

have been measured, and not their absolute quantities. In evaluating such data several interpretations of increases and decreases in enzyme activities can be made including synthesis and degradation, activation or inhibition, and compartmentalization. Protease activity specifically measured by change in viscosity of a gelatin substrate, or hydrolysis of BAPNA, a synthetic peptide, is endopeptidase in nature (Table III). Both exo- and endopeptidase activity can be found in ungerminated caryopses (59,123).

Studies have been made of protease activity during early seedling growth. In pea cotyledons (15,60,203), chickpea (9), rice (147), wheat (149) and mung bean (29) protease activity is known to increase during seedling growth. In oats, proteases (178) reach their maximum activity when degradation of the storage proteins is almost complete, whereas in kidney bean cotyledons (158) protease activity fluctuated in a manner seemingly unrelated to reserve protein hydrolysis. As activities of several of these proteases do not appear to correlate with the observed rates of protein breakdown, they may not have a crucial role in hydrolysis of the stored protein reserves. Furthermore several seed protease activities have only

been demonstrated using casein, denatured proteins such as haemoglobin (62,170), or the synthetic endopeptidase substrate BAPNA (112,120). Such enzymes may not be able to attack undenatured storage proteins during germination. Harvey and Oaks (67) have identified an acid protease with a high endopeptidase component in maize endosperm. It increases in activity from day 3 to day 8 after imbibition and then declines. In addition to being active against native proteins (ie. haemoglobin, gliadin, edestin and bovine serum albumin) results indicated that partially denatured zein and glutelin could also be degraded efficiently under standard assay conditions. Time course studies with the standard assay or with agar gels showed that the enzyme was not detectable initially in germination and hence is probably not responsible for the early degradation of zein and glutelin. On the other hand, the increase in activity does coincide with the main period of protein breakdown in the endosperm. Calculations showed that even when compensating for less favourable *in vivo* conditions enough enzyme was present to degrade all the storage protein in the endosperm in less than six days. More recent investigations by Feller *et al.* (51) demonstrated the presence of one carboxypeptidase and two

additional caseolytic endopeptidases in endosperms excised from intact corn seedlings during germination. Similarly they found very little activity in the proteases until 24 to 48 hours after imbibition of the dry endosperms. With this in mind maize endosperm proteolytic activity has been investigated in developing, dry and early germinating caryopses (0-4 days). Endopeptidase activity of the protease preparation has been established during the crucial period of initiation of reserve protein hydrolysis. Development of activity has been traced through development and early growth (0-4 days) of the grain.

Localization of Protease Activity

Research has indicated a localization of at least some of the enzymes involved in storage tissue hydrolysis. Localization approaches include (i) isolated aleurone layers; (ii) differential sucrose gradient separation of organelles; (iii) gelatin film substrate methods; and (iv) isolated protein bodies.

Most work has been conducted with isolated protein bodies from ungerminated seeds since protein body stability naturally decreases as germination proceeds.

In mature caryopses, evidence is available to suggest that enzymes may be bound to or associated with storage proteins. Acid proteases have been detected in protein bodies from barley aleurone tissue (137), hempseed (175), cottonseed (197), and germinating sunflower cotyledons (166). Likewise Ory and Henningsen (137) have demonstrated the association of acid phosphatase and acid protease with protein bodies isolated from dormant barley seeds.

Koroleva *et al.* (102) demonstrated that the cotyledons of ungerminated vetch seeds contained two endopeptidases in the protein bodies as well as two different endopeptidases in the cytoplasmic fraction. Enzyme activities were differentiated by pH optima.

Harris and Chrispeels (65) have assayed a protein body preparation and a cytoplasmic fraction from ungerminated mung bean seeds for several enzymes which might be involved in the degradation of storage proteins. The protein bodies contained all, or nearly all, of the carboxypeptidase and endopeptidase (5), whereas the cytoplasm contained all, or most of the leucine amino peptidase, and trypsin-like activity. More recently, Chrispeels et al. (29) have demonstrated, using

fluorescent antibodies, that the endopeptidase is transferred, early in seedling growth, from its site of synthesis in the cytoplasm to the protein bodies in vesicles that merge with the protein body.

During this study, localization of maize endospermic proteases has been studied in developing, dry and germinating caryopses.

MATERIALS AND METHODS

Plant Material (*Zea mays*)

The maize hybrid W64A x W182E was used throughout this study. Caryopses were purchased in batches from Wisconsin Foundation Seed, Madison, Wisconsin. Maize hybrid WF9 x M14 (1966) was a gift from Professor R.H. Hageman of the University of Illinois, or was purchased from Wisconsin Foundation Seed (1976). Barley was supplied by the Crop Science Department of the University of Guelph, Guelph, Ontario.

Preparation of Caryopses

Commercial maize caryopses were received coated with fungicide dust. This was removed by repeated rinsings with distilled water. For experiments requiring sterile conditions the caryopses were left in a 5 part sterile distilled H_2O; 1 part Javex (a commercial bleach containing 6% sodium hypochlorite) solution for fifteen minutes. The caryopses were then bathed with 0.01 M HCl for an additional ten minutes followed by a thorough rinsing with sterile distilled water (2). This treatment served to surface sterilize the caryopsis.

To reduce internal fungal contamination, caryopses were infiltrated with a commercial fungicide (0.5%), Captan or karathane, for two hours using dichloromethane (DCM) as solvent, 24 hours before use (6,42,122,181). Times of infiltration were determined so that affect on germination or hydrolysis of the endosperm starch reserves, as determined by amylase activity, (Table V, Figure 7) were minimal.

Germination of Maize Caryopses

Caryopses were rinsed thoroughly in distilled water before planting in a moist sand and vermiculite mixture. The caryopses were germinated at 28°C and grown with a daily cycle of 12 hours illumination, 12 hours dark. Samples of fifty seedlings each were harvested as required. At all stages of seedling growth the endosperm could be easily excised and quick frozen in liquid nitrogen. The endosperm were stored at -20°C until required, with no loss of enzyme activity. A 1 part sand to 1 part vermiculite mixture was used.

Developing Maize Endosperm (W64A x W182E)

Plants were grown at 28°C with 16 hours illumination. Cobs were harvested at set times after pollination. At all

stages of caryopses development the endosperm could be easily separated from the scutellum and embryonic axis before freezing in liquid nitrogen. The endosperm were stored at -20°C until required.

Incubation of Excised Maize Endosperm

Caryopses were infiltrated with karathane 24 hours before use. The caryopses were surface sterilized in diluted Javex, then rinsed with 0.01 M HCl and sterile distilled water. After imbibing for two hours in sterile distilled water the embryos and scutella were dissected out under sterile conditions. The endosperms were transferred to sterile 50 ml Erlenmeyer flasks, 10 endosperms per flask. Each flask routinely contained 10 ml of acetate buffer, 1.0 mM pH 5.0, which contained 10^{-3} M calcium chloride. Freshly prepared penicillin (10 µg/ml), chloramphenicol (10 µg/ml) and streptomycin (250 µg/ml) were also included to minimize bacterial contamination. A total of 50 endosperms were used per treatment. The flasks were incubated in a water bath at 28°C with shaking. After the required incubation time aliquots of incubation medium were plated on nutrient agar to test for contamination. Infected samples were discarded. The endosperms were frozen in liquid nitrogen and stored at

-20°C. The incubation medium was assayed immediately for hydrolytic enzymes and reducing sugars.

Products of Endosperm Starch Hydrolysis

Reducing sugars, released into the medium by starch hydrolysis, were measured by the Anthrone or Nelson method (131,198).

Extraction and Assay of Protease from Germinating Maize Endosperm

Extraction and assay procedures were essentially the same as those described by Harvey (68). Frozen endosperm were homogenized for two minutes at full speed in an Omnimix cooled in an ice bath. Two ml of 0.2 M acetate buffer, pH 3.8, which contained 5 mM β-mercaptoethanol were used per gram fresh weight of endosperm tissue. The resulting homogenate was centrifuged at 28,000 x g for 30 minutes at 0°C. The supernatant was assayed for proteolytic activity with a 5% solution of haemoglobin as substrate. Each assay routinely contained 50 mg of substrate, 1 ml acetate buffer, 0.05 M, pH 3.8, with 2.5 mM EDTA, enzyme (0.01 - 0.5 ml) together with distilled water to give a total volume of 2.5 ml. The mixture was incubated at 37°C for 10 minutes, and the reaction stopped

by adding an equal volume of 5% TCA. Undigested protein was allowed to precipitate for 10 minutes at room temperature. The precipitated haemoglobin was removed by filtration. Activity was routinely measured by the increase in absorbance at 280 nm of the TCA-soluble fraction. This was arbitrarily calibrated against the absorbance of tryptophan at 280 nm.

Protease activity released into the medium by excised incubated endosperm was also measured. An aliquot of fresh medium, 0.25 - 0.5 ml, was incubated with 50 mg haemoglobin and 1.5 ml assay buffer in a total volume of 3 ml. The reaction was stopped by the addition of 3 ml of 5% TCA and activity determined as above.

Extraction and Assay of Protease from Developing Maize Endosperm

Frozen endosperm were homogenized by grinding in a cold porcelain mortar with sand and buffer (0.1 M tris pH 7.5). Two mls of buffer were used per gram fresh weight of tissue, and the homogenate was centrifuged at 28,000 x g for 20 minutes at 0°C. The supernatant was used as crude protease. Protease activity was determined by the methods of Melville and Scandalios (1972) with slight modifications using α-N-benzoyl DL-arginine p-nitroanilide (BAPNA) as substrate. Substrate was prepared by dissolving 43 mg of BAPNA in 1.0 ml

of warm (60°C) dimethyl sulfoxide (DMSO) and then diluting to 100 ml with warm 0.1 M tris, pH 7.5. Routinely 50 λ of enzyme extract was incubated for 3 minutes with 0.77 ml of buffer (0.1 M tris, pH 7.5, 35°C) in a 1.0 ml quartz cuvette with a 1 cm light path. The reaction was started by the addition of 0.3 ml of warm substrate (35°C). The reaction was monitored by following the increase with time in absorbance (A) at 410 nm with a Unicam SP 1800 Spectrophotometer. This was arbitrarily compared to the increase with time in optical density at 410 nm when a trypsin standard was used. Trypsin at a concentration of 0.1 mg/ml was dissolved in 1.0 mM HCl and was used as an enzyme standard.

The supernatant was also assayed for activity using a 5% solution of haemoglobin as substrate. Each assay routinely contained 50 mg of haemoglobin, 1 ml of tris buffer, 0.1 M pH 7.5, and 0.2 - 0.5 mls of enzyme in a total volume of 2.5 ml. The mixture was incubated for 10 minutes at 35°C and the reaction stopped by adding an equal volume of 5% TCA. Undigested protein was allowed to precipitate for 10 minutes at room temperature. The precipitated haemoglobin was removed by filtration. Activity was measured by the increase in absorbance at 280 nm of the TCA-soluble fraction.

Isolation of Maize Protein Bodies

Extraction of protein bodies were carried out by methods described by Chrispeels with slight modifications (29). All procedures were carried out at 0 - 5°C. Fresh endosperms were homogenized in an Omnimix with 40% (w/v) sucrose in 25 mM citrate phosphate buffer, pH 4.5, or 0.1 M Tris-HCl buffer, pH 7.5; with 10 mM β-mercaptoethanol. One ml of buffer was used per gram fresh weight of endosperm tissue. The homogenate was filtered through miracloth, and centrifuged at 500 x g for ten minutes to remove starch and cell debris. Five ml of the resulting supernatant was layered on a step gradient routinely consisting of 25 ml of 50% (w/v) sucrose and 5 ml of 80% (w/v) sucrose in the same buffer. The gradients were centrifuged for 60 minutes at 75,000 x g in a SW27 Beckman rotor. Protein bodies of endosperms from germinated seedlings formed a sharp band on top of the 80% sucrose. Other cytoplasmic organelles plus dissolved proteins remained on top of the 50% sucrose. After centrifugation the gradient was fractionated by pumping it from the bottom; 16-21 fractions of approximately 1.5 to 2.0 ml each were collected. The A_{280} of the eluate was measured continuously using an ISCO model UL-4 absorbance reader. The fractions containing protein bodies were combined

and the sucrose concentration was lowered to about 0.6 M using extraction buffer. The protein bodies were then sedimented by centrifugation using a SW50L Beckman rotor at 160,000 x g for 15 minutes. This pellet was fixed for electron microscopy.

Identical fractions of protein bodies were combined and used directly to test protease activity. Protease activity was also measured for the fractions containing other cytoplasmic organelles plus dissolved proteins.

Electron Microscopy

The protein bodies were fixed in 2% glutaraldehyde buffered with 130 mM phosphate buffer containing 1% sucrose (Millonig's Buffer) for two hours on ice (124). The pellets were then rinsed once, for 10 minutes in the same buffer on ice, and a second time for 10 minutes at room temperature with slow agitation. Agitation on a rotor continued throughout all remaining steps until embedding in 100% Spurr's. The pellet was post fixed for two hours in 1% osmium tetroxide dissolved in the same buffer. Post fixation was followed by two buffer washes of 10 minutes each. The pellet was then dehydrated in a graded ethanol-water series consisting of 30, 50, 70, 95 and 100% steps. Each dehydration step was performed twice for a time duration of 10 minutes each. Final dehydration

was accomplished by two washes of 100% propylene oxide, both 10 minutes in duration. The pellet was then placed in a 1:1 mixture of Spurr's epoxy resin : propylene oxide for one hour. The pellet was transferred to a 3:1 mixture of Spurr's epoxy resin : propylene oxide for an additional 2 hours then left in pure Spurr's overnight. The next day the pellet was cut into 1 mm square pieces and embedded into blocks of pure Spurr's. The blocks remained in a 32°C oven for 17 hours, and later in a 60°C over for 24 to 48 hours. The blocks were sectioned with a glass knife on a Reichert ultramicrotome. The sectioned material was post stained with uranyl acetate for twenty minutes followed by 1 minute lead citrate. The grids were then examined in a Philips EM 300 electron microscope.

Extraction and Assay of α-Amylase from Germinating Maize Endosperm

Frozen endosperm were homogenized for two minutes at full speed in an Omnimix cooled in an ice bath. Two ml of 5 mM acetate buffer, pH 5.0, which contained 10^{-3} $CaCl_2$, were used per gram fresh weight of endosperm tissue (68). The homogenate was centrifuged at 28,000 x g for 30 minutes at 0°C. The supernatant was assayed for amylase activity with a starch solution as substrate. Each assay routinely contained

1 ml of the supernatant fraction and 1 ml of the starch solution. The enzyme was diluted if necessary with extraction buffer before assaying. The mixture was incubated for 10 minutes at 28°C. The reaction was stopped by addition of 1.0 ml of iodine reagent. To this final reaction mixture, 5.0 ml of distilled water was added, mixed and the absorbance read at 600 nm. Activity was expressed as mg starch degraded per minute per endosperm.

α-Amylase activity released into the medium by excised endosperms incubated in buffer was also measured. Aliquots of medium, diluted if necessary, were substituted for endosperm extracts in the routine assay.

Polyacrylamide Gel Electrophoresis, Protein Extraction Procedure

Proteins were extracted from frozen maize endosperm by grinding the endosperms in a cold mortar with 10 mM $CaCl_2$. One ml of buffer was used per gram fresh weight of endosperm tissue. The homogenate was centrifuged at 28,000 x g for 15 minutes at 0°C. To the supernatant sucrose was added to 20% and then used as an enzyme extract. Routinely 20 - 40 λ of enzyme was used per pocket.

Polyacrylamide Gel Electrophoresis

Electrophoresis, using a 6% gel (41), was carried out

for 3 to 4 hours at 4°C under a voltage gradient of 15V per cm. Running buffer was 0.1 M tris borate, pH 8.9. Gels were stained either for total protein or for specific amylase enzymes. When looking for amylase activity the slabs were incubated for 24 hours at room temperature on an Eberbach shaker (20 oscillations min^{-1}). The medium contained 0.8% (w/v) soluble starch in 70 mM phosphate buffer (pH 5.8) containing 10 mM $CaCl_2$. The slabs were then washed with water several times and placed in dilute Iodine-potassium iodide solution (58). Proteins were stained with Coomassie Blue. Protein bands were recorded as interpretive drawings using stain intensity as an index of relative protein concentration.

Preparation of Barley Half Seeds

Barley caryopses were cut transversely and the embryo containing halves were discarded. The extreme tips of the distal halves were likewise cut off and discarded. Half seeds were sterilized for 15 minutes in 1% sodium hypochlorite (commercial liquid bleach, diluted 1:5 with distilled H_2O), washed for 10 minutes in 0.01 M HCl, rinsed several times in sterile water and princubated in Petri dishes on moist sterile sand. The petri dishes containing the half seeds were

wrapped in aluminum foil and incubated at room temperature for 3 days. The endosperms were then transferred to sterile 25 ml Erlenmeyer flasks, 10 half seeds per flask and fifty endosperm per treatment. Each flask contained 20 µmoles of succinate buffer (pH 4.8), 200 µmoles of $CaCl_2$ and either 10^{-6} M GA or distilled H_2O. The final volume was 2.0 ml (188). The flasks were incubated in a shaking water bath (40 oscillations min^{-1}) at 25 C for 24 hours.

Extraction and Assay of α-Amylase from Incubated Barley Half Seeds

After the required incubation time aliquots of incubation medium were plated on nutrient agar to test for contamination. Infected samples were discarded. One ml of distilled H_2O was added to each flask and the medium decanted. The half seeds were rinsed with 2.5 ml of distilled H_2O and this was combined with the medium.

The half seeds were ground to a thick paste in a porcelain mortar with a little sand and 0.8 ml of 0.2 M sodium chloride. The homogenate was diluted with 4.0 ml of the same solution and centrifuged at 2,000 x g for 10 minutes at 4 C. The resulting supernatant fraction (referred to as extract) and the medium were assayed for α-amylase activity as described above.

RESULTS

I. Endosperm Hydrolytic Enzymes

A: Proteases

An assay for measuring proteolytic activity in endosperm extracts from maize hybrid Wf9 x 38-11 with natural substrates (gliadin, glutelin, and zein) or haemoglobin was developed by Harvey (66). The protease(s) was characterized as a high temperature, acid endopeptidase. Preliminary experiments were carried out to see whether the trend in protease activity was the same in maize hybrid W64A x W182E. The assay was optimised so that activity in crude extracts could be reliably measured with the substrate haemoglobin in endosperms of different ages. The changes in endosperm proteolytic activity during germination were followed.

(a) pH of the Assay

The pH optimum for assaying of the protease(s) was determined. Citrate buffer was used from pH 3.0 to 4.0 and 5.0 to 6.0, acetate from pH 3.6 to 5.2, phosphate from 6.0 to 7.0 and Tris from 7.5 to 9.0. All assay buffers were 0.05 M. The results in Figure 2 show that the highest activity was

obtained at pH 3.0. Lower pH buffers were not used. Extractions and assays were routinely conducted at pH 3.8.

(b) Linearity of the Assay

Figure 3A shows that with 0.3 ml enzyme per assay, product formation, measured by the increase in absorbance at 280 nm, increased linearly for 15 minutes. At this time the change in absorbance was 0.47 units. With 0.1 ml enzyme per assay product formation continued linearly for 25 minutes, until absorbance of the TCA-soluble fraction had increased by 0.20. The results in Figure 3B show that activity was proportional to enzyme concentration (0.1 - 0.3 ml/assay) between 0 - 15 minutes incubation. Hence in all subsequent assays enzyme concentration was adjusted so that the increase in absorbance would be between 0.1 and 0.4 units after 10 minutes incubation. Within these limits product formation should depend directly on the activity and concentration of the enzyme extract used in the assay.

(c) Optimal Substrate Concentration

Optimal substrate concentration per assay was determined. Figure 4 shows that with 0.2 ml enzyme extracted from seeds germinated for 5 days, 15 mg of haemoglobin per assay was necessary to achieve the maximum rate of hydrolysis.

Figure 2: Effects of Buffer pH on Protease Activity

Endosperms were extracted in 0.2 M acetate buffer containing 5 mM β-mercaptoethanol. Activity was assayed at 37 C using 0.05 M buffers in the following pH ranges; pH 3.0 to 4.0, citrate; pH 3.6 - 5.2, acetate; pH 5.0 - 6.0, citrate; pH 6.0 to 7.0, phosphate; and pH 7.5 - 9.0, Tris.

Endosperms of caryopses germinated for 6 days were used. Haemoglobin (5.0% solution) was used as substrate and activity is expressed as μM tryptophan equivalents released/hr/endosperm.

