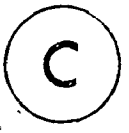
PEPTIDE HYDROLASES

IN MAIZE ENDOSPERM

By



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ABSTRACT

The enzymes responsible for hydrolysis of reserve protein in endosperm tissue during early growth of maize seedlings were investigated. The main areas of research included:

(1) studies to optimize endopeptidase, carboxypeptidase and aminopeptidase reactions.

(2) a partial purification of the endopeptidase protein.

(3) an investigation of the effects of certain plant hormones on the formation of the peptide hydrolase activities in endosperm tissue of maize (W64 x W182E).

Assays which could measure endopeptidase, carboxypeptidase and aminopeptidase activities were studied in extracts of endosperm tissue prepared from Zea mays. As a substrate for the endopeptidase assay haemoglobin was more reactive than gliadin, BSA, azocoll or azocasein. Synthetic substrates were used to assay carboxypeptidase (Z-CBZ-Phe-Ala) and aminopeptidase (Leu-para-nitroaniline). Interference with measurement of carboxypeptidase activity, due to autolysis, could be eliminated by dialysis of the extract. The dependence of the activities upon pH, concentration of substrate, volume of extract and temperature were investigated. Optimum conditions were established. Routinely, saturating protein concentrations were used in the acidic endopeptidase assays whereas the peptidase assays were performed at

less than saturating substrate concentrations due to limited solubility of the substrates.

The effects of selected enzyme inhibitors on the enzyme activities were tested. Para-chloromercuribenzoate gave the greatest inhibition of all activities. Aminopeptidase was most sensitive, followed by carboxypeptidase and then acid endopeptidase. Other inhibitors (PMSF and EDTA) had slight effects. Aminopeptidase was sensitive to 5 mM phenylmethylsulphonylfluoride whereas the carboxypeptidase and endopeptidase were not significantly affected.

A number of techniques were used in an attempt to purify the acid endopeptidase. Acid endopeptidase activity was clearly separated from carboxypeptidase activity on Sephadex G-50. The best partial purification of an endopeptidase fraction was achieved with Sephadex G-50 chromatography, followed by CM-cellulose chromatography. A thirteen fold increase in specific activity was attained. The molecular weight of the most pure fraction was estimated to be 21,000 daltons, using a Sephadex G-200 column.

The development of acid endopeptidase, carboxypeptidase and aminopeptidase was followed in the post pollination and post germination periods. The exopeptidase activities were high in the post pollination tissues, while acid endopeptidase was low. The latter activity rose after imbibition, reaching a peak at five to eight days. Carboxypeptidase also increased in activity following imbibition, while aminopeptidase activity declined from the value found in the mature caryopsis.

Endosperms, from which the embryo and scutellum had been removed were incubated in the presence of plant hormones and chemical effectors. Unlike barley (Hordeum vulgare), in which GA_3 is required to induce development of a protease activity in halfseeds, GA_3 is not required for development of endopeptidase activity in de-embryonated caryopses of maize (Zea mays). In the present study, it was found that GA_3 did not affect the appearance of exopeptidase activities either. Incubation with 2 μM abscisic acid (ABA) caused partial inhibition of the development of endopeptidase and carboxypeptidase activities. At a concentration of 10 μM, significant inhibition of aminopeptidase development was also seen. GA_3 (30 μM) could partially reverse the inhibition of acid endopeptidase and carboxypeptidase caused by the lower concentration of ABA. Cordycepin, an inhibitor of RNA formation, was found to have no effect on either the development of protease activity, or the inhibition brought about the ABA, although it did inhibit development of α-amylase in barley and maize endosperm pieces. Cycloheximide, a protein synthesis inhibitor drastically decreased the production of all peptide hydrolase activities.

PREFACE

The experiments described in this thesis were carried out in the Department of Biology, McMaster University, from May 1978 to May 1980. Except where others are specifically mentioned, this thesis consists entirely of my own work.

I would like to express my sincere appreciation to my supervisor, Dr. B.A. Oaks, for her helpful advice and boundless patience which were valuable aids in completion of this thesis. In addition, I would like to thank faculty members, staff personnel and co-workers who provided their time and expertise in assisting me. Special thanks to Miss Cathy Anderson for her very skillful typing of this thesis.