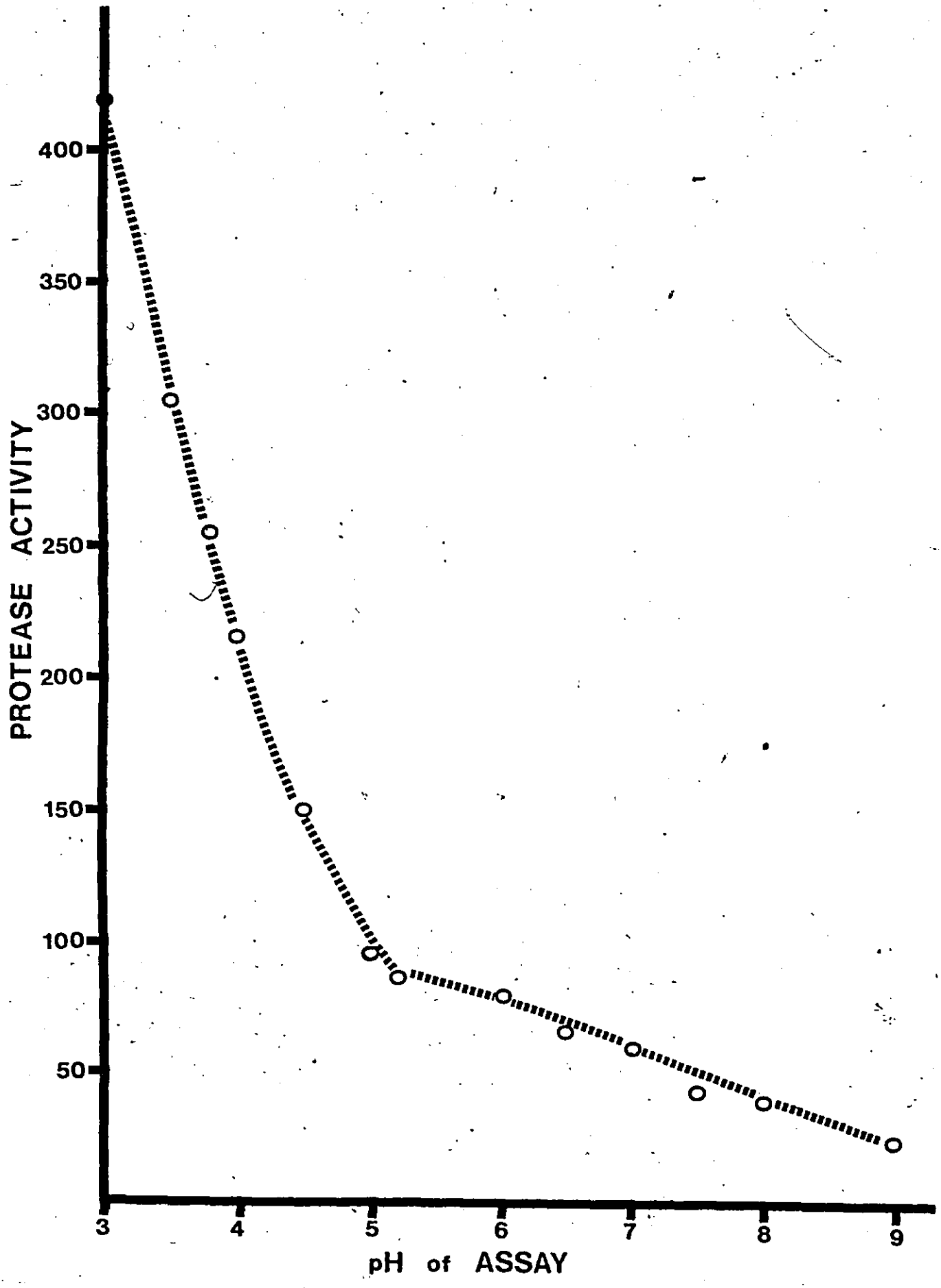
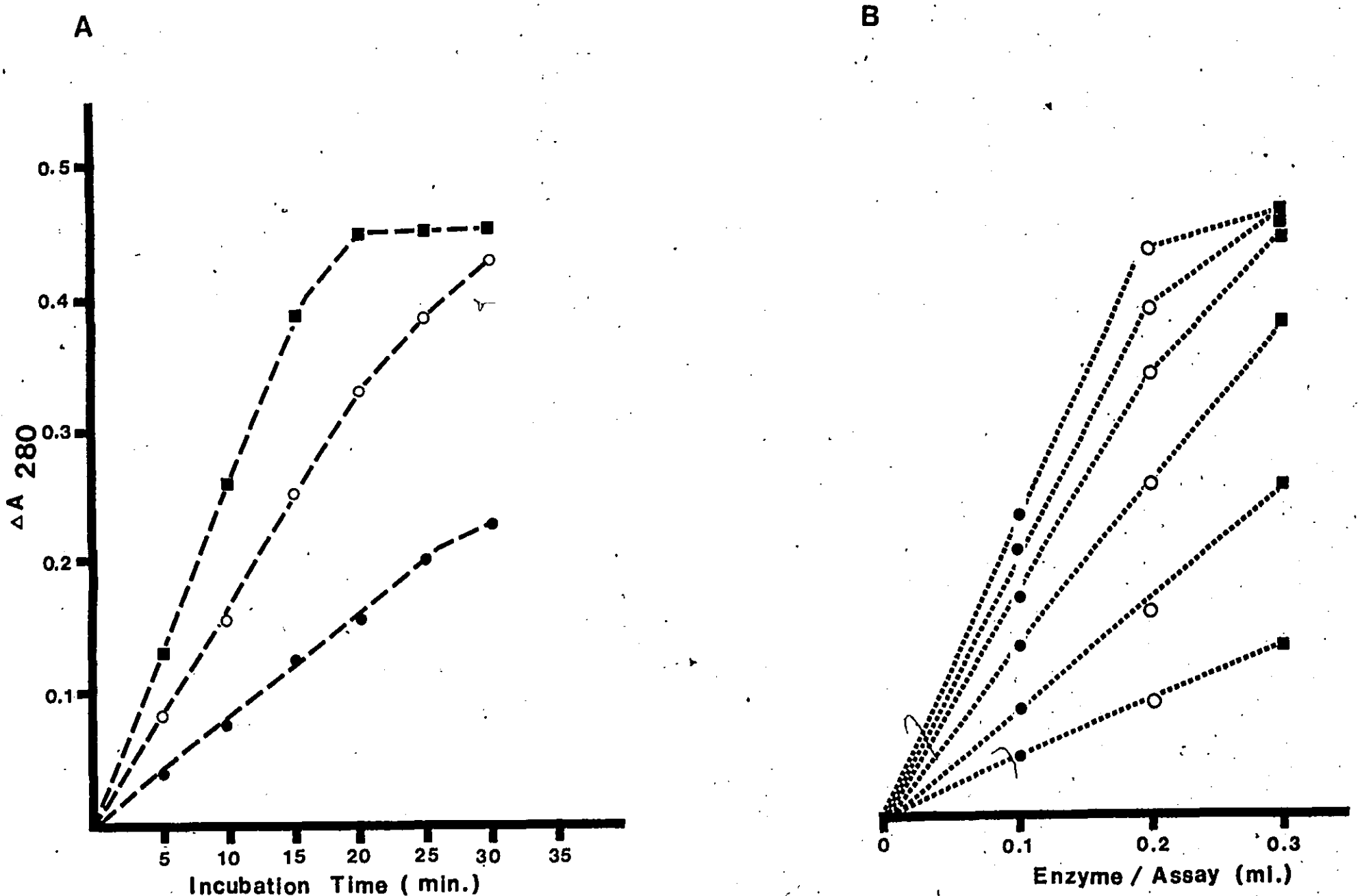

PROTEASE ACTIVITY
400
350
300
250
200
150
100
50
3
4
5
6
7
8
9
pH of ASSAY

Figure 3: Linearity of the Protease Assay

Enzyme was extracted in 0.2 M acetate buffer, pH 3.8, containing 5 mM β-mercaptoethanol, from endosperm of caryopses germinated for 4 days. Activity with haemoglobin substrate (5.0% solution) was assayed in 0.05 M acetate buffer, containing 2.5 mM EDTA, at the same pH, at 37°C. Activity was measured as the increase in absorbance at 280 nm of the TCA soluble fraction between 0 and 30 minutes incubation. Activity of 0.1 (●), 0.2 (o), and 0.3 (■) ml of enzyme per assay was measured.

Figure 3A shows the relationship between incubation time and product formation for the three enzyme concentrations.

Figure 3B shows the relationship between enzyme concentration and product formation after 5, 10, 15, 20, 25 and 30 minutes incubation.


A
0.5
0.4
0.3
0.2
0.1
ΔA 280
5
10
15
20
25
30
35
Incubation Time (min.)
B
0
0.1
0.2
0.3
Enzyme / Assay (ml.)

Routinely 50 mg haemoglobin (1 ml of 5% solution) was used to provide excess substrate in standard assays. No substrate inhibition was observed at high substrate concentrations.

(d) Standard Assay

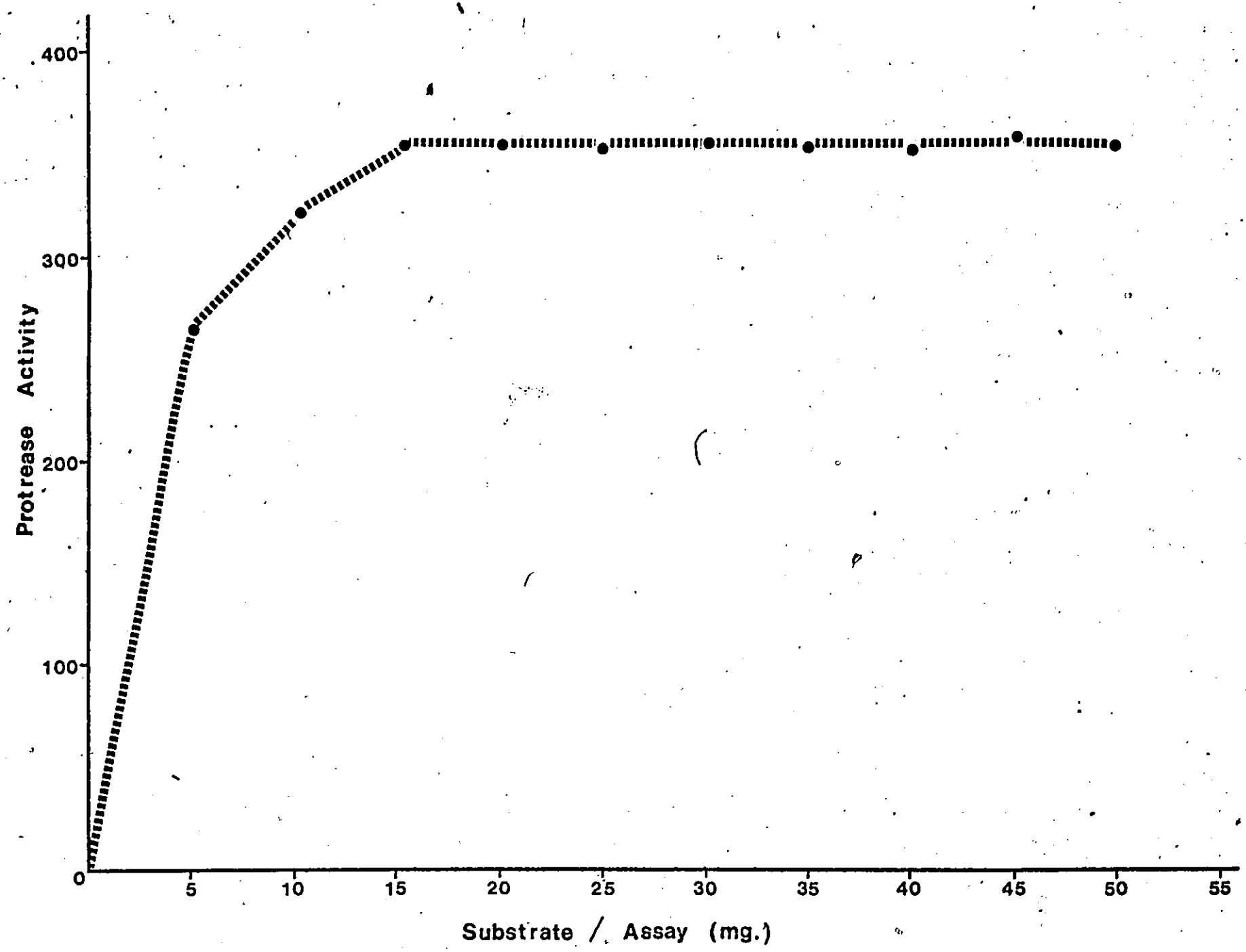
Routinely 0.2 ml of enzyme extract was incubated with 50 mg of substrate (1 ml of 5% haemoglobin), 1 ml of acetate buffer, 0.05 M, pH 3.8, with 2.5 mM EDTA, together with distilled water to give a total volume of 2.5 ml, for 10 minutes at 37°C. The reaction was stopped by adding an equal volume of 5% TCA. Activity was measured by the increase in absorbance at 280 nm of the TCA-soluble fraction. This was arbitrarily calibrated against the absorbance of tryptophan at 280 nm.

Protease Activity During Germination

Changes in activity of the protease during germination were investigated. Batches of 50 endosperm were harvested at 24 hour intervals for 11 days after imbibition. All endosperms were frozen in liquid nitrogen and stored at -20 C prior to extraction and assay. Protease activity was measured by the standard assay with haemoglobin as substrate. With this assay no activity was detected until 3 days after

Figure 4: Optimal Substrate Concentration for Assay of Protease Activity

Endosperms of caryopses germinated 5 days were extracted in 0.2 M acetate buffer, pH 3.8, containing 5 mM β-mercaptoethanol. The assays were conducted at 37°C in 0.05 M acetate buffer, pH 3.8, containing 2.5 mM EDTA. The substrate concentrations (5.0% haemoglobin solution) were 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mg per assay. Activity was expressed as µM Tryptophan equivalents released/hr/endosperm.


Protrease Activity
400
300
200
100
0
5
10
15
20
25
30
35
40
45
50
55
Substrate / Assay (mg.)

imbibition of the grain. A maximum was reached at 8 days, after which the protease activity declined (Figure 5). In all experiments activity has been expressed on a per endosperm basis because soluble protein per endosperm changes during germination. All values represent the means of three replicates from three individual experiments. The changes in endosperm proteolytic activity during germination in maize hybrid W64A x W182E followed the same trend as reported by Harvey (68) for the maize hybrid WF9 x 38-11.

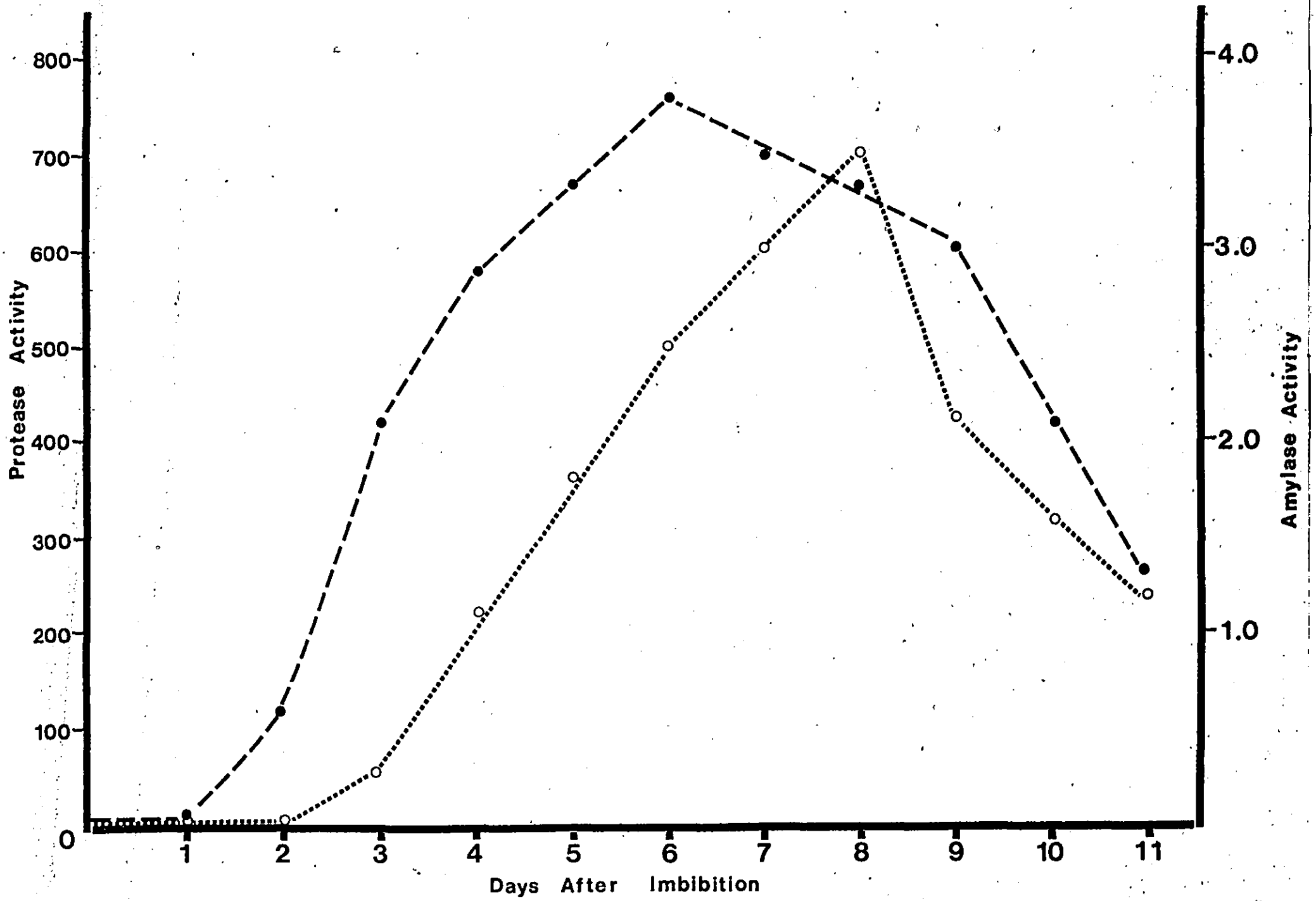
B: Amylase

(a) Total Activity

Another endosperm hydrolytic enzyme α-amylase was also measured for comparison of its pattern of activity during germination. Amylase activity was measured by the method of Jones and Varner (87), with slight modifications. The standard assay was as described in the Materials and Methods. With this assay, amylase activity was detected about two days after imbibition of the caryopses. This was 24 hours earlier than initial protease activity. It reached its maximum activity at 6 days, 48 hours earlier than the protease. All values represent the means of three replicates from three individual experiments (Figure 5). Again, these results are very similar to those reported by Harvey for the maize hybrid

Figure 5: Changes in Endosperm Hydrolytic Enzyme Activity During Germination

Endosperms were harvested between 0 and 11 days after imbibition. Alpha-amylase and protease were extracted and assayed as described in Materials and Methods. Protease activity (o) is expressed as µM tryptophan equivalents released/hr/endosperm, and amylase activity (●) as mg starch degraded/min/endosperm.


Protease Activity
800
700
600
500
400
300
200
100
0
Amylase Activity
4.0
3.0
2.0
1.0
1
2
3
4
5
6
7
8
9
10
11
Days After Imbibition

WF9 x 38-11 (68).

(b) An Electrophoretic Assay

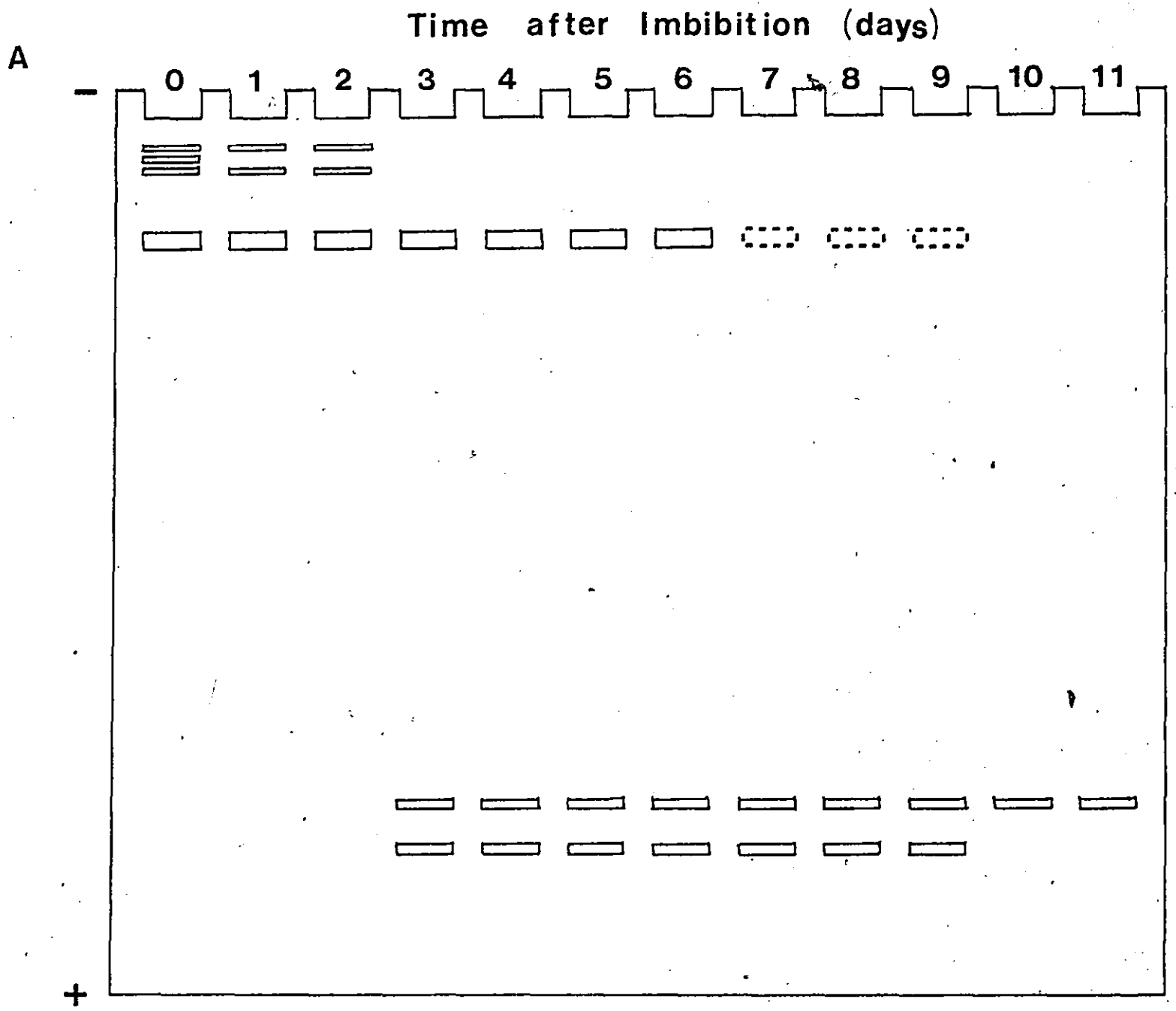
An electrophoretic assay was developed to investigate the changes in activity of specific amylases during germination. Batches of 50 endosperms were harvested at 24 hour intervals for 11 days after imbibition of the caryopses. The endosperms were frozen in liquid nitrogen and stored at -20°C prior to extraction and assay. Amylases were extracted as described in Materials and Methods. Crude enzyme preparations were added to the pockets of the gel. Application of an electric field to the buffered, heterogeneous protein solution resulted in a differential migration of proteins. During incubation of the gel in a starch solution, amylases present were able to degrade the substrate. Staining the gels specifically for starch then revealed clear bands in areas of the gel which had contained active amylases. Gels were also stained for total protein (Figure 6A). The pattern of the starch degrading enzymes show 2 bands (Figure 6B). The slowest of the two amylase bands is detected first, at two days. A second, faster band appears at 3 days after imbibition. As activity increases the bands become more pronounced. A maximal band intensity occurs at 5 to 6 days. Band strength then starts to wane. The faster band disappeared 24 hours earlier

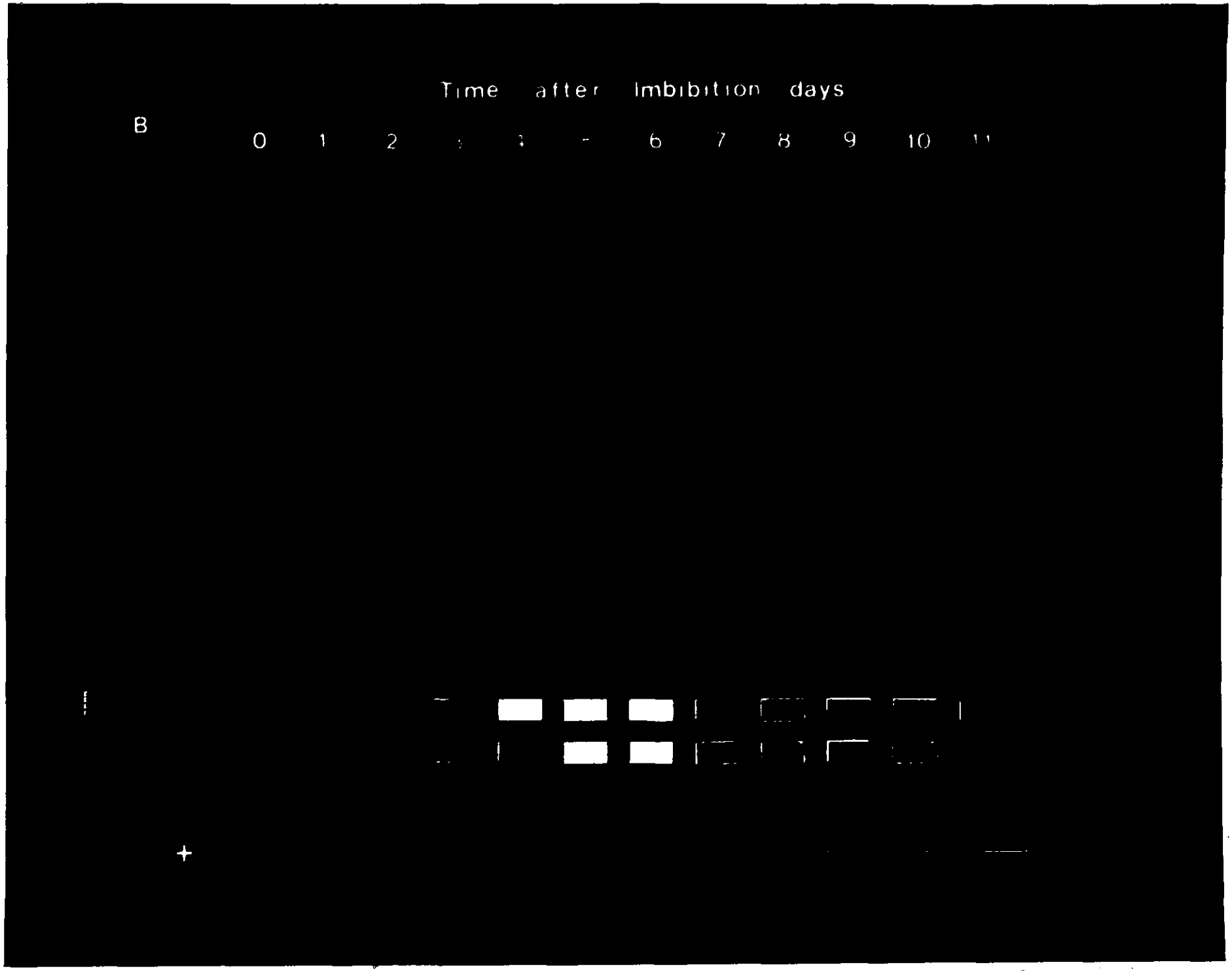
Figure 6: The Electrophoretic Gel Method of Assaying Amylase Activity

Endosperms were harvested between 0 and 11 days after imbibition. Amylase was extracted and electrophoresis conducted as described in Materials and Methods.

(A) Gel stained for protein with Commassie blue.

(B) Gel incubated in 0.8% starch solution for 24 hours and stained with $I_2KI - H_2O$ solution. Clear bands indicate activity of amylase.


A
Time after Imbibition (days)
0
1
2
3
4
5
6
7
8
9
10
11
−
+


Time after imbibition days
B
0
1
2
6
7
8
9
10

than the slower band. No activity was observed until the second day. The two lower bands in the protein stained gel (Figure 6A) were determined to be amylase bands. This was done by comparing migration distances with the bands found on the gel stained specifically for amylase activity (Figure 6B). The Commassie blue stained gel (Figure 6A) also contained a number of slow moving protein bands. These bands were present upon imbibition of the grain, but selectively disappeared as time progressed. Disappearance of the fastest of the slow bands coincided with the appearance of hydrolase activity (Figure 5 and 6).

II. Amylase Production by Excised Endosperms

During incubation of excised endosperms hydrolytic enzymes and hydrolysis products are released into the medium (69). Total enzyme production is obtained by measuring activities in both the medium and the endosperm. In Table IV the level of activity of amylase obtained from excised endosperm and their incubation medium is compared with amounts of amylase obtained from endosperm of intact germinated caryopses of the same age incubated at the same temperature. Thus, it is valid to compare total enzyme production by excised endosperms with enzyme activity in endosperm of germinated caryopses.

Table IV

Comparison of Amylase in Extracts of Intact Germinated Caryopses and Amylase in Extracts of Excised Endosperms and Their Incubation Medium.

Extracts were prepared from endosperms of caryopses germinated for 3 or 4 days and from endosperms incubated for 3 or 4 days. Activities of these extracts and of the 3 or 4 day incubation medium were measured with starch as a substrate. Amylase activity is expressed as mg starch degraded/min/endosperm. Activity was measured as described in Materials and Methods.

	Level of α-amylase activity (starch degraded/min/endosperm)			
	Intact Caryopses	Excised Endosperm		
	Endosperm Extract	Endosperm Extract	Incubation Medium	Total
3 Day Amylase Activity	2.12	1.00	0.96	1.96
4 Day Amylase Activity	2.90	1.1	1.78	2.88

(a) Contamination of Samples

In early experimentation, routine surface sterilization of dry caryopses with Javex, as described in Materials and Methods, proved inadequate in controlling fungal and/or bacterial contamination of excised endosperm samples. Sample loss due to contamination could be as high as 70% after a 96 hour incubation period. Hence new methods of sterilization were tried.

(b) Dichloromethane and Maize Germination

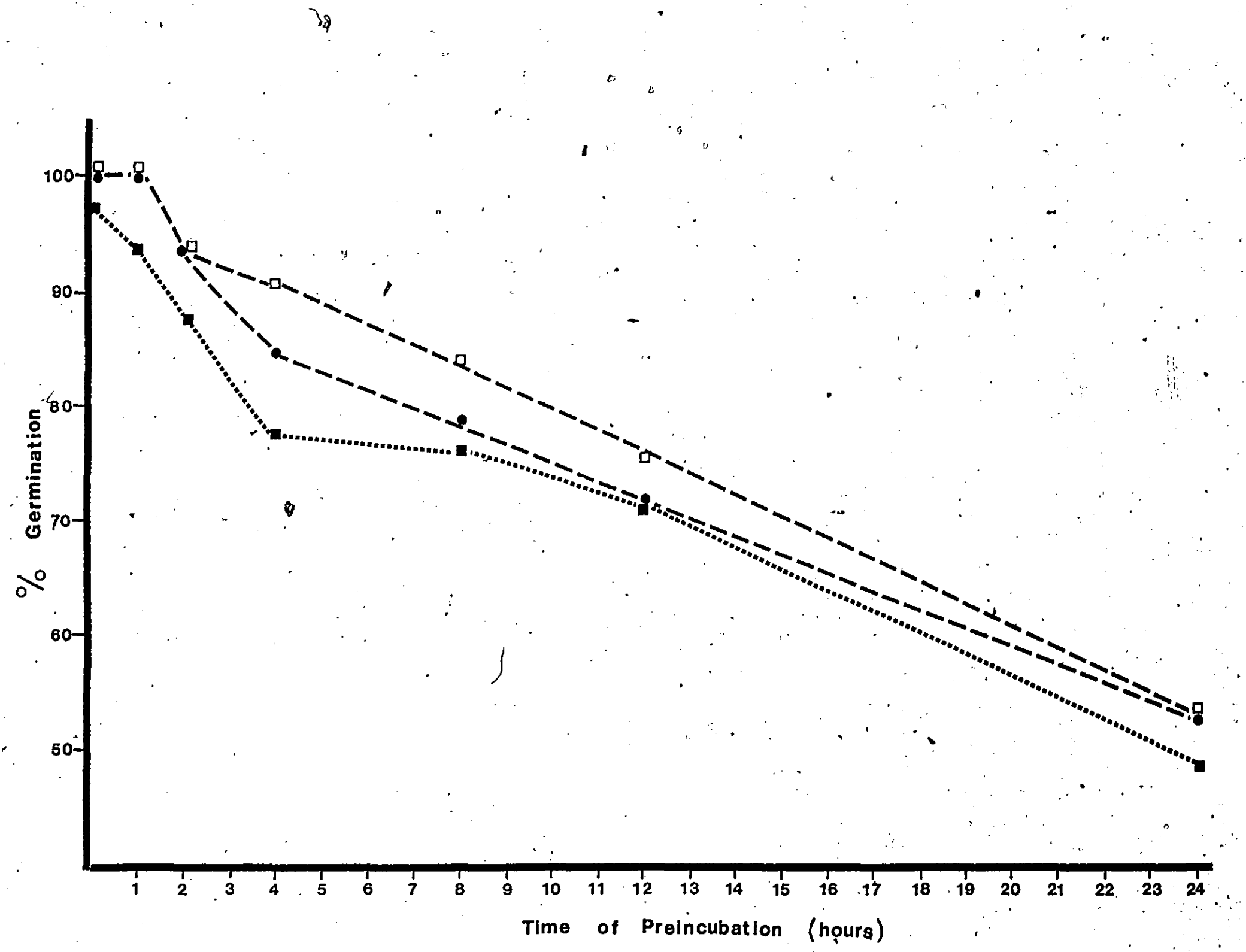
Meyer and Mayer (122) have proposed the use of dichloromethane (DCM) for introducing substances into dry seeds. Maize caryopses were soaked in DCM (2 ml per seed) for varying amounts of time up to 24 hours. After soaking, residual DCM was removed under vacuum and the treated caryopses left in the presence of Drierite ™, a desiccant, for 24 hours. The caryopses were then planted on wet vermiculite as before. Germination results (Figure 7) show that as time of incubation with DCM increases germination decreases. After two hours of incubation in DCM, germination is still almost normal (93%) whereas after 24 hours germination has dropped to almost half (53%).

(c) Permeation of Caryopses with Fungicide Using DCM

Infusion of either 0.5% Captan or 0.5% karathane was

Figure 7: Effect of Dichloromethane Preincubation on Subsequent Germination

Dry caryopses were soaked in DCM (□), DCM plus 0.5% Captan (■), or DCM plus 0.5% karathane (●), 2 mls per seed for 0, 1, 2, 4, 8, 12 or 24 hours. After soaking residual solvent was evaporated in a vacuum and the treated caryopses left in the presence of a desiccant for 24 hours. Fifty caryopses were used per treatment. The treated caryopses were planted on moist vermiculite and subsequent germination recorded after 144 hours.


100
90
80
70
60
50
% Germination
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
Time of Preincubation (hours)

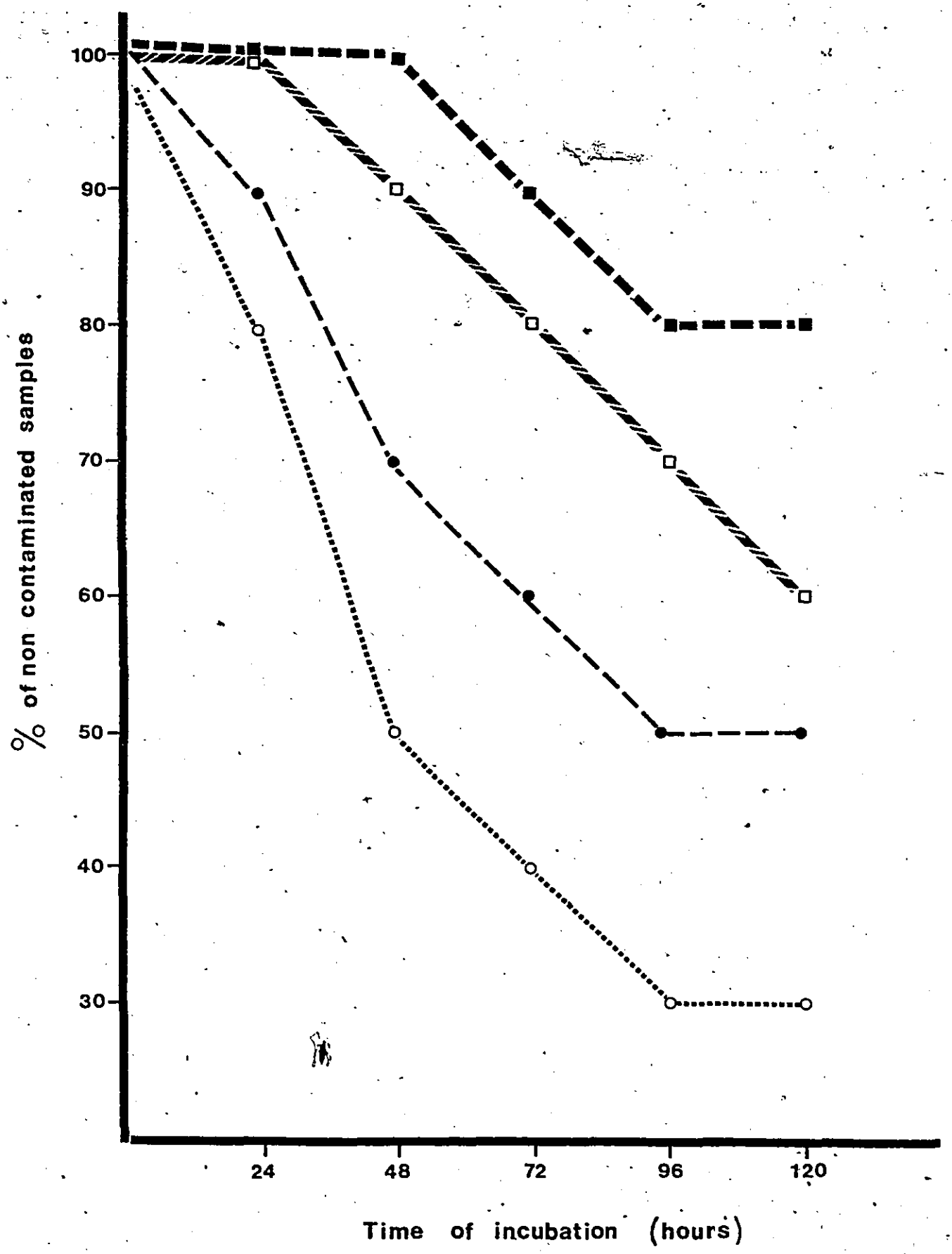
attempted using DCM as solvent. Results showed that Captan had an immediate adverse affect on germination when compared to the DCM control, whereas karathane had no additional affect up to two hours of treatment (Figure 7). At longer times karathane also had an adverse effect on germination. Hence caryopses were routinely princubated with 0.5% karathane for two hours using dichloromethane as solvent.

(d) Effect of Infusion of Karathane on Subsequent Contamination

The effect of infusing karathane, using DCM as solvent, into dry maize grain on the subsequent contamination of the excised endosperm pieces was examined. Dry caryopses were soaked in DCM (2 mls per seed) with 0.5% karathane, for 0, 1, 2 and 4 hours as described above and then the endosperms were dissected out using sterile conditions. The results in Figure 8 shows that as time of preincubation in 0.5% karathane increased so did the number of non-contaminated samples. Generally, in all samples tested loss due to contamination increased as time of incubation increased. However with non-treated seeds contamination accounted for a 60% loss of sample after 72 hours of incubation while only 20% of the sample was lost when the caryopses were treated for two hours with 0.5 karathane. In Table V the total amylase production from karathane infused excised endosperm is compared with control non-treated excised

Figure 8: Effect of Time of Karathane Infusion on Subsequent Contamination of Excised Endosperm Samples

Dry caryopses were soaked in DCM plus 0.5% karathane for 0 (○), 1 (●), 2 (□) and 4 (■) hours. After soaking residual solvent was evaporated in a vacuum and the treated caryopses left in the presence of a desiccant for 24 hr. Endosperms were then excised under sterile conditions and incubated in sterile 50 ml Erlenmeyer flasks, 10 endosperms per flask, as described in the Materials and Methods. Ten flasks were used per treatment. At 24 hr intervals aliquots of incubation medium were aseptically transferred to sterile nutrient agar plates. These nutrient agar plates were in turn incubated at 26°C. The number of non-contaminated plates are expressed as a percentage of the total number of plates inoculated per treatment at a given time of incubation.


100
90
80
70
60
50
40
30
% of non contaminated samples
24
48
72
96
120
Time of incubation (hours)

endosperm. Results indicate that preincubation with karathane in DCM for two hours has no effect on subsequent starch hydrolysis in excised endosperm.

(e) Amylase Production by Excised Endosperms

Changes in activity of α-amylase with time in excised endosperm and their incubation media were investigated. Treated endosperms were transferred to sterile 50 ml Erlenmeyer flasks, 10 endosperms per flask. Each flask routinely contained 10 ml of acetate buffer, 1.0 mM pH 5.0, with 10^{-3} M calcium chloride, penicillin (10 µg/ml), chloramphenicol (10 µg/ml) and streptomycin (250 µg/ml). This was known as the incubation media. The flasks were incubated in a water bath at 28°C with shaking. At 24 hr intervals 5 flasks, or fifty endosperm, were removed from the water bath. All endosperm were frozen in liquid nitrogen and stored at -20°C prior to extraction and assay of α-amylase. The incubation medium was assayed immediately for amylase activity. With this assay activity was detected in the endosperm extract as early as 24 hr after imbibition (Figure 9A). By 48 hr, activity in the endosperm extract had reached its maximum level. Activity in the incubation medium increased steadily after 48 hr. The total enzyme production during incubation was obtained by summing the activities extracted per endosperm and the activity

Table V

Comparison of Amylase Levels Obtained from Karathane-DCM Treated Excised Endosperms and From Control Non-Treated Excised Endosperms.

Extracts were prepared from excised endosperm preincubated for 2 hours in 0.5% karathane in DCM and incubated for 3 or 4 days, and from non-preincubated excised endosperm incubated for 3 or 4 days. Activities of these extracts and of their respective incubation medium were measured with starch as a substrate. Amylase activity is expressed as mg starch degraded/min/endosperm. Activity was measured as described in Materials and Methods.

	Level of α-amylase activity (starch degraded/min/endosperm)					
	Control			Treated		
	Endosperm Extract	Incubation Medium	Total	Endosperm Extract	Incubation Medium	Total
3 Day Amylase Activity	1.00	0.96	1.96	1.03	0.89	1.92
4 Day Amylase Activity	1.1	1.78	2.88	1.06	1.79	2.85

released per endosperm into the incubation medium (Figure 9A). Figure 9B shows the increase in total amylase production in endosperms incubated in buffer compared with amylase production in endosperms of intact caryopses germinated in vermiculite at the same temperature. Amylase activity appeared 24 hr after imbibition in the excised endosperm and increased rapidly. Amylase activity increased more slowly in the intact caryopses at first, but reached an activity level equivalent to that observed in the excised endosperm by the third and fourth days.

The results in Figure 9 B also show that linear increases of enzyme activity are similar in excised endosperms and germinating caryopses. This result is in agreement with those of Harvey (66) and suggest that α-amylase is produced in the endosperm. It should be noted that Dure (38), also working with maize, did not see an increase in α-amylase in excised endosperms, a result which suggested that α-amylase is produced by the scutellum, not the endosperm. Total production cannot be compared, since α-amylase activities were still increasing at the end of the experiment.

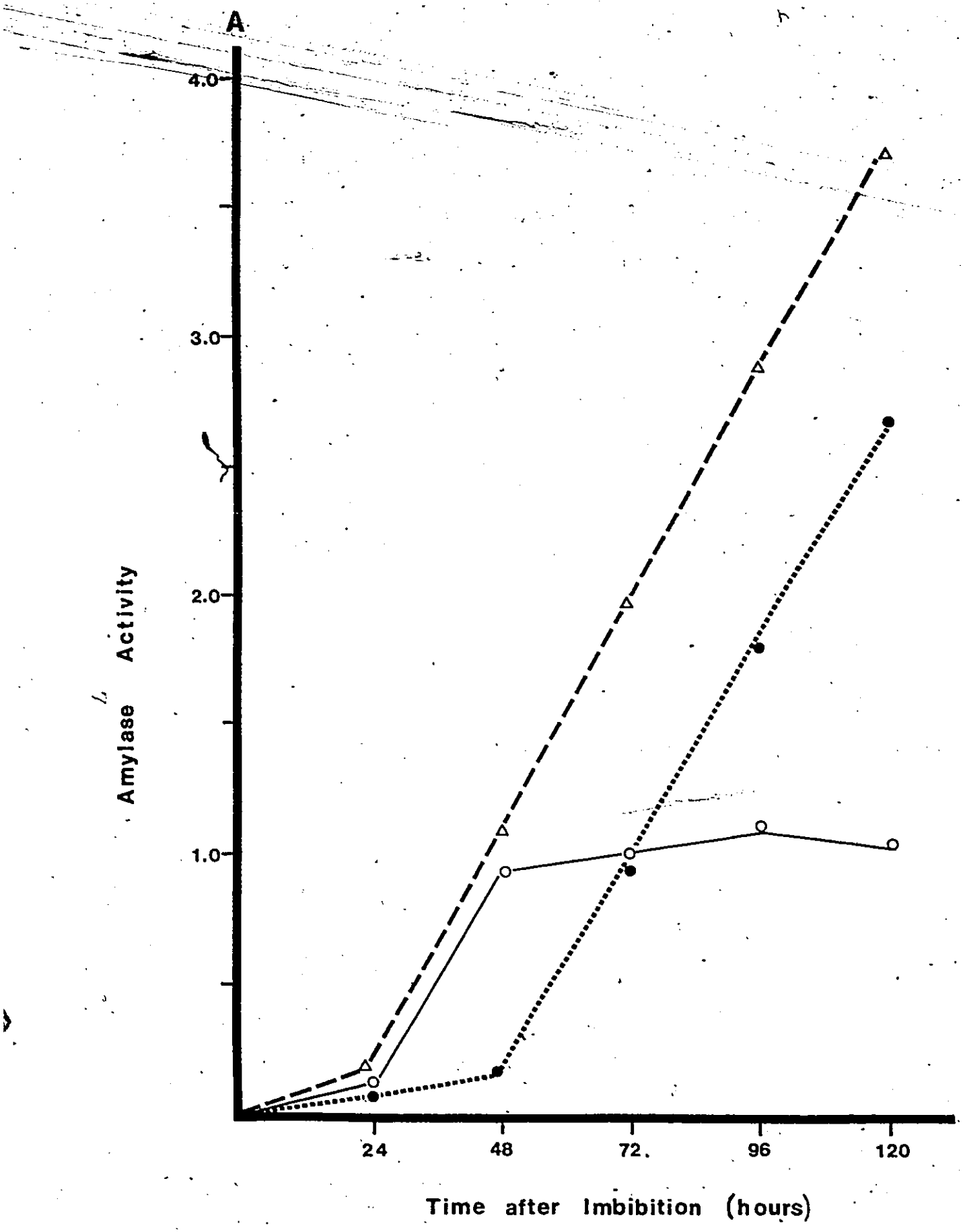
This comparison of α-amylase production and endosperm starch breakdown in intact and embryoless caryopses demonstrates that the presence of the embryo or an embryo factor is not necessary for initiation of continuation of hydrolytic

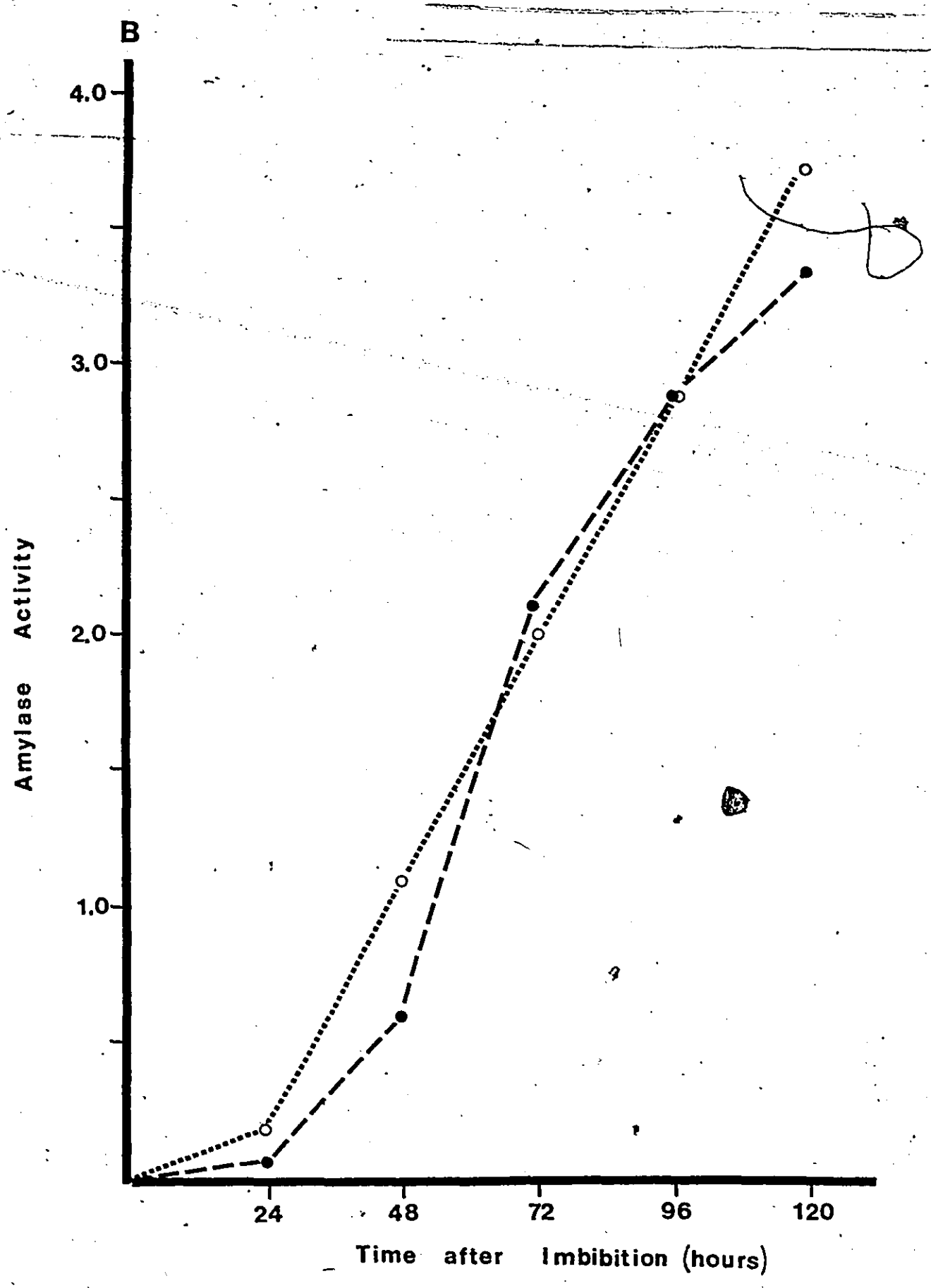
Figure 9: (A) Amylase Production by Excised Endosperms

Excised endosperms (W64A x W182E) were incubated at 28°C. Samples were removed from incubation at 24 hr intervals. Amylase in the endosperms was extracted and assayed (o), and amylase released into the medium by the excised endosperms was also measured (●). Starch was used as substrate in the amylase assay. Total amylase per endosperm was calculated and expressed as mg starch degraded/min/ endosperm (Δ).