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ABBREVIATIONS

A	Absorbance
ABA	Abscisic Acid
BAEE	α-benzoyl-L-arginine ethyl ester
BANA	α-benzoyl-L-arginine-β-naphthylamide
BAPA	α-benzoyl-L-arginine-P-nitroanilide
BPB	Bromophenol blue
C	Degree Celsius
CBZ	N-carbobenzoxy
CM	Carboxymethyl
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminetetraacetic Acid
g	Gram
xg	Gravity
GA_3	Gibberellic Acid
Hbase	Haemoglobinase
hr	Hour
mg	Milligram
ml	Millilitre
mRNA	Messenger RNA
PCMB	Parachloromercuribenzoate
PHMB	Parahydroxymercuribenzoate

ABBREVIATIONS (continued)

PMSF	Phenylmethylsulphonylfluoride
SDS	Sodium dodecylsulfate
TCA	Trichloroacetic Acid
Ve	Elution Volume
Vo	Void Volume

INTRODUCTION

Structure of Maize Caryopsis

The mature maize caryopsis is typical of the fruit of Graminaceae. It consists of embryo, scutellum and endosperm with a fused pericarp and testa; the testa or seed coat arising from the remnants of the integuments and the nucellar membrane. The proportions are roughly 85% endosperm, 10% embryo and scutellum, and 5% testa and pericarp (47, 71).

Within the fused testa and pericarp lies the triploid endosperm tissue, which arises from the fusion of one sperm nucleus of the pollen grain with the two polar nuclei of the embryo sac. At the outer edge of the endosperm lies a layer of living cells, which are called the aleurone cells. The remainder of the endosperm, known as the starchy endosperm consists of thin-walled, non-respiring cells. These contain large numbers of starch granules and protein bodies (in which are stored the alcohol soluble prolamine, zein) in a matrix of the alkali soluble glutelin proteins (47, 71).

On one of the faces of the caryopsis, near the base, the embryo can be seen embedded in the endosperm. Diverging from the embryonic axis at the scutellar or cotyledonary node and directly in contact with the endosperm is the scutellum, a large structure considered to be a modified cotyledon. The embryonic axis itself consists of the radical

Figure 1

Structure of Maize Caryopsis (Leonard, W.H. and J.H. Martin; Cereal Crops; MacMillan Publishing Co. Inc., New York, 1963, p. 154.

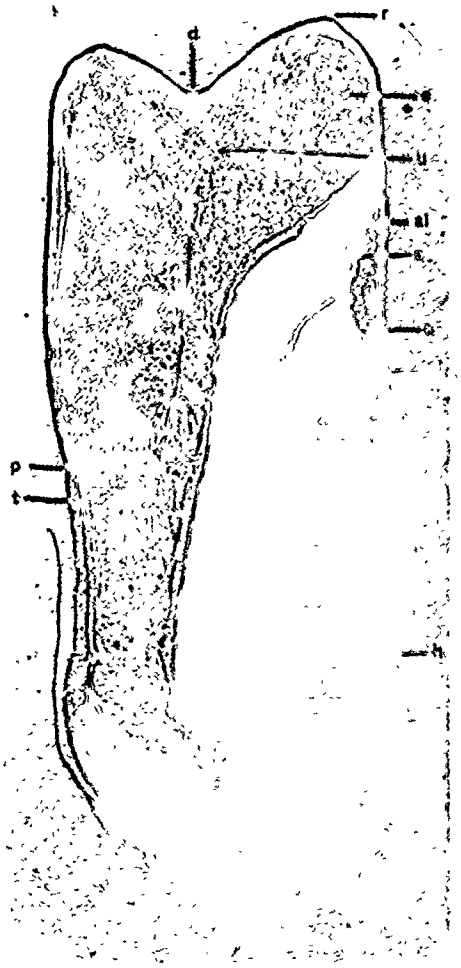


FIGURE 1 : Half of nearly mature dent corn kernel showing dent (d), remnant of style (r), endosperm (e), unfilled portion of immature endosperm (u), aleurone (al), scutellum (s), plumule (pl), hypocotyl and radicle (h), testa or true seed coat (t), and pericarp (p). The germ comprises the entire darker area including the scutellum (s), coleoptile (c), and rootcap (ro).