(B) Amylase Activity from Excised and Intact Endosperms

Caryopses (W64A x W182E) were germinated at 28°C (●) and excised endosperms were incubated at 28°C (o). Samples were harvested at 24 hour intervals. Amylase in the endosperms was extracted and assayed. Amylase released into the medium by incubated endosperms was also measured. Starch was used as substrate. Total amylase per endosperm was calculated and activity expressed as mg starch degraded/min/ endosperm.


A
4.0
3.0
2.0
1.0
Amylase Activity
24
48
72.
96
120
Time after Imbibition (hours)


B
4.0
3.0
2.0
1.0
Amylase Activity
24
48
72
96
120
Time after Imbibition (hours)

processes in maize endosperm.

III. Influence of Gibberellic Acid on Endosperm Hydrolysis

The effect of gibberellic acid (GA), a plant growth substance, on hydrolytic processes in the endosperm were investigated using excised endosperms. GA_3 was included in the incubation medium. Total sugar and α-amylase production by the excised endosperms were assayed as convenient indicators of GA effects on endosperm hydrolytic processes.

(a) Gibberellic Acid Concentrations

Excised endosperms were incubated with a range of gibberellic acid concentrations, from 0 to 80 μM. Six separate experiments were performed with various hybrids. The amount of sugar and α-amylase in the excised endosperm and released into the incubation medium during the incubation period were variable from hybrid to hybrid and between different batches of the same hybrid (Table VI and VII). The response to gibberellic acid appeared to depend on the batch of caryopses tested. No GA_3 stimulation of α-amylase or sugar release was observed with W64A x W182E (1976), WF9 x M14 (1976) or WF9 x M14 (1966). High concentrations of GA were generally inhibitory. In barley endosperm (1975) gibberellic acid concentrations of 1.0 μM caused an 8.5 fold increase in

Table VI

The Effect of Gibberellic Acid on the Activity of α-Amylase in Incubated Endosperms

In separate experiments excised endosperms from various maize hybrids and one barley line were incubated in buffer for 1, 3, 4, or 5 days. Alpha-amylase in the excised endosperm and incubation medium were assayed and the total activity expressed as mg of starch degraded/min/endosperm.

Gibberellic Acid (μM)	α-Amylase Release per Endosperm into the Incubation Medium					
	W64A x 182E[a] 1976	WF9 x M14[a] 1976	WF9 x M14[a] 1966	WF9 x M14[b] 1966	WF9 x M14[c] 1966	Barley[d] 1975
0	1.116	0.314	0.0427	0.0327	0.0344	0.012
1.0	1.043	-	-	-	-	0.093
5.0	0.825	-	-	-	-	-
10.0	-	0.279	0.0404	0.0327	0.0310	-
20.0	0.741	-	-	0.0305	-	-
80.0	-	0.170	0.0375	-	-	-
	α-Amylase Activity per Excised Endosperm					
0	0.913	0.281	0.0093	0.0075	0.0081	0.075
1.0	0.899	-	-	-	-	0.640
5.0	0.620	-	-	-	-	-
10.0	-	0.222	0.0074	0.0063	0.0067	-
20.0	-	-	-	-	-	-
80.0	-	0.136	0.0078	-	-	-

continued...

Table VI (continued)

Gibberellic Acid (μM)	Total α-Amylase Activity per Endosperm					
	W64A x 182E[a] 1976	WF9 x M14[a] 1976	WF9 x M14[a] 1966	WF9 x M14[b] 1966	WF9 x M14[c] 1966	Barley[d] 1975
0	2.029	0.595	0.052	0.0402	0.0425	0.087
1.0	1.942	-	-	-	-	0.733
5.0	1.445	-	-	-	-	-
10.0	-	0.492	0.0478	0.039	0.0377	-
20.0	-	-	-	-	-	-
80.0	-	0.306	0.0453	-	-	-

(a) = 3 day incubation

(b) = 4 day incubation

(c) = 5 day incubation

(d) = 1 day incubation

Table VII

The Effect of Gibberellic Acid on the Release of Sugar in Incubated Endosperms

Excised endosperm from maize hybrid WF9 x M14 (1966) was incubated in buffer for 4 or 5 days. Sugar (µg/endosperm) in the excised endosperms and the incubation medium were assayed, and values per endosperm calculated. Dry caryopses from the maize hybrid WF9 x M14 (1966) were also grown in wet vermiculite for 5 days. Sugar (µg/endosperm) in these endosperms were also calculated.

Gibberellic Acid (µM)	Sugar Released per Endosperm Into the Incubation Medium				Intact Caryopses	
	WF9 x M14 (1966) (a) (1)	WF9 x M14 (1966) (a) (2)	WF9 x M14 (1966) (b) (1)	WF9 x M14 (1966) (b) (2)	WF9 x M14 (1966) (1)	WF9 x M14 (1966) (2)
0.0	1201	3123	1097	2850	-	-
10.0	941	2450	1835	2171	-	-
	Sugar per Excised Endosperm					
0.0	183	446	-	-	-	-
10.0	184	515	-	-	-	-
	Total Sugar per Endosperm					
0.0	1384	3569	-	-	1468	3817
10.0	1125	2965	-	-	-	-

(a) = 4 day incubation

(b) = 5 day incubation

(1) = Nelson's determination

(2) = Anthrone's determination

α-amylase release, but "background" values for release in the absence of hormone are very low. In earlier experiments Harvey (69) observed that the addition of 10 μM gibberellic acid to various maize imbred and hybrid lines had a marginal effect on α-amylase production. In one case, however, dwarf-5, a maize mutant deficient in the ability to synthesize gibberellic acid, GA stimulated α-amylase activity in the excised endosperm by over 300%. These results indicate that GA is required for hydrolytic enzyme production and hydrolysis of endopserm storage products. It is reasonable to assume therefore, that the addition of exogenous hormone to barley or dwarf-5 endosperms replaces an endogenous GA deficiency, whereas sufficient gibberellic acid may already be present in most maize varieties.

(b) Influence of Low pH on Gibberellic Effects

Palevitch and Thomas (1976) demonstrated that the action of exogenously added GA on α-amylase activity measured by the release of reducing sugars from barley seed endosperm was enhanced by decreasing the pH of the incubation medium to below the pKa point (Table VIII). Excised endosperms from two maize hybrids, W64A x W182E (1976) and WF9 x M14 (1966) were incubated with 20 μM GA at pH 3.5, 4.1, and 5.0. The pKa value for GA_3 is 3.8 (Cross 1954). Controls contained no

Table VIII

The Effect of Low pH on GA Enhancement in Excised Endosperm

Excised endosperms from maize hybrid W64A x W182E (1976) and WF9 x M14 (1966) were incubated in buffer for 3 or 4 days. The pH of the incubation medium was adjusted to 3.5, 4.1, or 5.0. Alpha-amylase activity in the incubation medium was assayed, and values per endosperm calculated. Activity expressed as mg of starch degraded/min/endosperm.

α-Amylase Release per Endosperm into the Incubation Medium					
Gibberellic Acid (μM)	W64A x W182E (1976)			WF9 x M14 (1966)	
	pH 3.5	4.1	5.0	3.5	5.0
0.0	0.755	0.811	0.988	0.0255	0.0327
2.0	0.721	0.727	0.741	0.0249	0.0305

gibberellic acid in the incubation medium. Amylase activity in the medium was assayed as an indicator of pH effect on the action of exogenously added GA. The results in Table VIII show that GA did not stimulate the production of α-amylase in either of the hybrids tested. Palevitch and Thomas (1976) postulated that reducing the pH eleviated problems associated with exogenous GA penetration through various permeability barriers in the fruit and seed coat, endosperm layers, or other tissue. In my system, with maize, penetration problems seem not to be important since changes in pH had no effect on the GA response.

IV. Location of Acid Protease Activity

(a) Isolation of Protein Bodies from Maize Endosperm

Batches of 50 endosperms were harvested at 24 hour intervals for 11 days after imbibition. All extractions were done with freshly harvested endosperms. Extraction and sucrose gradient isolation of protein bodies were performed as described in Materials and Methods. The protein bodies formed a sharp band on top of the 80% sucrose. The supernatant (cellular organelles and soluble proteins) remained on top of the 50% sucrose (Figure 10). Figure 11 shows a sharp 280 nm absorption peak at the 50% - 80% sucrose interface. This peak

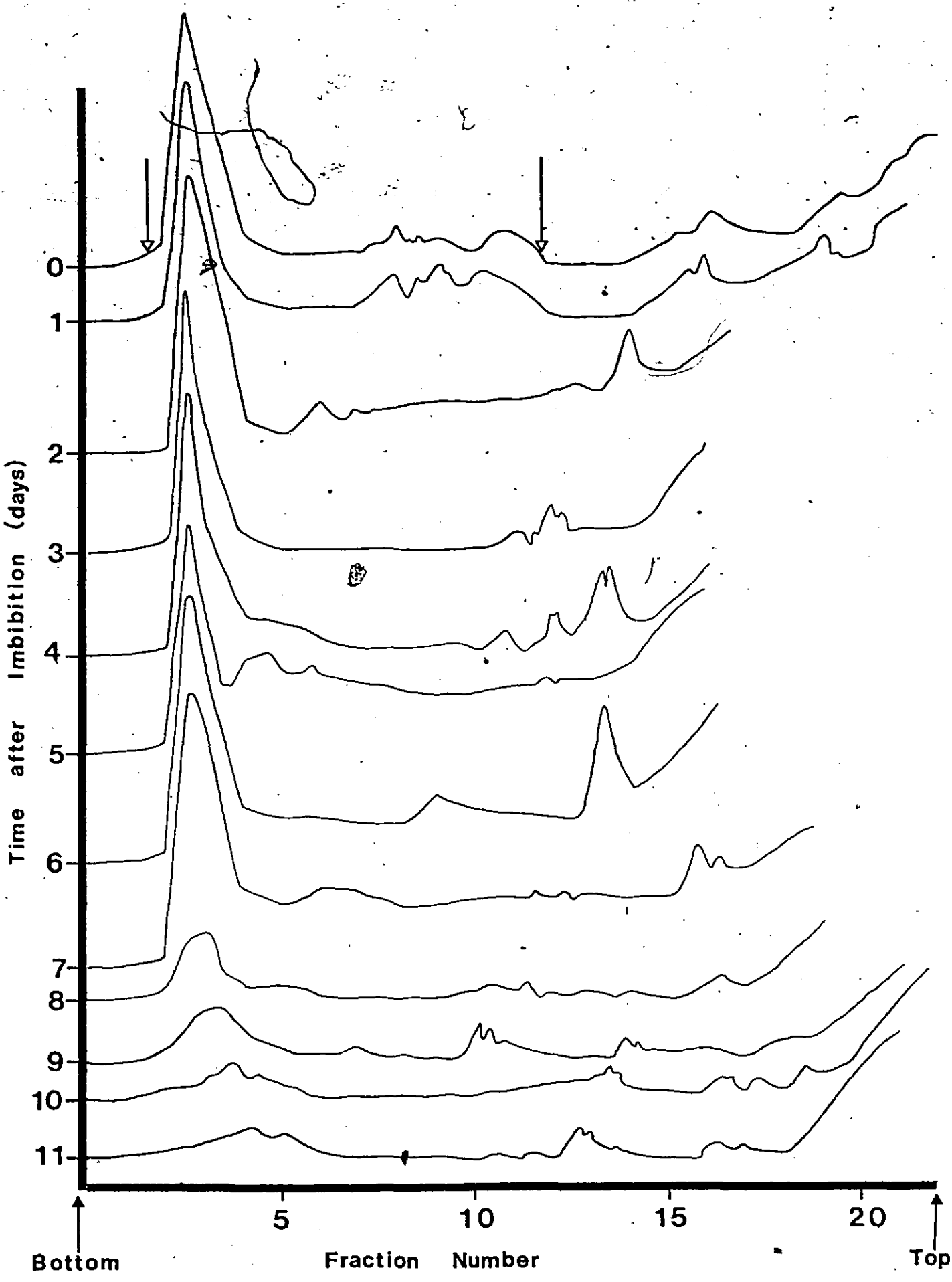
Figure 10: The Step Sucrose Gradient

Endosperms were harvested between 0 and 11 days after imbibition. An endosperm homogenate was prepared as described in Materials and Methods. The homogenate was placed on top of a 50/80% (W/V) sucrose step gradient. Figure 10 shows this gradient, loaded with a 8 day homogenate, after centrifugation. Two bands are apparent. Band A, sitting at the 50/80% sucrose interface, contains the isolated protein bodies (see Figure 13). Band B, sitting at the 40/50% sucrose interface, contains other cellular organelles (not determined). Above band B are the soluble proteins (C).


C
B
A

Figure 11: Isolation of Protein Bodies by Centrifugation in Step Sucrose Gradient

Endosperms were harvested between 0 and 11 days after imbibition. An endosperm homogenate was prepared as described in Materials and Methods. The homogenate was placed on top of a 50/80% (w/v) sucrose step gradient. The gradients were eluted after centrifugation at 75,000 x g for 1 hour and the absorbance of the fractions determined at 280 nm. The arrows left to right represent the 80 - 50% interface and the 50 - 40% interface respectively.


Time after Imbibition (days)
0
1
2
3
4
5
6
7
8
9
10
11
5
10
15
20
Bottom
Fraction Number
Top

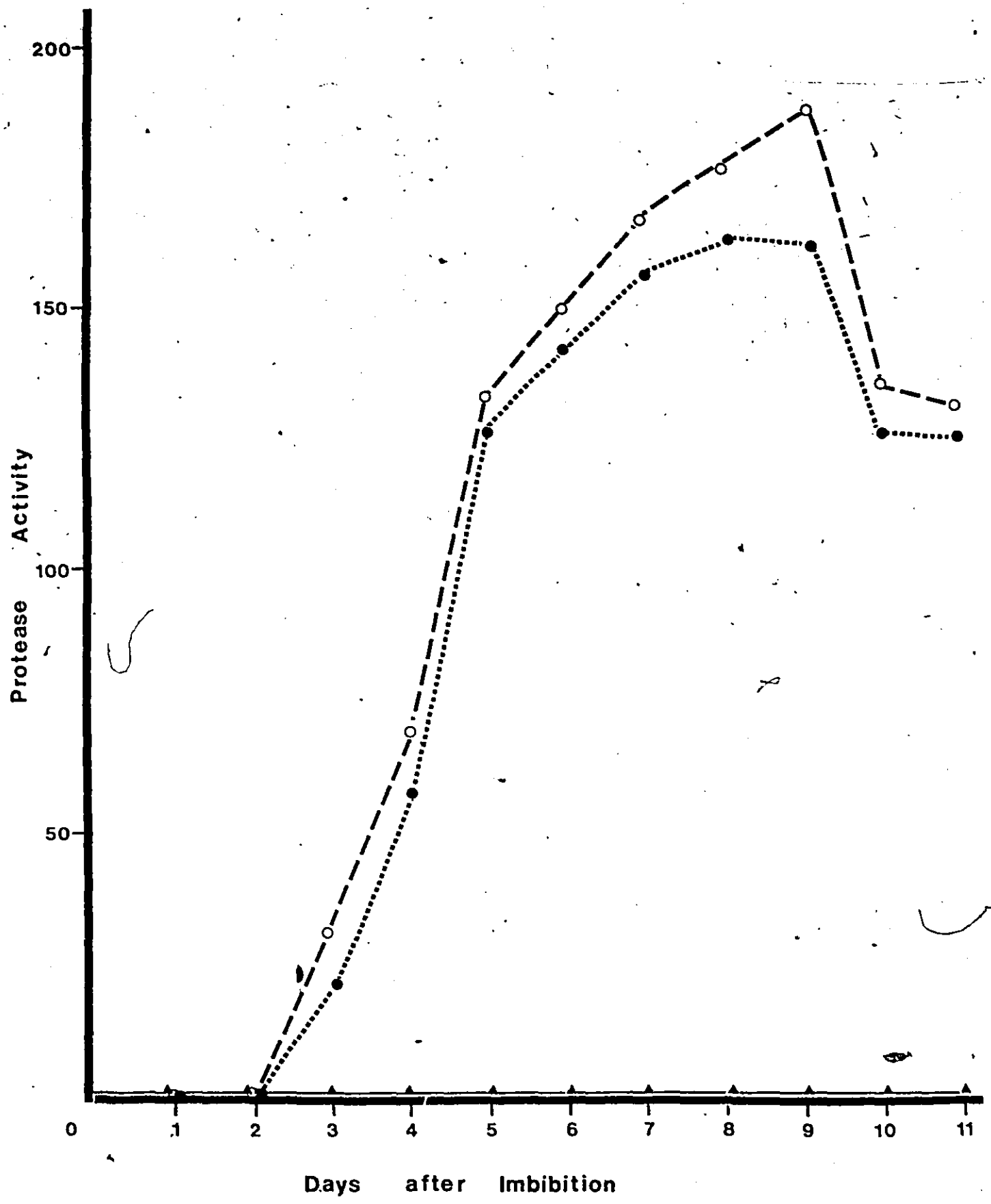
corresponds with those fractions containing protein bodies (see below). The protein body absorption peak remains relatively unchanged until day 7, after which time it disappears. The time of disappearance of the protein body peak (day 8) corresponds with the time of greatest protease activity (Figure 12). The second greatest absorption area occured on top of the 50% sucrose. Although the levels of absorption were not homogeneous within this layer, highest values were exhibited routinely towards the top of the gradient. It was assumed that this layer was composed of all other cellular organelles and soluble proteins which could not pass into the 50% sucrose (supernatant). In later experiments the fractions corresponding to the A_{280} peak on top of the 50% sucrose (Figure 11) were combined, and the organelles sedimented by centrifugation using a SW50L Beckman rotor at 160,000 x g for 15 minutes. The supernatant was pipetted off and tested for protease activity using the standard assay with haemoglobin as substrate. Results in Table IX and Figure 12 show that all activity associated with the crude extract appears in this supernatant.

(b) Electron Microscopic Identification of Protein Bodies

The fractions corresponding to the A_{280} peak on top of

Figure 12: Changes in Endosperm Protease Activity During Germination

Endosperm were harvested between 0 and 11 days after imbibition. An endosperm homogenate was prepared as described in Materials and Methods. The homogenate was placed on top of a 50/80% (w/v) sucrose step gradient. The gradients were eluted after centrifugation at 75,000 x g rpm for 1 hour. Two fractions of the sucrose gradient were assayed for protease activity; the protein body fraction (▲) and the supernatant fraction (other organelles and soluble proteins) (●); plus the crude extract (o) as described in the Materials and Methods. Protease activity is expressed in μM tryptophan equivalents released/hr/endosperm. Haemoglobin was used as substrate in the protease assay.


200
150
100
50
Protease Activity
0
1
2
3
4
5
6
7
8
9
10
11
Days after Imbibition

the 80% sucrose (Figure 11) were combined, and the organelles were sedimented by centrifugation as described in the Materials and Methods. This pellet was fixed for electron microscopy. The spheres correspond to what has been called protein bodies in maize (Khoo and Wolf, 1970). Many of the granules, measuring 1μ or more in diameter, stain with a dark, narrow, irregular, peripheral band from which there are projections into the interior (Figure 13, see appendix), and occasionally there is a darker core. Small granules, as well as a few large ones, stain uniformly. A granular component, plus other unidentified cellular debris, can be seen dispersed throughout the isolate.

(c) Protease Activity

Changes in activity of the acid endopeptidase during germination, in sucrose separated cell fractions, were investigated. All fractions containing either the protein bodies or supernatant (cellular organelles and soluble protein) were collected and combined. In each of the two fractions protease activity was measured by the standard assay with haemoglobin as substrate. Activity was also measured in the crude extract. With this assay no activity was detected in any fraction until 3 days after imbibition of the grain. In the crude extract a maximum was reached at 9 days, after which the protease activity declined (Figure 12). Activity

levels in the fraction containing soluble proteins and other cytoplasmic organelles, although slightly lower, paralleled the activities observed in the crude extract. At no time was activity observed in the protein body fraction (Figure 12, Table IX).

(d) Protease Activity in Protein Bodies

An attempt was made to uncover any protease activity that might be associated with protein bodies isolated by centrifugation on discontinuous sucrose gradients. A number of tests were tried. Osmotic shock and high salt treatments were carried out by methods described by Huang and Beevers (1973), detergent treatments were carried out by methods described by Kagawa *et al.* (1973), both with slight modifications. A 2 ml protein body fraction was added to 4 ml of 25 mM citrate phosphate buffer, pH 4.5, with or without 0.15 M KCl, shaken in a vortex mixer and incubated for 30 minutes on ice. To 2.0 ml of fresh protein body isolate was added 0.2 ml of 5% Triton X-100 solution. The mixture was allowed to stand for 30 minutes on ice with occasional stirring. After incubation each fraction was tested for protease activity using the standard assay. Results showed (Table X) that none of the treatments used had any effect on releasing protease activity from the protein body

Table IX

Location of Protease Activity in Sucrose Density Gradient

Extracts were prepared from endosperms of caryopses germinated for 8 days as described in the Materials and Methods. The homogenate was placed on top of a 50/80% (w/v) sucrose step gradient. The gradients were eluted after centrifugation at 75,000 x g for 1 hour and 1.2 ml fractions collected. Fractions exhibiting acid protease activity were combined (top 6 fractions) and the sucrose concentration lowered to about 0.6M using extraction buffer. Organelles and other cellular debris was then sedimented by centrifugation at 45,000 rpm for 15 minutes. The resulting supernatant was collected and tested for acid protease activity with haemoglobin as substrate. Activity is expressed as µM tryptophan equivalents per endosperm per hour.

	Level of Acid Protease Activity (µM tryptophan equivalents/endosperm/hour		
	Crude Extract (before sucrose density gradient)	Combined Fractions With Protease Activity (before Sedimentation)	Supernatant (after sedimentation)
8 day endosperm	225.00	214.17	206.43

fraction. In addition a protease body isolate, prepared from 4 day old endosperms, was tested for activity over a range of assay buffers, using the standard assay with haemoglobin as substrate. Citrate buffer was used from pH 3.0 to 4.0 and 5.0 to 6.0, acetate from pH 3.6 to 5.2, phosphate from 6.0 to 7.0 and tris from 7.5 to 9.0. All assay buffers were 0.05 M. Again, no protease activity was observed in the fractions tested (Table X).

(e) Location of Acid Protease Activity in Sucrose Gradient

Fourty endosperms were harvested 8 days after imbibition of the caryopses. They were homogenized by grinding in a cold porcelain mortar with sand and extraction buffer containing 25 M citrate phosphate buffer, pH 4.5 with 10 mM β-mercaptoethanol and 40% sucrose (w/v). One ml of buffer was used per gram fresh weight of tissue. The homogenate was filtered through miracloth, and centrifuged at 500 x g for ten minutes to remove starch and cell debris. Five ml of the resulting supernatant was layered on top of a sucrose gradient consisting of 20 ml of 50% (w/v) sucrose and 6.5 ml of 80% (w/v) sucrose in the same buffer. The gradient was centrifuged for 60 minutes at 75,000 x g in a SW27 Beckman rotor. The gradient was fractionated by pumping it from the top of the centrifuge tube. Twenty-five 1.2 ml

Table X

The Effect of Osmotic Shock, High Salt, Detergent Treatment and pH on the Release of Protease Activity from Protein Body Isolates

	Protease Activity				Protease Activity (using 4 Day Endosperm Extracts)	
Age	Gradient Protein Body Fraction	Osmotic Shock	High Salt (KCl)	Detergent (Triton X-100)	pH	
0	nil	nil	nil	nil		nil
1	nil	nil	nil	nil	3.0	nil
2	nil	nil	nil	nil	3.5	nil
3	nil	nil	nil	nil	3.8	nil
4	nil	nil	nil	nil	4.0	nil
5	nil	nil	nil	nil	5.0	nil
6	nil	nil	nil	nil	6.0	nil
7	nil	nil	nil	nil	6.5	nil
8	nil	nil	nil	nil	7.0	nil
9	nil	nil	nil	nil	7.5	nil
10	nil	nil	nil	nil	8.0	nil
11	nil	nil	nil	nil	9.0	nil

fractions were collected and protease activity measured in each by the standard assay using haemoglobin as substrate. Results in Figure 14 show that only those fractions above the 40% - 50% interface exhibited any protease activity. Protein bodies are represented by absorption at 280 nm at the 50 - 80% sucrose interface. Ninety-seven percent of the original protease activity layered on the sucrose gradient in the crude extract was recovered after centrifugation and fractionation.

V. Proteolytic Enzyme Activity in Developing Endosperm

A: Proteases

An assay for measuring endopeptidase activity, developed by Erlanger *et al.* (1961) and Burger (1968), was used by Melville and Scandalios (1972) to measure protease activity in germinating maize plant. This assay, with slight modifications, was used to measure protease activity in developing endosperms. The assay was optimized so that activity in crude extracts could be reliably measured with the substrate α-N-benzoyl DL-arginine p-nitroanilide (BAPNA) in developing endosperms of different ages. The changes in endosperm proteolytic activity during development and early germination were followed.

(a) pH of the Assay

The pH optimum for assaying of the protease was

Figure 14: Location of Acid Protease Activity in Sucrose Gradient

Endosperm of caryopses harvested 8 days after imbibition were extracted in 25 mM citrate-phosphate buffer, pH 4.5, with 10 mM β-mercaptoethanol and 40% sucrose. Five ml of the extract was layered onto a step sucrose gradient as described. After centrifugation and fractionation of the gradient each fraction was assayed by the standard assay with haemoglobin as substrate. Protease activity (□) was expressed as release of μM tryptophan equivalents/hr/endosperm. The A_{280} (●) of the eluate was measured continuously using an ISCO model UL-4 absorbance reader. The arrows left to right represent the 80/50% interface and the 50/40% interface respectively.

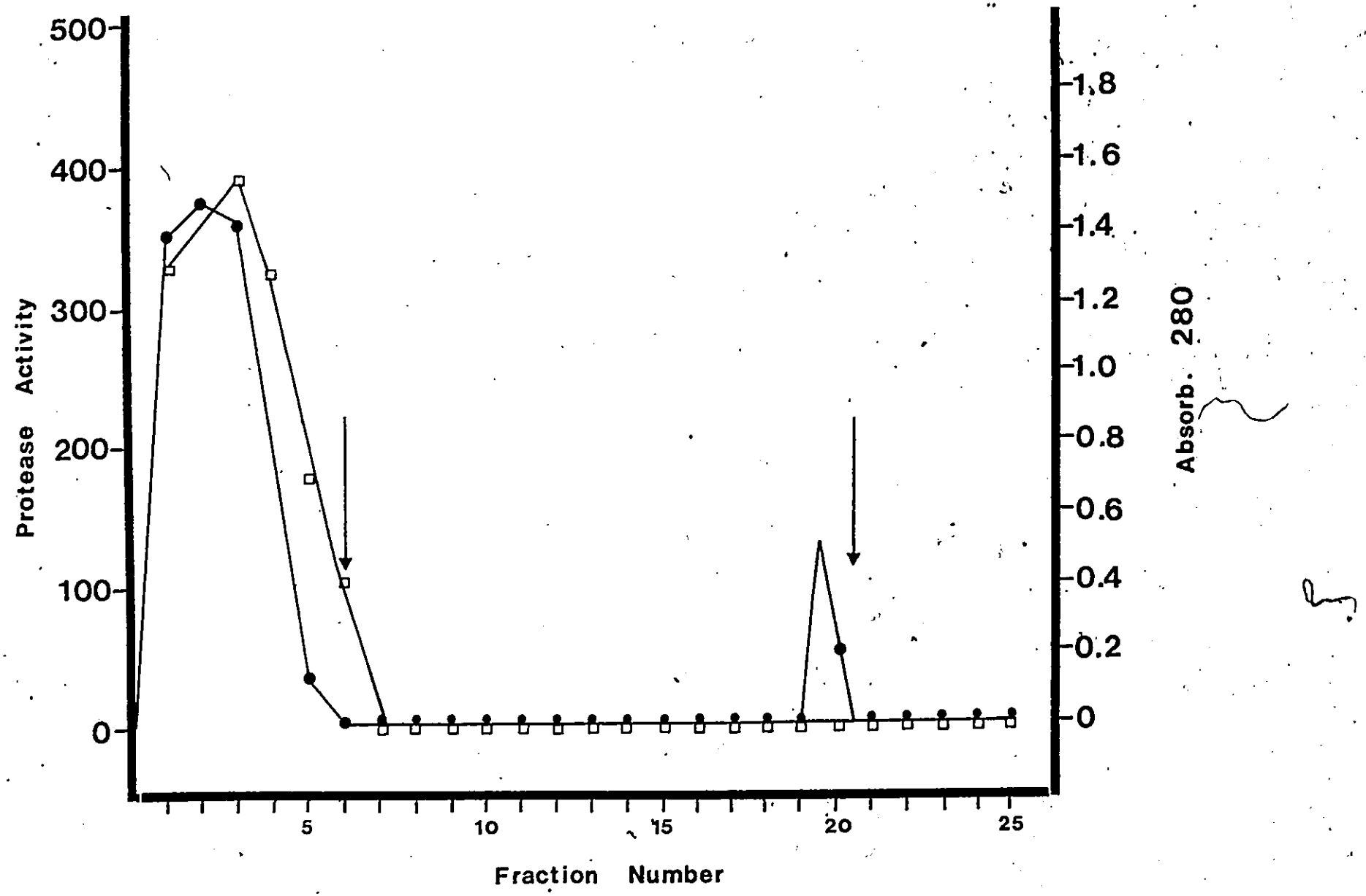
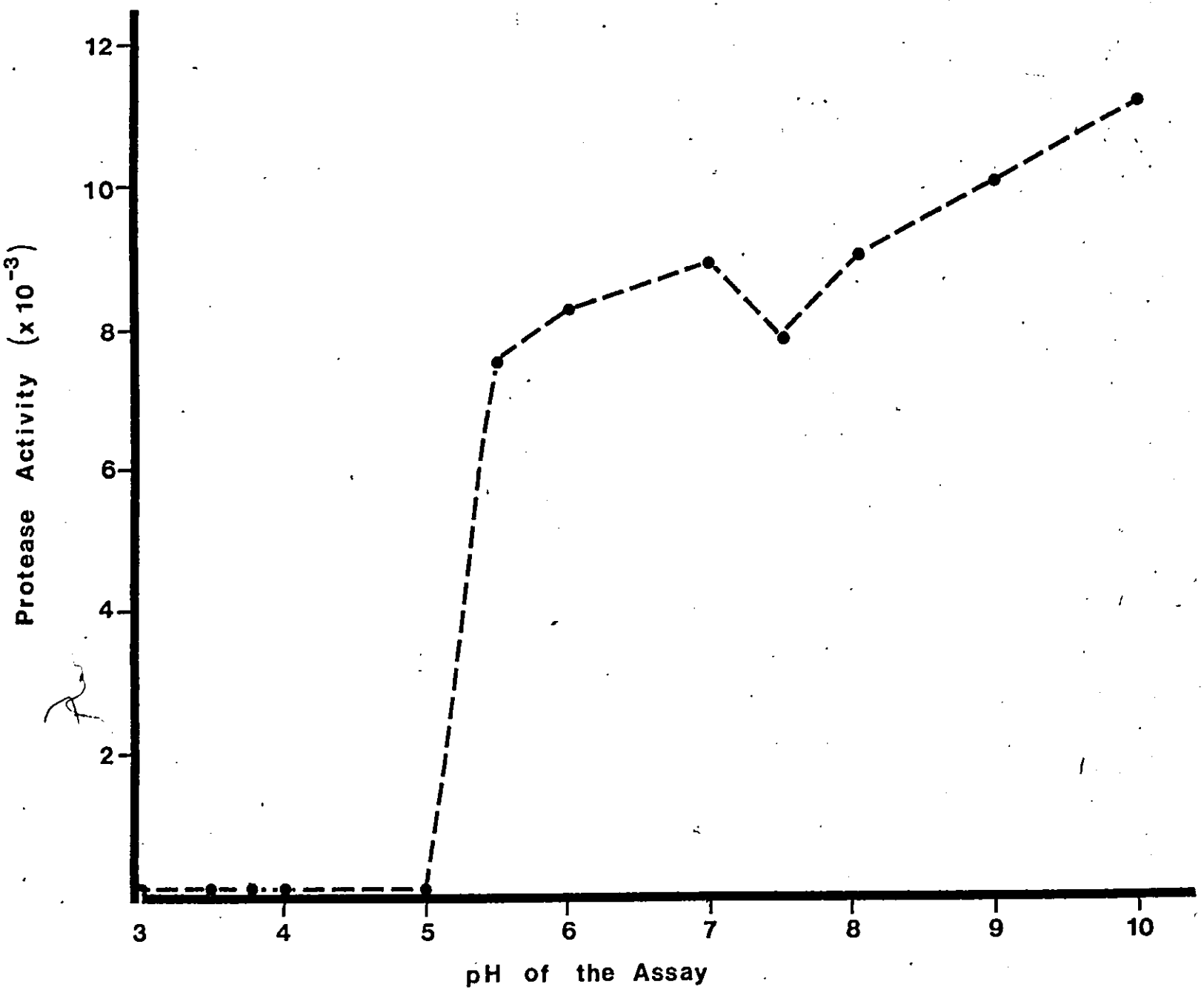

500
400
300
200
100
0
Protease Activity
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Absorb. 280
5
10
15
20
25
Fraction Number

Figure 15: Effects of Buffer pH on Protease Activity

Endosperm were extracted in 0.1 M Tris-HCl buffer, pH 7.5. Activity was assayed at 35°C using 0.1 M buffers in the following pH ranges; pH 3.0 to 4.0, citrate; pH 5.0 to 6.0, citrate; pH 6.0 to 7.0, phosphate; pH 7.0 to 9.0, Tris; and pH 10.0, Borate.

Endosperms of caryopses harvested at 21 days after pollination were used. BAPNA was used as substrate and activity is expressed as increase in absorbance/min.


Protease Activity (x 10^{-3})
12
10
8
6
4
2
3
4
5
6
7
8
9
10
pH of the Assay

determined. Citrate buffer was used from pH 3.0 to 4.0 and 5.0 to 6.0, phosphate from 6.0 to 7.0, Tris from 7.0 to 9.0 and Borate at pH 10.0. All assay buffers were 0.1 M. The results in Figure 15 show a broad pH optimum from pH 5.5 to pH 10.0. Extractions and assays were routinely conducted at pH 7.5.

(b) Optimum Assay Temperature and Storage Stability

Activity was assayed at temperatures between 20°C and 70°C. The results in Figure 16A show that activity was highest at 55°C. The enzyme appeared to be inactivated at higher temperatures. Assays were routinely conducted at 35°C.

Stability of the protease was tested. Enzyme extracts were either frozen or left on ice, and tested for activity at some later date. Neither storage treatment resulted in a significant activity loss up to experiment termination at 18 days (Figure 16B).

(c) Linearity of the Assay

Figure 17A shows that with all enzyme concentrations used, product formation, measured continuously by the increase in absorbance at 410 nm, increased linearly. With 50 λ of enzyme per assay, product formation continued linearly for 15 minutes until absorbance due to colour release from the substrate BAPNA had increased by 0.129. The results in

Figure 16: Temperature Optimum for Assay of Developing Endosperm Protease and Lability of this Enzyme

A: The assay temperature was varied between 20°C and 70°C. Endosperms of caryopses harvested 21 days after pollination were extracted with 0.1 M Tris-HCl buffer, pH 7.5. Activity was assayed with 0.1 M Tris-HCl buffer, pH 7.5, with BAPNA as substrate. Activity is expressed as the increase in absorbance/min.

B: The enzyme was allowed to stand for 0-18 days on ice (o) or frozen (●) before assaying its activity at 35°C. Activity of the stored enzyme is expressed as a percentage of the fresh extract activity (100%).

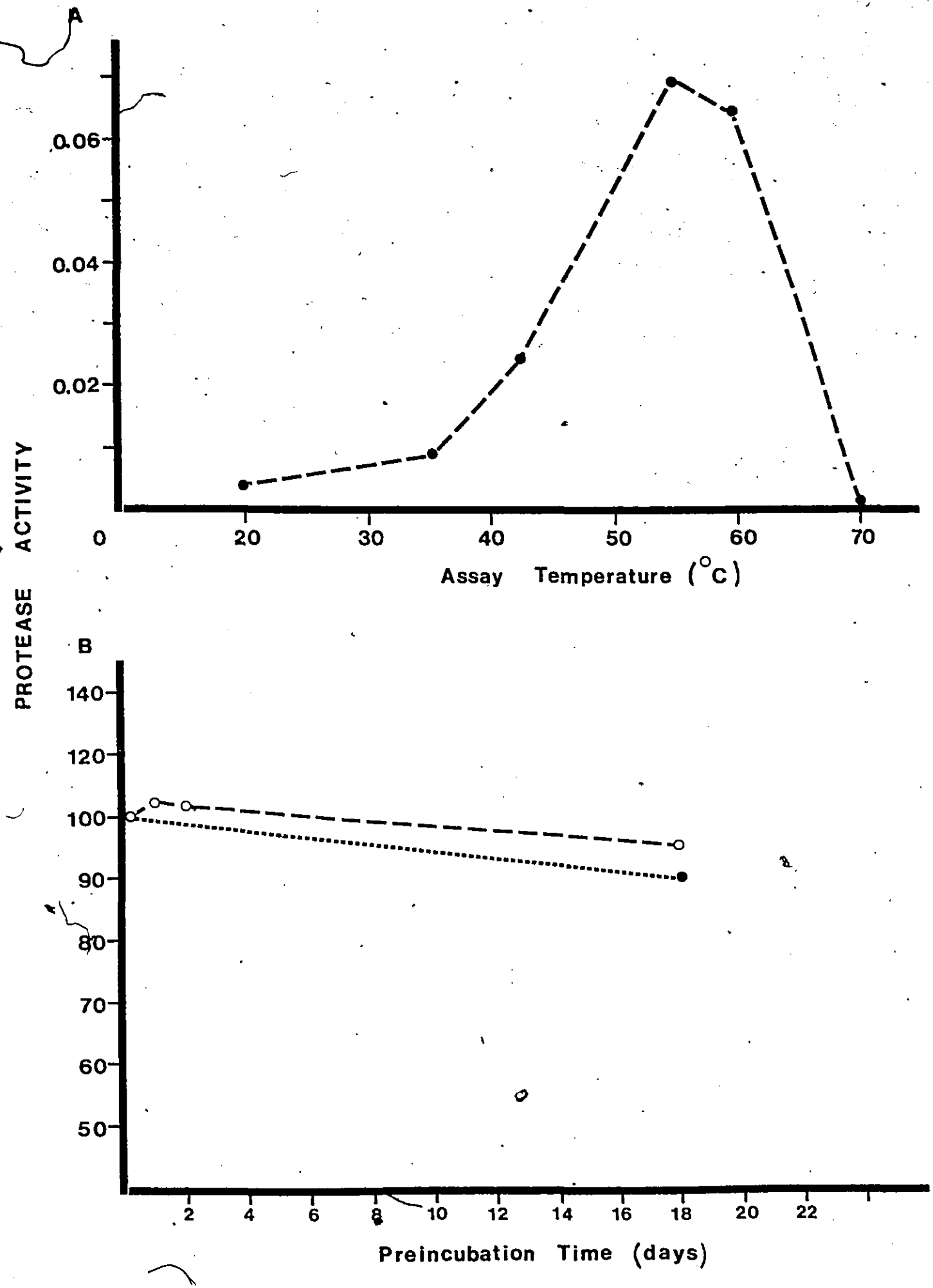

A
0.06
0.04
0.02
0
20
30
40
50
60
70
PROTEASE ACTIVITY
Assay Temperature (°C)
B
140
120
100
90
80
70
60
50
2
4
6
8
10
12
14
16
18
20
22
Preincubation Time (days)

Figure 17B shows that activity was proportional to enzyme concentration (0-50 λ/assay) between 0-5 minutes incubation. Hence in all subsequent assays enzyme concentration was adjusted so that the increase in absorbance would be between 0.01 and 0.04 units after 5 minutes incubation. Within these limits product formation should depend directly on the activity and concentration of the enzyme extract used in the assay.

(d) Optimal Substrate Concentration

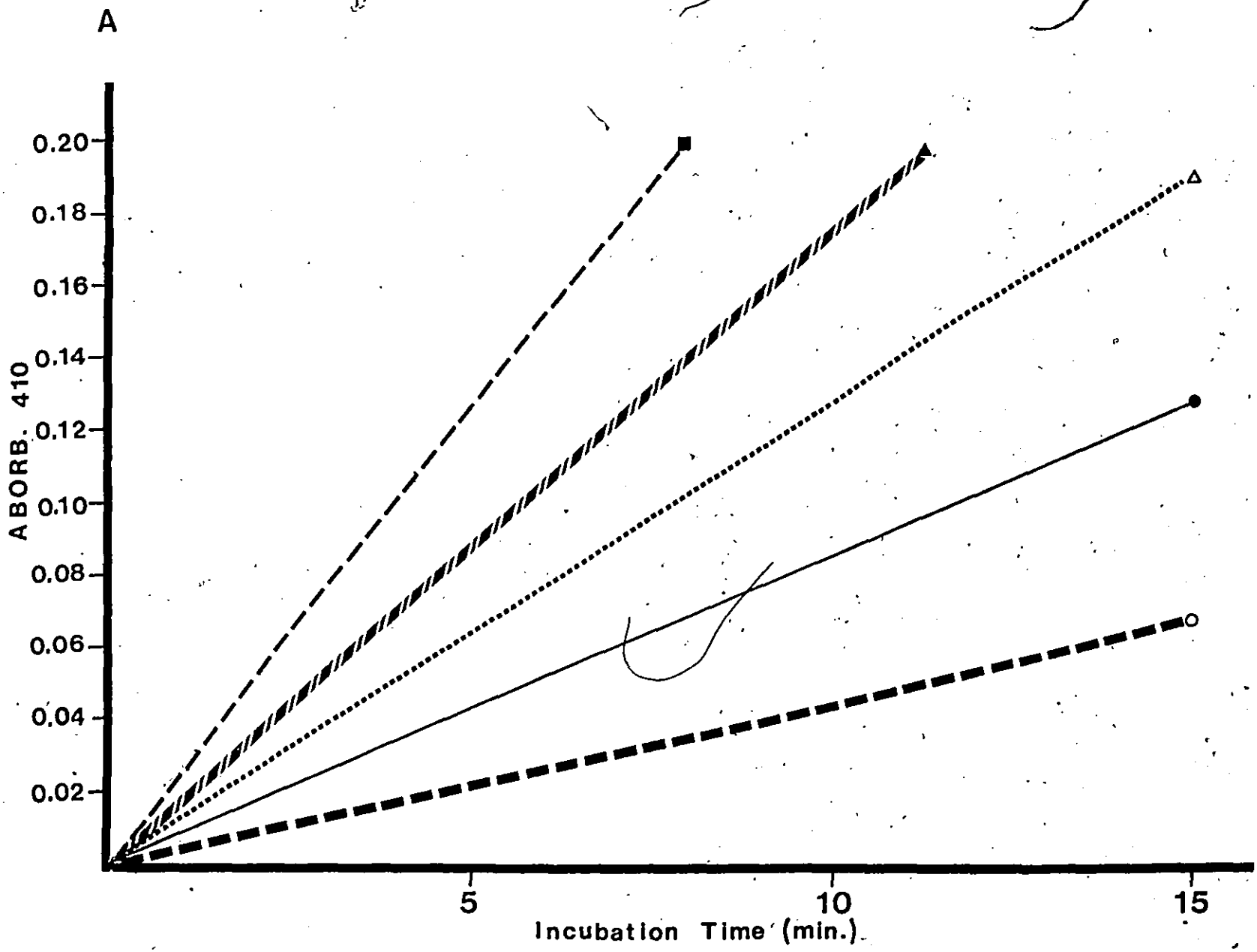
Optimal substrate concentration per assay was determined. Figure 18 shows that with 50 λ enzyme extracted from endosperm harvested 21 days after pollination 43 µg of BAPNA per assay was necessary to achieve the maximum rate of hydrolysis. Routinely 0.129 mg BAPNA (0.3 ml of 0.43 mg/ml solution) was used to provide excess substrate in standard assays. No substrate inhibition was observed at high substrate concentrations.