(Courtesy US Department of Agriculture)

or primary root enclosed in the coleorhiza and facing the base of the caryopsis; and the plumule, with four or five small foliage leaves, enclosed in the coleoptile and directed away from the base (47, 71).

Germination and Early Seedling Growth

Germination in seed plants can be defined as 'the sequential series of morphogenetic events that result in the transformation of an embryo into a seedling' (8). Germination is an early event in the reactivation of the embryo. The digestion of the endosperm reserves is initiated 2 or 3 days afterward. The physical events of germination include the imbibition of water; activation of the seed; growth of the embryo by cell division, cell elongation or both; emergence of the radical; and establishment of the seedling. However, the exact point at which germination concludes and early seedling growth commences is not well defined. Many of the biochemical events following imbibition of the seed have been at least partially defined (8, 80). These events will be outlined in the introduction and some questions still unanswered will be discussed.

(1) Reserve Protein and Carbohydrate

In cereals, the main reserve material in caryopses is carbohydrate and the principal carbohydrate reserve is starch. For maize, this means that 50-70% of the dry weight of the mature caryopsis is starch, whereas there may be 5% lipid and 1-4% sugar (80).

Starch is a polymer of glucose and is made up of both the linear chain amylose and the branched form amylopectin. It is hydrolyzed

by amylases. Alpha-amylase attacks internal parts of the starch molecule, liberating oligosaccharides while β-amylase cleaves maltose units from the terminal points of the polymer. Debranching enzyme hydrolyzes the branch points in amylopectin. β-amylase is the form present in the dry maize caryopsis. Within three days after imbibition α-amylase begins to be synthesized and by the time it reaches its highest activity it accounts for the majority of amylase activity (80).

Proteins which make up a substantial fraction of the dry weight of cereal caryopses (approximately 10%) have been divided into groups on the basis of solubility (80, 95). Water soluble albumins and salt soluble globulins are considered to be the metabolically active fractions. In maize, the storage fractions are a prolamine, zein (soluble in 70% alcohol) and glutelin (soluble in alkali). Some people have recently questioned the designation of glutelin as a storage protein, preferring to consider it a structural protein (Miflin, unpublished). Nevertheless, in this thesis storage proteins will refer to both zein and glutelin. The relative distribution and amino acid content of each fraction is shown in Table I.

The storage proteins zein and glutelin are of major interest because they are hydrolyzed to provide essential nutrients for early seedling growth. The hydrolysis products are transported via the scutellum to the seedling where they are used as the preferred nitrogen source for protein synthesis (3, 5). Table I makes clear the distinction of zein and glutelin in terms of amino acid composition, with the

TABLE 1

Amino acid composition of Osborne fractions from a normal maize variety (124)

	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	3.5
Percent of Grain Total Nitrogen	0.9	1.5	36.9	41.4

prolamine being rich in leucine, and low in lysine and other basic amino acids, whereas glutelin has a more balanced distribution of amino acids. Both zein (129) and glutelin (90) have been shown to be composed of a number of subunits which aggregate to form the parent fractions. Mutants, opaque-2 (83) and floury-2 (89), which have increased levels of lysine and tryptophan, are known. This is caused by a reduction in the relative amount of zein.

(2) Hydrolysis of Reserve Protein

Harvey (45) investigated the hydrolysis of the storage proteins of Zea mays. Within two days after imbibition the storage proteins begin to be hydrolyzed. By eight days the reserves are almost depleted. In the batch of seeds examined glutelin degradation was most rapid initially but later both zein and glutelin disappeared at similar rates. By examining batches of caryopses with different zein to glutelin ratios she showed that the most abundant type of protein is degraded most rapidly. Recently, Moreaux (86) reported a similar pattern for hydrolysis of endosperm proteins, although in that case hydrolysis was complete by about seven days.

Peptide hydrolases are the enzymes responsible for the hydrolysis of the storage proteins. These enzymes have not been as thoroughly studied as the starch degrading enzymes. Although many peptide hydrolase activities have been found during germination of seeds and early seedling growth (Table II) and some of the proteins responsible for these activities have been partially or wholly purified (Table III), many questions regarding the function and

specificity of the peptide hydrolases remain to be answered.

(3) Peptide Hydrolases

Peptide hydrolases are implicated in protein turnover in all metabolizing cells and in some cases in protein degradation outside cells. For example, in mammalian systems, experimentalists have determined the sequence by which a large number of peptide hydrolases co-operate to hydrolyze ingested proteins (35). The peptide hydrolases involved in this process have been studied, isolated and substrate specificities determined (11, 12), so that a fairly complete understanding of the process of mammalian protein digestion has been attained. A wide range of peptide hydrolase activities, eg., cathepsins, rennin, thrombin, lysozyme are known to be involved in protein turnover in mammalian tissues (7, 116).

Early work in plants elucidated the mechanism of commercially important peptide hydrolases: papain, bromelain and ficin (11, 12). During digestion of endosperm reserve proteins of cereals, most study as yet, has been concerned with describing and classifying activities which have been found in crude extracts (114). The common criteria for classification are substrate specificity, pH optimum and active site residue (134).

Acid peptide hydrolases, described to date (eg., pepsin, chymosin) require aspartyl groups at the active site, other enzymes (eg., trypsin, chymotrypsin) have essential serine groups, some (eg., papain, bromelain, cathepsin B) are sulphydryl sensitive proteins and

others (eg., carboxypeptidase A) are metal requiring enzymes. Regarding substrate specificity, hydrolases active on proteins (proteinases or proteases) are distinguished from those active on polypeptides (peptidases). Some proteins show both specificities. Enzymes which hydrolyze internal bonds of a polypeptide are called endopeptidases whereas those which only remove terminal residues are known as exopeptidases (134). These terms will be used to describe the activities studied in this thesis.

Barley (Hordeum vulgare) is one cereal in which the development of peptide hydrolases has been extensively studied. Early work, done for the brewing industry, showed that proteinase activity increased in the barley caryopsis during malting, ie., during germination (74). Further work showed that the main area of synthesis was the endosperm (99) and Varner (19, 55) proved that the aleurone layer of the endosperm was the site of synthesis of proteinase in addition to a number of other hydrolases. Other workers have followed the synthesis of peptidases in barley. Mikola's group reported eight peptidases in various parts of the germinating barley caryopsis (84). Those which increased most in endosperm during germination had carboxypeptidase specificity. Recently, Schroeder and Burger (115) reported that the development of one particular carboxypeptidase occurred in the aleurone layer.

A number of groups have shown increased endo- and exopeptidase activity when wheat (Triticum) is germinated (85, 102, 103, 111). Recently the localization of peptide hydrolase activity within the wheat caryopsis has been studied in the developing caryopsis (109,

69) as well as during germination (103, 69). Prentice et al. (103) reported increases in neutral and acidic peptidase activity (BAPAase and BAEEase) during germination. The increase occurred in embryo, aleurone and starchy endosperm but was greatest in the embryo. Kruger and Preston (69) reported a three fold increase in carboxypeptidase upon germination of wheat. Activity was concentrated in the aleurone layer and starchy endosperm.

In maize (Zea mays), Harvey (44) described a protein degrading enzyme present in extracts of endosperm of germinating maize. It had a pH optimum near 3.8, a temperature optimum for the assay at 46°C and was characterized as an endopeptidase from the ratio of total nitrogen to α-amino nitrogen of the products. The ability of the enzyme to degrade the natural substrates zein and glutelin was demonstrated. It was seen that the activity increased to a maximum eight days after imbibition and the level of activity was calculated to be sufficient to account for the observed rate of hydrolysis of the endosperm proteins zein and glutelin (45). Results with the RNA synthesis inhibitor 6-methylpurine and the protein synthesis inhibitor cycloheximide suggested de novo synthesis of the protein (45). More recently Feller et al. (29) have investigated endopeptidase (hydrolysis of casein) as well as exopeptidase activity. They found the highest activities in the endosperm and scutellum. Profiles of the pH dependence of the activities were obtained as well as the effects of active site regents. Endopeptidase and carboxypeptidase activity

showed substantial increases in endosperm and scutellum tissue during germination while aminopeptidase activity tended to decrease.