(e) Standard Assay

Routinely 50 λ of enzyme extract was incubated for 3 minutes with 0.77 ml of buffer (0.1 M Tris, pH 7.5, 35°C) in a 1.0 ml quartz cuvette with a 1 cm light pass. The reaction was started by the addition of 0.3 ml of warm (35 C substrate BAPNA 0.43 mg/ml). The reaction was monitored by following

Figure 17: Linearity of the Protease Assay

Enzyme was extracted in 0.1 M Tris-HCl buffer, pH 7.5 from endosperm of caryopses harvested 20 days after pollination. Activity with BAPNA substrate was assayed in 0.1 M Tris-HCl buffer, pH 7.5, at 35°C. Activity was measured as the continual increase in absorbance at 410 nm of the assay mixture between 0 and 15 minutes incubation. Activity of 20 (o), 50 (●), 100 (Δ), 200 (▲) and 400 (■) λ of enzyme per assay was measured. A shows the relationship between incubation time and product formation for the five enzyme concentrations. B shows the relationship between enzyme concentration and product formation after 2.5, 5.0, 7.5, and 10.0 minutes incubation.


A
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
ABORB. 410
5
10
15
Incubation Time (min.)

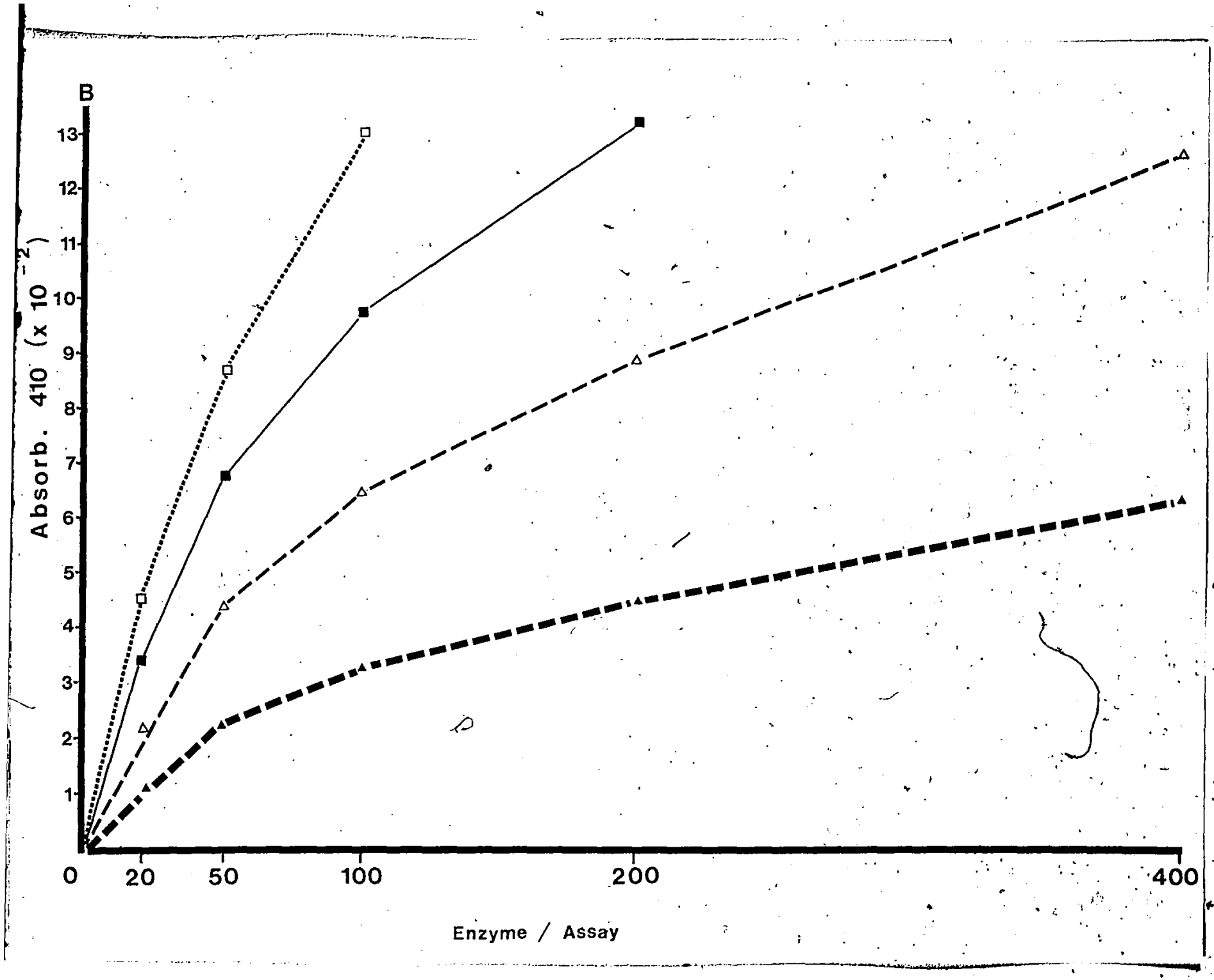
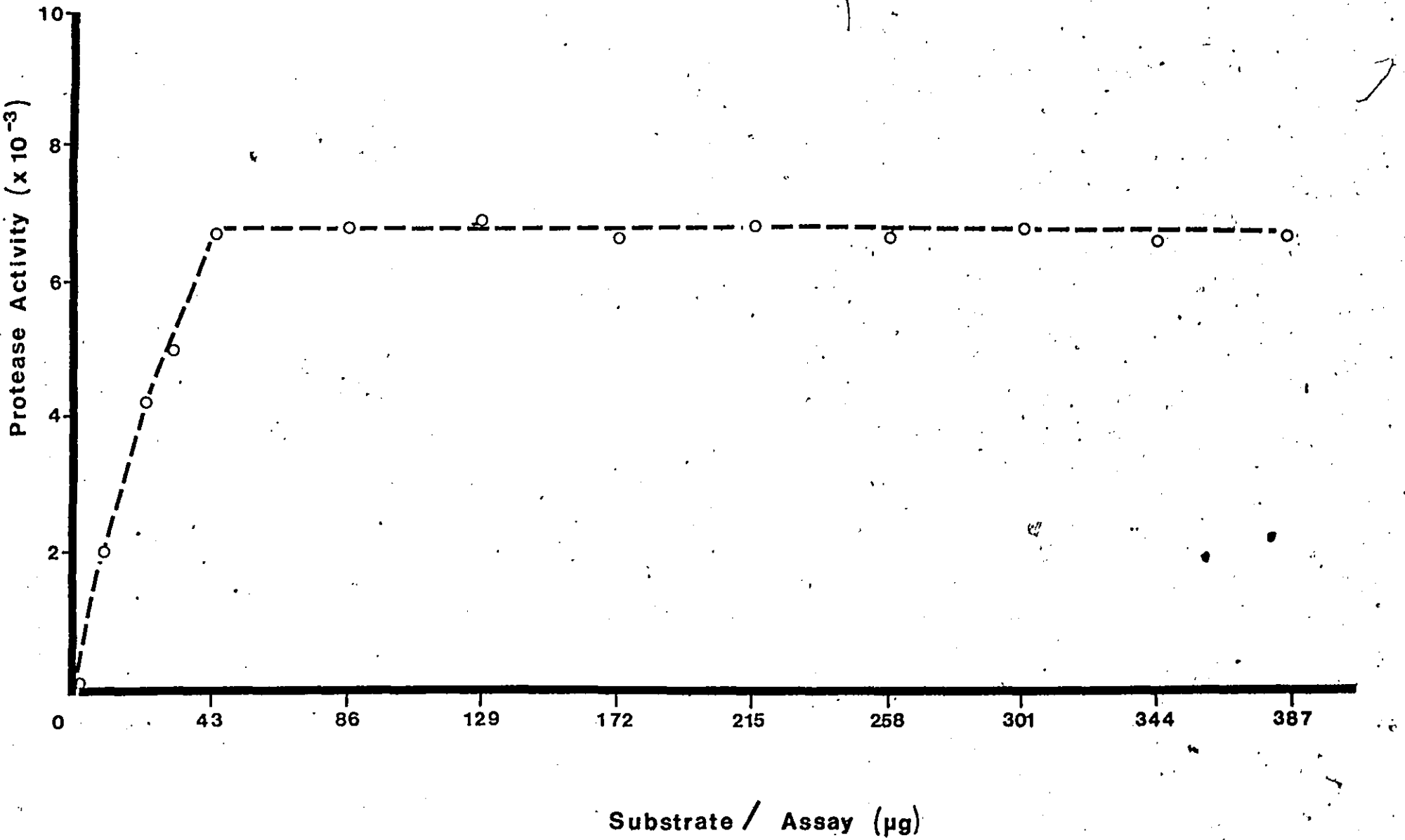

B
Absorb. 410 (x 10⁻²)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
20
50
100
200
400
Enzyme / Assay

Figure 18: Optimal Substrate Concentration for Assay of Protease Activity in Developing Endosperm

Endosperm of caryopses harvested 21 days after pollination were extracted in 0.1 M Tris-HCl buffer, pH 7.5. The assays were conducted at 35°C in the same buffer. The substrate concentrations (BAPNA/assay) were 0, 0.02, 0.05, 0.07, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 ml. of a 43 mg/ml BAPNA solution. Activity was expressed as increase in absorbance/min.


Protease Activity (x 10⁻³)
10
8
6
4
2
0
43
86
129
172
215
258
301
344
387
Substrate / Assay (µg)

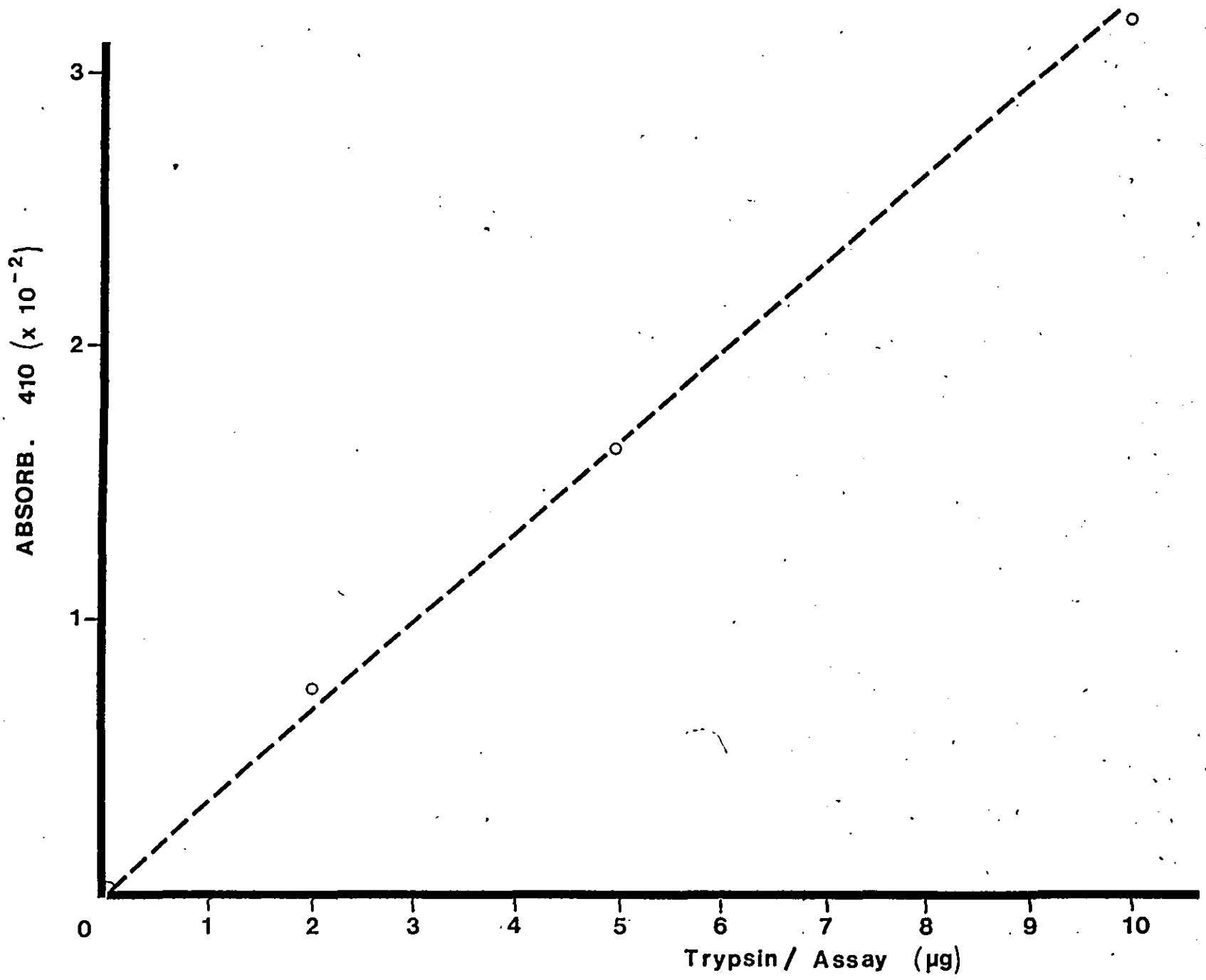
the increase with time in absorbance at 410 nm with a Unican SP 1800 spectrophotometer. This was arbitrarily compared to the increase with time in optical density at 410 nm when a trypsin standard was used (Figure 19).

Protease Activity During Development

Changes in activity of the protease during development were investigated. Batches of 30 endosperms were harvested at intervals for 57 days after pollination. All endosperms were frozen in liquid nitrogen and stored at -20°C prior to extraction and assay. Protease activity was measured by the standard assay with BAPNA as substrate, and by a second assay method using haemoglobin as substrate. In the standard assay activity was measured by equivalent trypsin activity, and in the second assay method by the release of tryptophan as described in the Materials and Methods. With each of these assays activity was detected in the earliest tested samples of 8 days. Activity increased sharply until a maximum was reached at 25 days, after which the protease activity declined to the 43 day level. A second protease activity increase was seen in the 57 day endosperm. At this stage of development the caryopses were well dessicated. Activity has been expressed on an endosperm basis because soluble protein per endosperm

Figure 19: Trypsin Standard

Trypsin at a concentration of 0.1 mg/ml was dissolved in 1.0 mM HCl and used as an enzyme standard. Activity with BAPNA substrate was assayed in 0.1 M Tris-HCl buffer, pH 7.5 at 35°C. Activity was measured as the increase in absorbance at 410 nm of the assay mixture. Activity of 0, 2, 5, and 10 µg of trypsin per assay was measured. The relationship between trypsin concentration and rate of product formation is shown.


ABSORB. 410 (x 10⁻²)
3
2
1
0
1
2
3
4
5
6
7
8
9
10
Trypsin / Assay (µg)

changes during development (Figure 20A and 20B).

Location of Neutral-Basic Protease Activity in Sucrose Gradient

Twenty-five endosperms were harvested 21 days after pollination. Endosperm were homogenized by grinding in a cold porcelain mortar with sand and extraction buffer containing 0.1 M Tris HCl pH 7.5 and 13% sucrose. Two mls of buffer were used per gram fresh weight of tissue. The homogenate was filtered through miracloth, and centrifuged at 500 x g for ten minutes to remove starch and cell debris. Five ml of the resulting supernatant was layered on top of a sucrose gradient made up in the following way from the top toward the bottom of the tube: 5 ml 13% (w/v) sucrose, 20 ml linear gradient 20-60% (w/v) and a cushion of 5 ml 60% (w/v) sucrose. The gradient was centrifuged for 60 minutes at 75,000 x g in a SW27 Beckman rotor. After centrifugation two bands were present in the gradient. A broad yellowish band was present between the center and the bottom of the gradient and a second more defined milky band sat on top of the 13%, 20-60% sucrose interface. A small pellet was on the bottom of the tube. The gradient was fractionated by pumping it from the bottom of the centrifuge tube. Thirty-two 1 ml fractions were collected and protease activity measured in

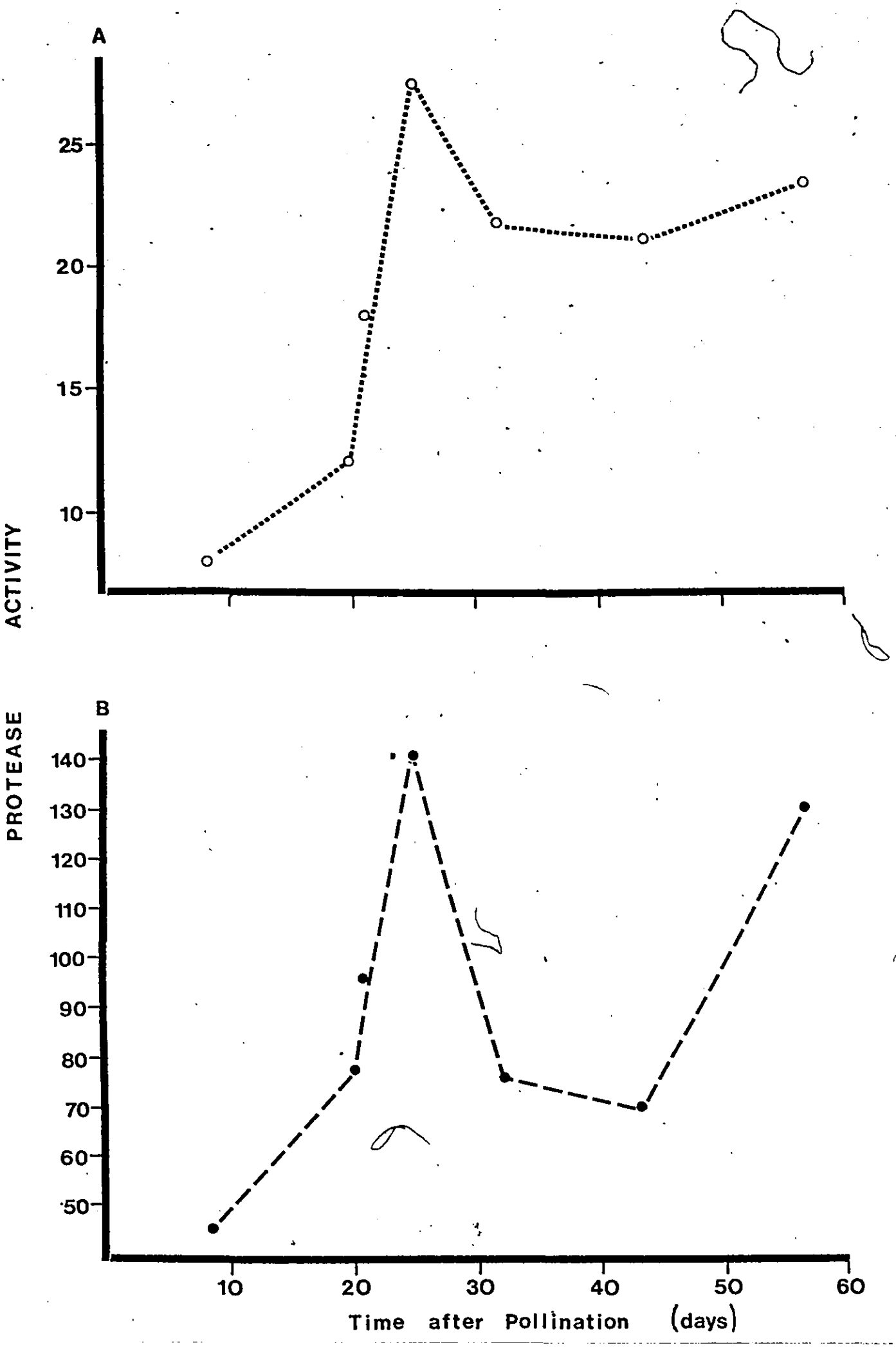
LEAF 95 OMITTED IN PAGE NUMBERING.

Figure 20: Changes in Endosperm Protease Activity During Development

Endosperm were harvested at intervals for 57 days after pollination. Protease was extracted and assayed as described in Materials and Methods.

In A, BAPNA was substrate and activity is expressed as µg trypsin equivalents per endosperm.

In B, haemoglobin was substrate and activity is expressed as µM tryptophan equivalents released/hr/endosperm.


A
ACTIVITY
25
20
15
10
B
PROTEASE
140
130
120
110
100
90
80
70
60
50
10
20
30
40
50
60
Time after Pollination (days)

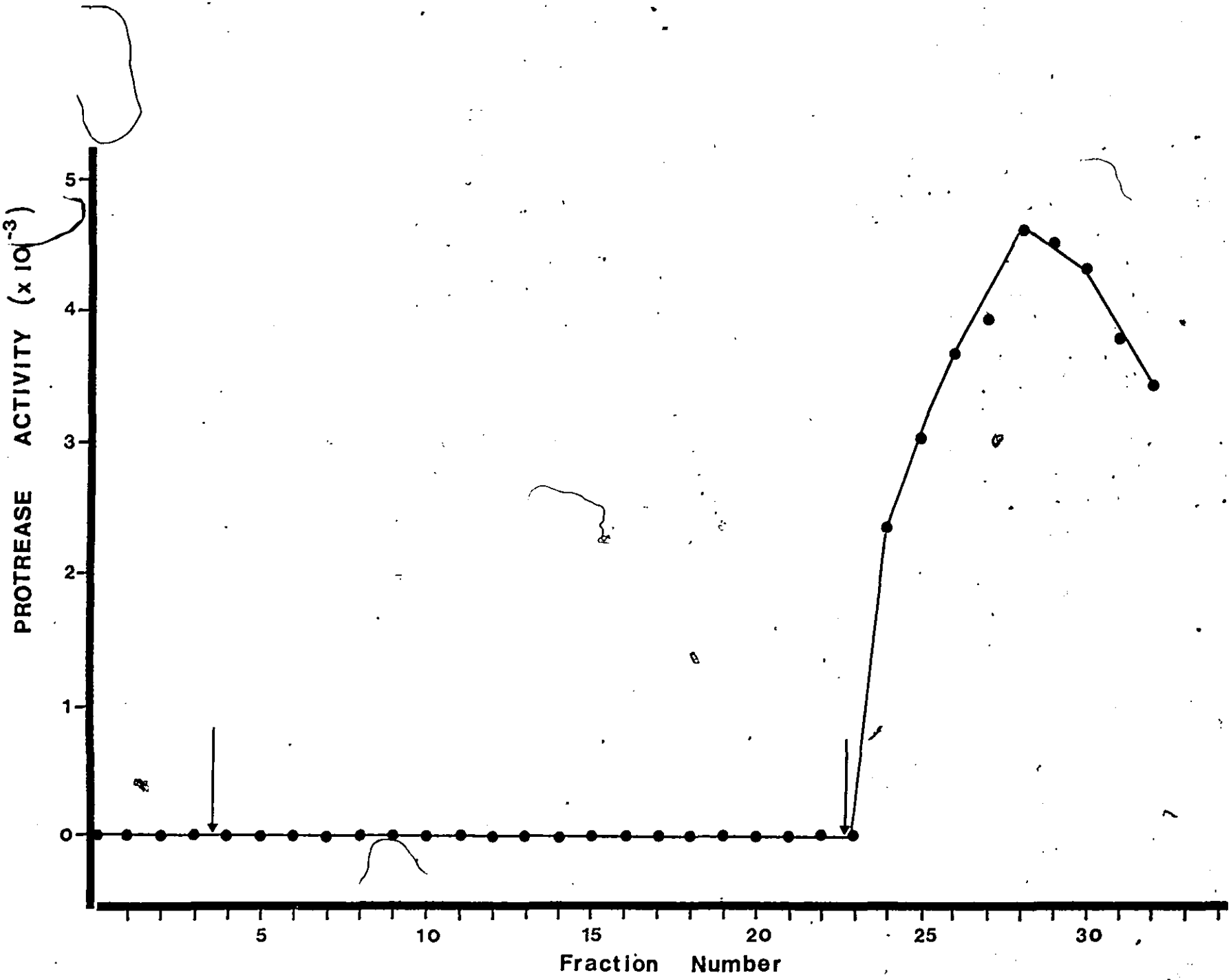
each by the standard assay using BAPNA as substrate. Results in Figure 21 show that only those fractions above the 13%, 20-60% interface exhibited any activity. The pellet was resuspended in 4 mls of Tris HCl buffer, pH 7.5 (ie, extraction buffer) and tested for protease activity using the standard assay. No activity was recorded in the resuspended pellet. Ninety-six percent of the original protease activity layered on the sucrose gradient in the crude extract was recovered after centrifugation and fractionation.

Protease Activity During Germination

Changes in activity of the neutral-basic protease during early germination were investigated. Batches of 15 endosperms were harvested at 24 hour intervals for 4 days after imbibition. All endosperm were frozen in liquid nitrogen and stored at -20^{o}C prior to extraction and assay. Protease activity was measured by the standard assay (Buffer, Tris pH 7.5) with BAPNA as substrate. With this assay maximum activity was detected in the dry caryopses. It declined steadily until day 4 (Figure 21). Activity of the acid protease was also measured so that the relative amounts of the two proteases could be compared. As before,

Figure 21: Location of Neutral-Basic Protease Activity in Sucrose Gradient

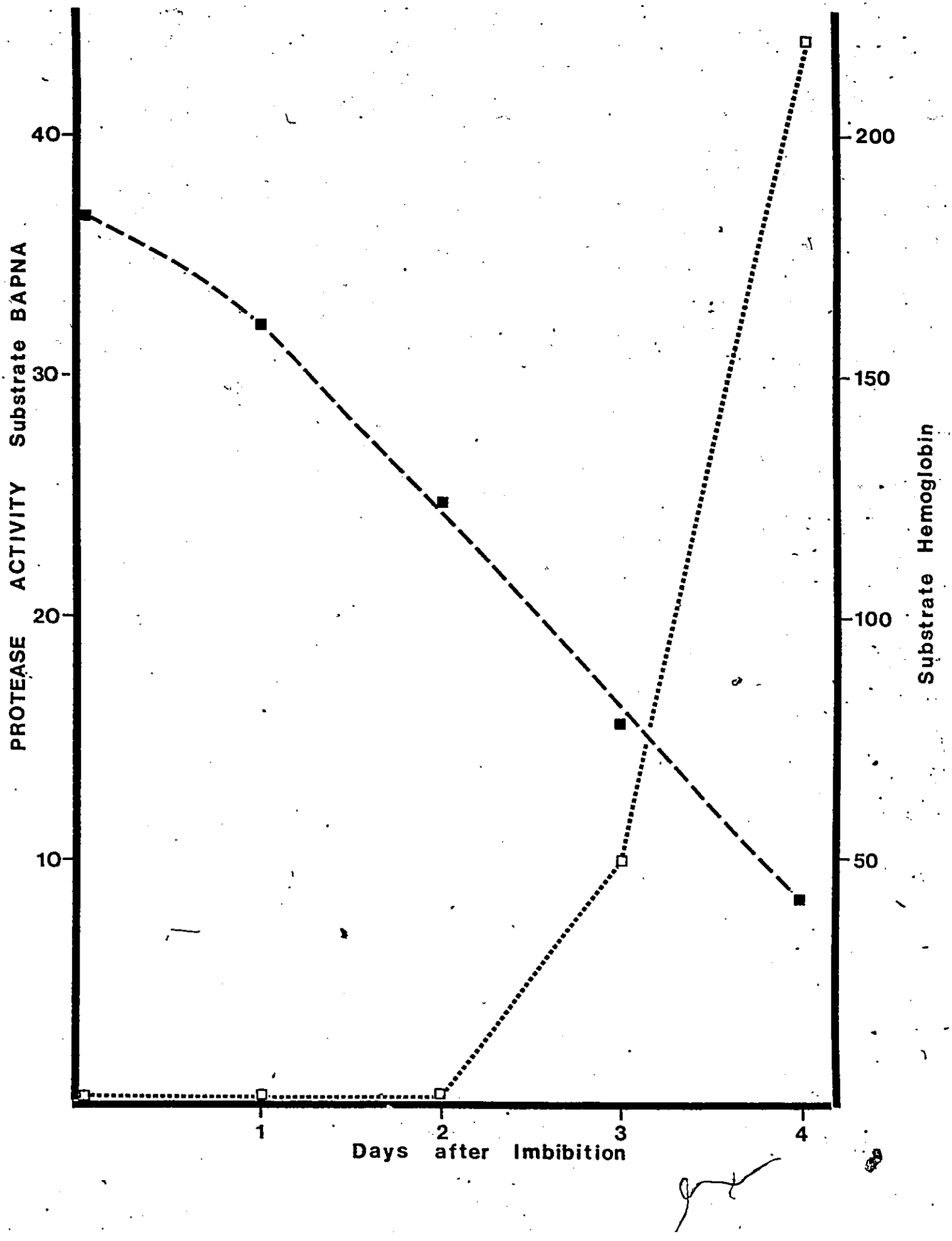
Endosperm of caryopses harvested 21 days after pollination were extracted in 0:1 M Tris-HCl buffer, pH 7.5 with 13% (w/v) sucrose. Five ml of extract were layered onto a linear sucrose gradient as described. After centrifugation and fractionation of the gradient each fraction was assayed by the standard assay with BAPNA as substrate. Activity was expressed as increase in absorbance/min. Fraction #0 represents the resuspended pellet. The arrows left to right represent the 60% cushion, 60-20% interface and the 60-20%, 13% interface respectively.


PROTREASE ACTIVITY (x 10^{-3})
0
1
2
3
4
5
5
10
15
20
25
30
Fraction Number

activity is expressed as μM tryptophan equivalents released per endosperm per hour. Results in Figure 22 show that only after activity of the neutral-basic protease has decreased significantly is there any detectable activity of the acid protease at day three.

Figure 22: Changes in Endosperm Protease Activity

Endosperms were harvested between 0 and 4 days after imbibition. Protease was extracted and assayed as described in Materials and Methods. BAPNA was used as substrate in the neutral-basic protease assay and haemoglobin was used as substrate in the acid protease assay. The neutral-basic protease activity (■) was measured as µg trypsin equivalents per endosperm and the acid protease activity (□) as µM tryptophan equivalents/ endosperm/hr.


PROTEASE ACTIVITY Substrate BAPNA
40
30
20
10
200
150
100
50
Substrate Hemoglobin
1
2
3
4
Days after Imbibition

DISCUSSION

Hydrolysis of the Endosperm Reserves

When excised barley embryos are allowed to grow on water or a mineral salt medium, the supplies of sucrose and raffinose are rapidly depleted (82,107). These two oligosaccharides, together with fructosan, constitute an important substrate for respiration and seedling growth in the time which elapses before mobilization of the endosperm reserves has been initiated.

In the endosperm, a dramatic increase in amylase, cellulase, and protease-peptidase activity begins shortly before the reserves of the embryo are exhausted (110). Thus the insoluble reserves of the endosperm are degraded to materials of low molecular weight which can be easily transported to the growing embryo.

Proteolytic and amylolytic activity has been studied in ungerminated wheat (62,94,116) and in resting and germinating barley (18,44,177) because of their relevance in both the baking, and the malting and brewing industries. Flour suspensions and crude extracts of germinating grain have been shown to have maximum proteolytic activity at

low pH (59,67,116,176) and high incubation temperatures, 40 to 50 C (67,116,191). Thus the major proteolytic enzymes in cereal endosperm appear to be acid proteases with high temperature optima (Table III).

Early investigations seemed to indicate that all endosperm proteases were of the papain-type, requiring free sulphydryl groups for their activity (10,45). Later workers showed that this was not necessarily the case. Sundblom and Mikola (177) have shown that barley endosperms produce not only several sulphydryl-activated enzymes, pH optima 3.9 and 5.0 to 6.5, but also enzymes that are insensitive to sulphydryl-blocking agents, metal-activated, and active in a higher pH range. All enzymes were shown to be endopeptidases. The sulphydryl enzyme with the pH optimum of 3.9 was the most active. The pH of the barley endosperm six days after imbibition of the caryopsis is 4.0 (20), hence this enzyme may account for most of the protein breakdown in the endosperm during early growth of the caryopsis.

Work by Harvey and Oaks (67) with maize endosperm has demonstrated a protease with characteristics comparable to other cereals (Table III). The maize endosperm protease activity was optimal at pH 3.8, was strongly inhibited by

sulphydryl-blocking agents, and had a high temperature optimum (46 C). Total nitrogen to α-amino ratios were high with each substrate tested, indicating that the endosperm protease has strong endopeptidase activity. In these aspects the maize protease is not unlike the sulphydryl enzyme which Sundblom and Mikola considered to be the principle barley endosperm protease (177).

Although such enzymes have been partially purified from germinated sorghum (4,56), wheat (155,166), and recently maize endosperms (3), most investigations have been performed with crude extracts. Furthermore several seed proteases have been found to degrade only denatured proteins such as haemoglobin (62,170), or the synthetic endopeptidase substrate BAPNA (112,120). Additional work with the maize endopeptidase indicates that this protease is capable of degrading partially denatured zein and glutelin, with efficiency similar to haemoglobin. Few seed proteases have been demonstrated to degrade their seed reserve proteins. For example, Shinano and Fukushimo (170) have purified a lotus seed protease which although increases in activity during early seedling growth, cannot degrade native protein, including lotus seed globulin.

The maize storage proteins, zein and glutelin, differ in their synthesis, their amino acid composition and location within the seed. These facts led to an early hypothesis that the hydrolysis of these storage proteins were likely under the control of different hydrolytic enzymes. However the maize protease has been demonstrated to display no specificity for either protein, since both are degraded with similar efficiency (67).

Using the maize hybrid W64A x 182E the endosperm endopeptidase was extracted using the method developed by Harvey (66). The protease exhibited similar kinetics as those reported by Harvey, although the maximal pH differed slightly (Fig. 2, 3 and 4).

Regulation of Endosperm Hydrolysis

Analysis of the endosperm protein of maize has shown that zein and glutelin degradation begins during the first 48 hours after imbibition of the caryopsis (66). The rate of loss of either protein appears to depend on the protein composition of the caryopsis. That is, the most abundant protein is preferentially degraded most rapidly (68). This is indicative of non-specific

proteolysis and is in agreement with observations that the endosperm protease preparation degrades zein and glutelin with equal efficiency.

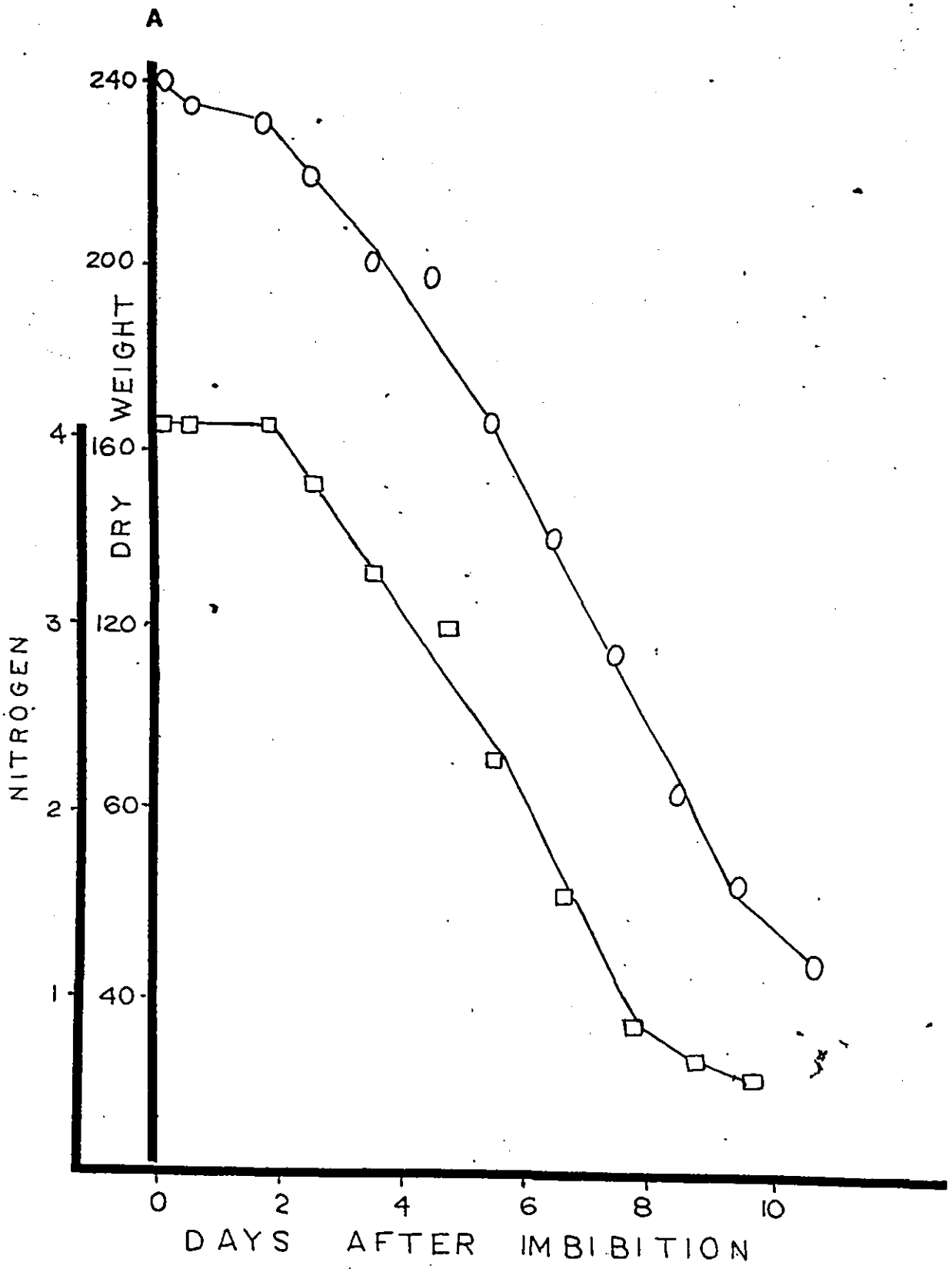
Time course studies with the developed standard assay (Fig. 5) show that the enzyme is not detected initially in germination and hence is probably not responsible for the early degradation of zein and glutelin. The protease appeared at day 3, and increased rapidly in activity until 8 days after imbibition. Comparison of protease activity with the reported course of zein and glutelin breakdown (Fig. 23) shows that the protease does not appear until after the initiation of protein hydrolysis, and reaches its maximum activity when degradation of zein and glutelin should almost be complete.

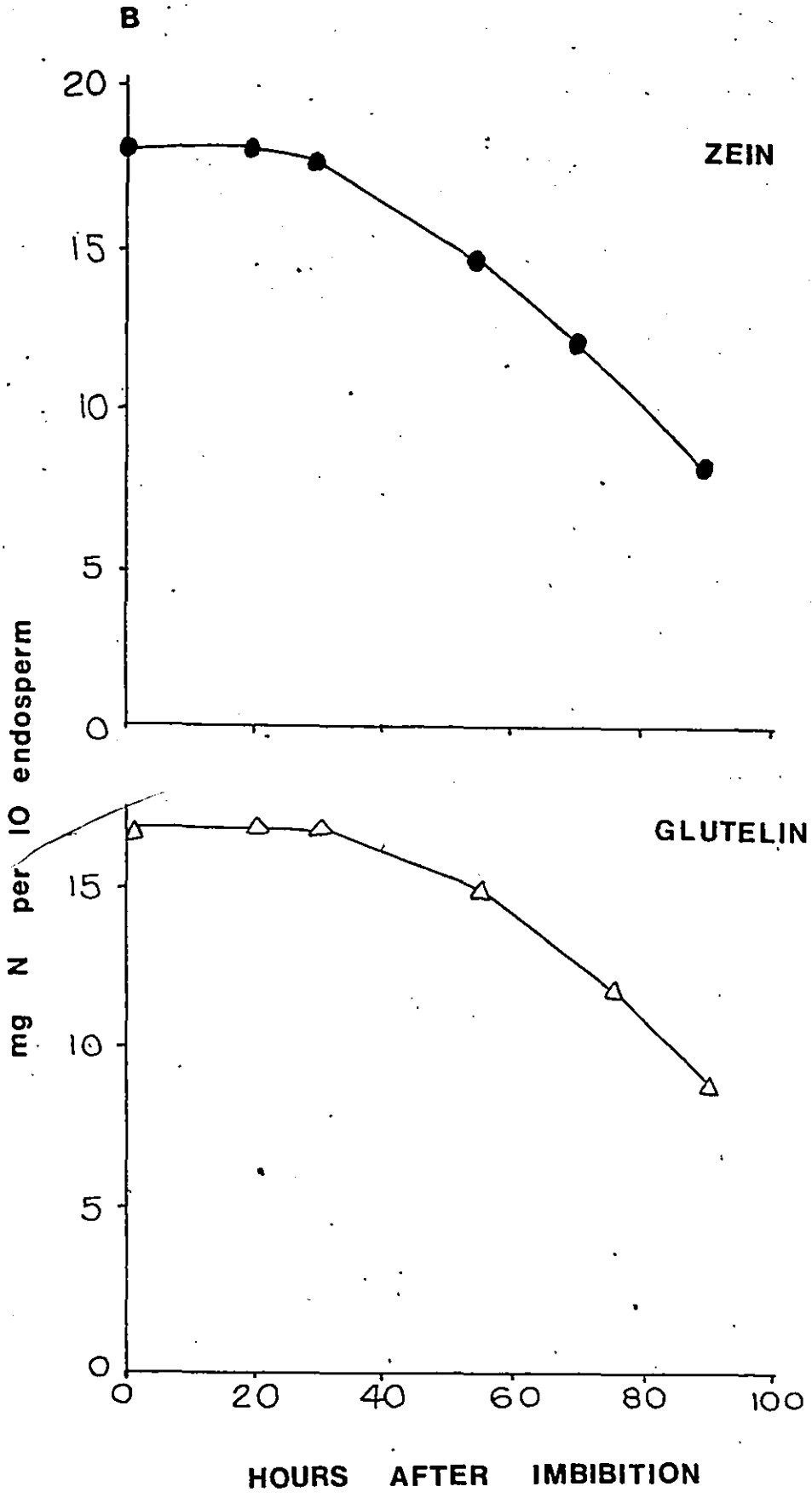
Similarly, development of α-amylase activity in the endosperm was measured at daily intervals after imbibition of caryopsis. Results (Fig. 5) show that amylase activity is not detectable until 48 hours after seed imbibition. Activity increases rapidly and is more or less maximal between 4 and 9 days after imbibition. Only after this time does the activity begin to wane. Comparison of α-amylase activity with respect to starch breakdown (69) shows that the appearance of α-amylase

Figure 23: Changes in Endosperm Total Nitrogen, Zein, Glutelin and Dry Weight During Germination (66).

(A) Endosperm of maize hybrid Wf9 x 38-11 were harvested between 0 and 11 days after imbibition. Dry weight per endosperm is indicated as (o), and endosperm total nitrogen as (□).

(B) Caryopses (WF9 x 38-11, 1969) were germinated at 28 C. Zein (●) and glutelin (Δ) content in the endosperm was measured at 0, 20, 30, 55, 75 and 90 hours after imbibition.


A
240
200
160
120
60
40
DRY WEIGHT
4
3
2
1
NITROGEN
0
2
4
6
8
10
DAYS AFTER IMBIBITION


B
20
15
10
5
0
ZEIN
GLUTELIN
mg N per 10 endosperm
15
10
5
0
0
20
40
60
80
100
HOURS AFTER IMBIBITION

activity coincided with the beginning of starch loss, while times of greatest recorded activity coincided with times of greatest substrate hydrolysis.

Analysis of amylase by electrophoresis (Fig. 6A and B) confirm those obtained by the standard assay. Activity is slight or absent until 48 hours after seed imbibition. Activity then increases rapidly and is strongest between 4 and 9 days, after which time it too begins to drop. Concomitant with the time course increase in activity was the appearance of two starch degrading bands as shown by polyacrylamide gel electrophoresis using either the protein or activity stain (Fig. 6A and B). On day 2 the first band became evident, and by day 3 the second starch degrading band was observed. Longer post imbibition times did not result in an increase in the number of bands although it did produce further increases in activity. It seems reasonable to assume that increases in the amount of one or both of the enzyme bands is responsible for the observed increase in activity. Goldstein and Jennings (58) also observed, concomitant with the increase in amylase activity, the appearance of a number of starch-degrading bands in extracts from de-embryonated maize kernels during a 10 day incubation

period.

Calculations of total proteolytic and amylolytic activities indicate that these hydrolases are produced in excess of requirements to degrade the endosperm protein and starch reserves (67). Theoretically it might be predicted that hydrolase activity would be highest during the rapid phase of substrate breakdown, and that a constant level of activity would be maintained while the rate of substrate hydrolysis is linear. In many instances there is a clear correlation between protein breakdown and protease activity (9,37,123). In contrast (Fig. 5) (51,68,156) massive increases in maize protease activity occur between 3 and 8 days after imbibition, whereas reported substrate loss is linear at this time (51,68). Maximum protease activity is obtained only when protein stores are depleted. Maximum protease activity after depletion of storage reserves has also been observed in germinating pea (14,60,203), rice (128), kidney bean (158) and oats (178).

Sutcliffe and Baset (178) observed that in oat endosperm, hydrolysis proceeds as a front from the dorsal to the ventral side of the caryopsis. To explain this phenomenon they assumed that activity must be confined to a specific zone at any given time. To support this

assumption they hypothesized that either:

1) As the substrate is depleted in a zone the enzyme molecules become inactivated and do not contribute to *in vivo* activity. Thus new enzyme would be produced to degrade substrate in adjacent substrate rich areas. *In vitro* however all enzyme would still be able to contribute to the total activity measured; or
2) As the zone of hydrolysis spreads the enzyme present is diluted, and so more is produced to maintain the active concentration necessary for substrate hydrolysis. Both models may explain the discrepancy produced when protease activity measured *in vitro* steadily increases during seedling growth (Fig. 5) while the rate of substrate hydrolysis in the endosperm apparently remains constant (68).

Maize experiments with cycloheximide show that continuous enzyme production is not necessary to maintain the normal rate of protein and starch hydrolysis (66). This simply indicates that excess enzyme is produced by germinated endosperms. This is not a phenomenon exclusively restricted to maize endosperm. Very large accumulations of hydrolytic enzymes in excess of requirements for mobilization of nutrients have been

observed in leaves during their senescence (117,127). Hence, loss of control of enzyme production may be a trait common to senescing tissues.

If activity of the protease is progressively inhibited *in vivo* evidence in the literature indicates two possible mechanisms; (1) accumulation of endogenous protease inhibitors; or, (2) amino acid (end product) inhibition of activity. The former appears improbable because no indication of maize protease inhibitors in the germinated caryopsis has been found (16). End-product inhibition of protein breakdown has been observed in maize (134) and pea (203). Oaks (134) found that if maize caryopses were grown with an amino acid mixture resembling the endosperm protein hydrolysate, protein breakdown in the endosperm was inhibited. As protease activities were not assayed, either protease activity and/or production may have been inhibited. Although it appears that additions of amino acids before the beginning of endosperm hydrolysis inhibited either protease production or activity (134), accumulations of similar or greater concentrations of amino acid during endosperm hydrolysis has not proven inhibitory (66). Additional work (66) indicates that there is little accumulation of protein hydrolysis products

in endosperms in the later stages of seedling growth, hence end-product inhibition by accumulation of amino acids does not appear to be a satisfactory explanation.

Initiation of Storage Protein Degradation

Harvey determined that the degradation of zein and glutelin in intact caryopsis begins between 30 and 50 hours after imbibition (66). Experimentation (Fig. 5) has shown that acid protease activity is not detectable until day 3. Low activities of the acid protease could be present between 0 and 3 days, but not detected by the assay method used. Available evidence to date (68) indicates this is not the case and justifies the hypothesis that this acid protease is not responsible for the initiation of storage protein degradation.

Protein hydrolysis in flour-suspensions of ungerminated maize occur in the absence of activating agents (66). Thus initial protein breakdown in maize only requires imbibition, whereas subsequent protein hydrolysis depends on endopeptidase production. This would indicate, as suggested by Harvey (68), that initial hydrolysis results due to the presence of inactive

protease(s) in the endosperm of the quiescent grain. Upon imbibition of the caryopsis the latent protease(s) would be activated and in turn denature the zein and glutelin. By day 3 synthesis of the acid protease(s) begins and in turn degrades the denatured zein and glutelin. If such a protease is present in mature dry grain it therefore must be synthesized during seed development. Melville and Scandalious (120) have reported finding an endopeptidase which *in vitro* is very active when isolated from immature liquid endosperm. Using their methods an endopeptidase was extracted from developing endosperm of maize hybrid W64A x W182E. Activity type was determined because of the enzyme's ability to degrade the synthetic endopeptidase substrate BAPNA (Fig. 17) (112,120). The protease was also able to degrade the substrate haemoglobin at a neutral pH. In agreement with Scandalious's results, maximum enzyme activity was achieved at a neutral-basic pH range (Fig. 15) and high incubation temperatures, 35 to 60 C (Fig. 16A).

Time course studies, through development, with the standard assay (Fig. 20A) show that the enzyme is detectable at the earliest test time (8 days). Activity rapidly increased until 25 days post pollination, after which activity decreased slightly. Again at 57 days post

pollination, activity increased to levels only slightly less than those recorded for day 25. By 57 days post pollination the caryopsis were considered mature and the experiment was terminated.

Melville and Scandalious (120) detected this endopeptidase in all tissues, except the pericarp and endosperm, of a large number (>900) of inbred maize strains tested during early seedling growth (0-17 days). Interestingly their method of seed treatment called for maize caryopsis to be soaked, or imbibed, in deionized water for 3 days before planting. Harvey maintains (68) that upon the imbibition of the caryopsis the latent protease(s) would be activated. It was therefore possible that Scandalious's protease was present and active in the endosperm during the first three days, becoming inactive again on the first day after planting. To test this possibility caryopsis were planted using our standard method. As always the grain was simply rinsed in tap distilled water to remove fungicide dust, and then directly planted in flats containing wet vermiculite and sand. Results (Fig. 22) show that the neutral-basic endopeptidase is present in the endosperm during very early seedling growth. Furthermore activity extracted from day 0

endosperm is greater than any recorded activity found during grain development (Fig. 20A). Activity steadily decreased until termination of the experiment at day 4. At this time activity was minimal.

Preliminary results (Fig. 21) suggests that this protease is also found in the cytoplasm. Experimentation was incomplete as only endosperm from developing caryopsis were tested. In addition, conclusions drawn from these results may be incorrect due to the harsh isolation techniques employed. Only careful study of the locations of this protease(s) in the mature maize grain, and during the initial hours and days of growth, would clarify this problem. In addition no work has been done to determine whether this endopeptidase (BAPNAse), is active with either zein or glutelin substrates. A wide pH optimum (Fig. 15) would tend to suggest the presence of more than one protease. Purification of the protease extract may produce a number of proteases with specific pH optima and substrate preference. At this stage, the results suggest that this dry grain protease may be responsible for the initiation of zein and/or glutelin degradation.

Preston, in cooperation with our laboratory, has

been able to identify three different protease activities in the maize hybrid W64A x 182E endosperm using extraction techniques developed for wheat (46,154,190). With each protease little or no activity was recorded during the first 24-48 hours. Patterns of protease activity increase are similar to those of the maize acid protease. Four additional proteases have also recently been extracted from maize endosperm during early growth by Feller *et al.* (51). They consider those proteases whose activities correlate well with the times of greatest endosperm nitrogen loss to be responsible for the major storage protein degradation. These proteases only become active 48-72 hours after planting. Again both investigators have failed to demonstrate whether these extracted protease could degrade their seed reserve proteins.

Starch hydrolysis in maize endosperm, determined by dry weight loss and sugar release (68), is not apparent until α-amylase activity appears at 48 hours (Fig. 5). However the methods used to determine endosperm starch breakdown were only approximate. Internal cleavages of starch polymers could occur without loss in dry weight, or the release of sugars. Hence starch breakdown could begin undetected before the appearance of α-amylase

activity in the endosperm. Therefore the experiments performed demonstrate the appearance of starch degrading enzymes which coincide with the initial apparent degradation of endosperm starch reserves. Results do not conclusively show that newly synthesized α-amylases (Fig. 6A) are responsible for the initiation of endosperm starch hydrolysis.

Location of Protease Activity

Both endo- and exopeptidases are known to be present in ungerminated wheat (191), barley (123) and maize (120). On imbibition of the grain these enzymes may initiate reserve protein hydrolysis.

Proteases have been demonstrated in protein bodies of ungerminated barley (137), hempseed (175), squash (1), vetch seeds (102), mung beans (29), and sorghum (5). Protein bodies extracted from ungerminated squash seeds undergo autolysis when endogenous proteases are activated with cysteine (1). Paleg (145) found that barley aleurone protein bodies begin to disintegrate after 18 hours gibberellin treatment, and Yomo and Varner (202) observed vacuolation of aleurone cells due to disintegration of protein and lipid bodies during the first 48 hours of

germination of barley. Similarly, I have observed significant change in maize protein body population and ultrastructure as early as 24 hours after imbibition (unpublished results). Thus in maize the protein bodies may also have their complement of enzymes, and may begin to disintegrate before the acid protease activity appears in 3 days. When the appearance of the acid protease is inhibited by inhibitors such as cycloheximide and 6-methylpurine, a small amount of total endosperm protein is still degraded (66,68). Although proteolysis is apparent, it is limited and inadequate to account for reserve protein degradation. This limited proteolysis may be adequate to denature the reserve proteins, since scission of only a small number of peptide bonds can cause extensive denaturation of storage protein (161).

Proteolytic activity of protein bodies isolated from ungerminated seeds of *Sorghum bicolor* has been studied by Adams and Novellie (4,5). Their results describe a protease with a pH optimum of 4.0 and a temperature optimum of 50° C. It is an exopeptidase present in dry sorghum endosperm protein bodies, capable of converting a small proportion of protein body protein (not prolamine) into free amino acids by a carboxypeptidase activity.

Therefore during early seedling growth the enzymes present in the mature maize seed may:

1) break down internal structures to make both the zein in the protein bodies and the cytoplasmic glutelin equally accessible and denature the proteins to facilitate further degradation by the protease(s) appearing later; and/or
2) digest a small fraction of reserve protein into free amino acids, thus supplying the amino acids used for *de novo* synthesis of the endopeptidase(s) responsible for the major utilization of the protein reserves.

Although Harvey's work (67) showed the acid endopeptidase was able to degrade extracts and thus partially denatured zein and glutelin with similar efficiency, this does not necessarily imply it can degrade native storage protein, or degrade both with equal efficiency in the intact grain. Early zein and glutelin degradation (68) and possible protein body autolysis (unpublished) suggest the later appearing acid endopeptidase may not be able to hydrolyse undenatured storage protein.

Assuming the acid endopeptidase is specific for neither native storage protein it is relatively safe to

suggest that it should not be specifically associated with either storage protein to the exclusion of the other. To investigate this hypothesis time course studies with the developed standard assay were used. A crude endosperm extract was tested for proteolytic activity and then layered on top of a sucrose density gradient. After centrifugation and subsequent protein body isolation (Fig. 10 and 13), fractions from the gradient were tested for proteolytic activity (Fig. 12 and 14). As before the enzyme is not detectable initially. The protease activity appeared at day 3, and rapidly increased in activity until day 9 after imbibition (Fig. 12). In all cases, all recovered protease activity was associated with the top soluble protein fraction of the gradient. At no time could activity be ellicited from those fractions identified as containing protein bodies (Table X). Protein body identification was made using electron microscope techniques (Fig. 13). Further experimentation (Table IX) indicates that protease activity is associated with no organelle or cellular structure and is free in the cytoplasm. Chrispeels now has evidence (in press) to support his claim that during protein degradation in mung bean cotyledons, the endopeptidase(s) are transported from their

site of synthesis in the cytoplasm to the protein bodies in vesicles that merge with the protein body. These results indicate an increased localization of the protease in the protein body and a concomitant decrease in the cytoplasm. However this does not seem to be the case with the acid endopeptidase found in maize endosperm. Activity always remains in the soluble fraction and closely mimics in increase and amount that activity found in the crude extract (Fig. 12). Understandably the stability of the protein body in the isolation media substantially decreases as early growth and subsequent storage protein hydrolysis proceeds. Figure 11 indicates that by eight days after imbibition the protein body fraction is all but gone. This result is in agreement with those results of Harvey (68) which show that at this time zein and glutelin degradation should almost be complete. It is therefore possible that the isolation procedures used, especially at later times of growth (day 6 on), may damage protein bodies and subsequently cause the release of contained protease. That all protein bodies would be synchronously damaged and all protease concurrently released seems improbable.

The Role of Gibberellic Acid

Isolated maize endosperms produce α-amylase and degrade their starch reserves in the absence of the embryo or exogenously added GA_3 (Fig. 9A and B; and Tables VI and VII). Autonomy of maize endosperm hydrolysis is noted in the literature (58,69,186), but conflicting reports also appear. Dure (38) observed that endosperm hydrolysis depended on the presence of the embryo, and α-amylase was principly produced by the scutellum, not the endosperm. Similarly, Ingle and Hageman (66) found that some protein but little or no starch breakdown occured in de-embryonated endosperms. They also demonstrated that GA_3 applied exogenously could replace the missing embryo factor required for starch breakdown, stimulating sugar release from starch. The exogenous hormone was found to replace a stimulus which moved from the embryo to the endosperm 36 hours after imbibition. On the other hand, all batches of maize which I have tested displayed no additional response when gibberellic acid was added to the medium. The maize hybrid (WF9 x M14 1966) initially used by Ingle and Hageman for his original experiments (Tables VIand VII) was one of the varieties tested here. Amylase activity was determined both directly by measuring enzyme activity

using the standard assay (Table VI) and more indirectly by the measurement of the production of sugar in the endosperm as Ingle and Hageman had done (Table VII). Exogenously added GA_3 was responsible for a substantial increase in α-amylase activity and release in barley endosperm. Hence the technique for measuring α-amylase induction is satisfactory.

Variability in response to exogenous GA_3 is also observed among batches of barley (79). Isolated endosperms or aleurone layers of some batches of grain produce no α-amylase in the absence of GA_3, while others produce considerable amounts of the enzyme. These are called "high background" grain, and respond less markedly to GA than the "low background" varieties.

Free and bound hormones have been found in many caryopses (11,115,162,168). Auxin esters and glycosides have been isolated from mature maize caryopses (43,184) and bound cytokinins and gibberellins from immature maize kernels (86,125,151). Ross and Bradbeer (163) have suggested that bound hormones may be liberated during germination to stimulate growth processes. An alternative explanation implied by Goldstein and Jennings (58) suggests that some endogenous inhibitor leaches out thus lowering

that level of endogenous GA needed to give a maximal GA response. This possibility has been outlined by Harvey and Oaks (69) who observed that addition of GA_3 is required to overcome the inhibitory effects of ABA on α-amylase development. For example, GA induction of α-amylase in barley aleurone is inhibited by absicsic acid, and the ABA inhibition can be recovered by increasing the concentration of GA_3 (28). Similarly in maize, addition of ABA to the incubation medium of excised endosperms inhibited α-amylase production and subsequent starch breakdown. Addition of exogenous GA_3 could reduce or completely overcome this inhibition (69). The specific interaction of ABA and GA_3 indicated that gibberellic acid has a special role in the regulation of hydrolase production in maize.

Work with barley (57) indicates that those conditions that produce deep dormancy in the grain also results in higher ABA levels. Conversely there is a defined correlation between low ABA equivalent content and a more pronounced tendency to sprout. The findings of various workers indicate that ABA may be involved in the regulation of ripening. Environmental factors such as temperature and water supply may effect the ABA content

of the grains, possibly leading to an acceleration or retardation of the ripening process.

All workers in maize agree that a GA-like factor is important in the regulation of hydrolase production in maize endosperm. The discrepancy between those results obtained by Ingle and Hageman and our laboratory may be as easily explained as resulting from an after ripening affect. I propose that when Ingle and Hageman performed their experiments (76) the ABA level of the excised endosperm was high enough to produce an ABA:GA ratio that prevented self hydrolysis. The addition of exogenous GA simply changed this ratio to one that favoured endosperm production of hydrolases. With after-ripening either: 1) the ABA level in the endosperm decreased, or 2) the endogenous GA level increased. Both processes would have the same immediate effect in changing the ABA:GA ratio in favour of a higher equivalent GA content. Now when the same endosperm is incubated, α-amylase activity increases without the addition of exogenously added GA. In agreement with this hypothesis is the fact that in both Ingle and Hageman's experiment (76) and our experiments high concentrations of GA_3 worked to inhibit α-amylase activity. Whereas all GA additions resulted in the inhibition of

α-amylase activity in my results (Table VI) only the higher concentration of exogenously added GA were inhibitory in their experiments. Lower concentrations (10 μM) did stimulate α-amylase activity (76). It is possible therefore that initial endogenous endosperm GA levels in their grain were lower, or that initial ABA levels were higher.

Events of Early Seedling Growth

In summary, without further experimental proof to the contrary, I would suggest the following sequence of events leading to the hydrolysis of endosperm storage reserves.

Day 0: The endosperm of the quiescent grain contains native zein and glutelin, inactive protease(s) (possibly BAPNAse), and a ABA:GA ratio which would permit germination.

Day 1: Imbibition of the caryopsis activates the latent protease(s) which denature the zein and glutelin. The protease(s) may be specific for and/or closely associated with one or the other of the storage proteins. Imbibition

may allow the release of bound GA.

Day 2: Alpha-amylase is synthesized *de novo*. Synthesis, stimulated by endogenous GA, occurs in the aleurone layer of the endosperm (27). Newly synthesized α-amylase initiates starch hydrolysis.

Day 3: Endogenous GA stimulates the *de novo* synthesis of acid protease(s) responsible for the massive hydrolysis of denatured zein and glutelin.

Day 4 to 8: Time of greatest zein and glutelin, and starch hydrolysis. Amino acids released are transported to the embryo or are incorporated into new endosperm proteins. Sugars released are transported to the embryo or remain in the endosperm. Protease(s) and α-amylase activities increase.

Day 9 to 11: Storage reserves are depleted and hydrolytic activities begin to decline.

SUMMARY

Early seedling growth in maize can only continue if storage reserves of the endosperm are hydrolyzed and transported to the embryo. Results indicate that endosperm starch hydrolysis is initiated and proceeds due to the production of α-amylase 48 hours after imbibition of the caryopsis. Concomitant with the time course increase in activity is the appearance of two starch degrading bands as shown by polyacrylamide gel electrophoresis. The relative specificity of activity of each band with native starch substrates remains to be determined.

Neither the embryo nor an embryo factor needs to be present for the production of α-amylase by the endosperm. Activity of α-amylase produced from untreated incubated excised endosperm is as great as that extracted from endosperm of intact seedlings. The inclusion of GA in the incubation medium has no additional affect.

Comparison of acid protease activity with the time course breakdown of zein and glutelin show that acid protease activity does not appear until after the initiation of protein hydrolysis. The acid protease activity is not associated with any cellular organelle but is free in the cytoplasm. Previous work (68) indicates that this endopeptidase is able

to degrade partial denatured zein and glutelin with similar efficiency. Together, these results suggest that this protease, although likely responsible for the major hydrolysis of endosperm protein reserves, does not initiate storage protein degradation.

Neutral-basic protease activity was extracted from developing, dry, and post imbibition maize endosperms. Activity with the synthetic substrate BAPNA indicates endopeptidase activity. Activity optima with the substrate BAPNA was at pH 7.5. Presence of the protease(s) in the dry endosperm and during very early seedling growth (0-4 days) suggests that this protease(s) may be responsible for the initiation of storage protein degradation. To test this possibility proposed experimentation would include: 1) the determination of the activity of the protease(s) against the native protein substrates, zein and glutelin, and; 2) the possible association of the protease(s) with either native protein in the dry grain or during early seedling growth.

Within the last year Preston (personal communication) and Feller et al. (51) have demonstrated the presence of several proteases in the endosperm of maize before and after germination. As is usually the case, activities were determined *in vitro* with protein substrates commonly used to assay for proteases. Unfortunately these substrates bear little resemblance to native protein. Further studies are

necessary to reveal which of these proteases are active *in vivo*, at what time during growth, and to what extent each is involved in storage protein mobilization.

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APPENDIX

Figure 13: Protein Bodies Isolated From Sucrose Gradient

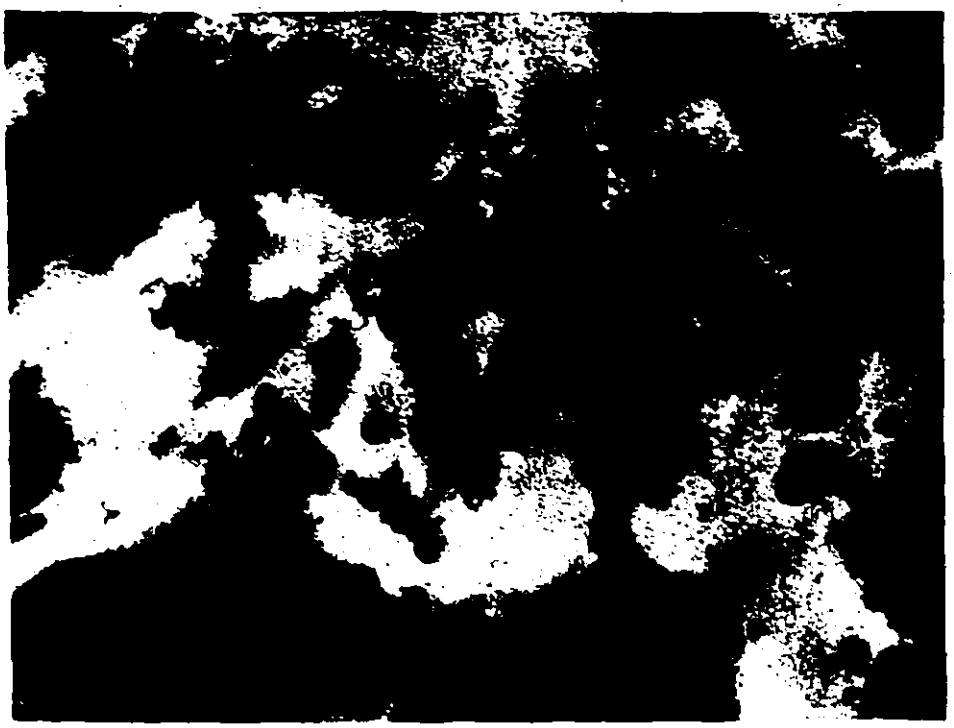
The fractions corresponding to the A 280 peak on top of the 80% sucrose (Figure 11) (ie, Band A, Figure 10) were combined and the organelles sedimented by centrifugation as described in the Materials and Methods. This pellet was fixed for electron microscopy.

Figure 13A (20,900 X) show spheres which correspond to what have been termed protein bodies. Many of the protein bodies measure 1 μ or more in diameter and stain with a dark narrow, irregular, peripheral band.

Figure 13B (49,000 X) shows that some contain darker staining particles arranged in concentric rings. Small protein bodies, as well as a few larger ones stain uniformly.

A granular component, plus other unidentified cellular debris, can be seen in both Figure 13A and B. Both micrographs are of protein bodies isolated from endosperms of maize seedlings 24 hours after imbibition.

A



B

Figure 24: Early Degradation of Protein Body Isolate

The fractions corresponding to the A 280 peak on top of the 80% sucrose (w/v) (Figure 11) were combined and the organelles sedimented by centrifugation as described in the Materials and Methods. This pellet was fixed for electron microscopy.

Figure 24A (21,600 X) is representative of the protein body population found in an isolate prepared from dry maize endosperm. The single black body is similar to that type of protein body found in the aleurone layer (ie, aleurone grains) as reported by Wolf *et al.* (99). Species of this type are found scattered throughout the isolate.

Figure 24B (21,280 X) is representative of the protein body population found in an isolate prepared from maize endosperm excised from caryopses 24 hours after imbibition. The aleurone grain type bodies show internal signs of degradation.

In isolates prepared from maize endosperms excised from caryopses 48 hours after imbibition, aleurone grain type bodies as depicted in Figure 24A and B, are never found (not shown).

(Above work from Graduate Course 764).

A

B