A summary of peptide hydrolase activities and some of their characteristics in crude extracts of germinating and resting plant seeds is shown in Table II. The large numbers of activities found and their varying characteristics have led to questions concerning their function. Regarding germination and early seedling growth the main question is 'Which peptide hydrolases are responsible for the hydrolysis of reserve proteins?' Harvey (46) suggests that in order to be judged capable of hydrolyzing reserve proteins a peptide hydrolase must have endopeptidase capability, be active on the native reserve proteins and be sufficiently active to account for the observed time scale of hydrolysis.

In view of the large number of peptide hydrolase specificities discovered in the endosperm tissue of dormant, quiescent and germinated seeds, it appears that the hydrolysis of reserve proteins could involve a number of enzymes. It might be expected that after imbibition the initial steps of hydrolysis of the insoluble reserve proteins would be accomplished by one or more endoproteases, capable of acting on the native proteins. However, once solubilized, it might be expected that a variety of endo- and exopeptidases could function to hydrolyze the intermediate products, producing amino acids ultimately. It is unclear how the reserve proteins are solubilized. It is conceivable that the hydrolysis of a few specific bonds could solubilize the protein

TABLE II

Development of Peptide Hydrolase Activities in Germinating Seeds

	Plant	Specificity and Assay	pH	Ref.
(1)	Quiescent Wheat	Protease - change in nitrosamine staining of haemoglobin	5.0	63
		Protease - tyrosine release from haemoglobin	3.8	81
		Endopeptidase - peptide release from haemoglobin	3.8	38
		Exopeptidase - amino acid release from haemoglobin	3.8	38
(2)	Germinated Wheat	Dipeptidase - hydrolysis of Leu-gly	7.5	
		Protease - release of amino and carboxyl groups from edestin	4.1	85
(3)	Quiescent and Germinated Wheat and Barley	Endopeptidase - hydrolysis of BAPA and BAEE	8.6	13, 103
(4)	Quiescent Barley	Endopeptidases - hydrolysis of BAPA	8.6	
		- change in gelatin viscosity	4.0 5.0	94

TABLE II (Continued)

	Plant	Specificity and Assay	pH	Ref.
(5)	Germinated Barley	Protease - release of α-amino N from gliadin	4.8	55
		Endopeptidases - change in gelation viscosity	3.8, 7.0 5.0, 6.5	121
		Endopeptidase - release of Tyr and Trp from haemoglobin	3.8	
		- hydrolysis of BAPA	8.6	10
		Carboxypeptidases - hydrolysis of CBZ-Phe-Ala	4.8	
		CBZ-Phe-Phe	5.2	84
		CBZ-Pro-Tyr	5.7	
(6)	Quiescent Maize	Endopeptidase - hydrolysis of BAPA	7.5	82
(7)	Quiescent and Germinated Maize	Endoprotease - α-amino N from casein	3.8, 5.4	
		Carboxypeptidase - CBZ-Phe-Ala	5.0	29
		Aminopeptidase - Leu-p-nitroaniline	7.0	
(8)	Germinated Maize	Endopeptidase - release of Tyr and Trp from haemoglobin	3.8	44, 45
(9)	Germinated Oats	Protease - release of Tyr and Trp from haemoglobin	8.0	122

TABLE II (Continued)

	Plant	Specificity and Assay	pH	Ref.
(10)	Quiescent and Germinated Rye	Endopeptidase - hydrolysis of casein yellow and haemoglobin	4.3, 6.7	
		Carboxypeptidase - CBZ-Arg-p-nitroaniline	6.8	91
		Aminopeptidase - Leu-p-nitroaniline	6.5	
(11)	Quiescent and Germinated Peanuts	Protease - hydrolysis of peanut storage proteins	5.0	78
(12)	Quiescent and Germinated Castor Bean	Protease - hydrolysis of haemoglobin	3.5 - 4.0	
		Endopeptidase - hydrolysis of BANA	6 - 8	
		Carboxypeptidase - CBZ-Phe-Ala and CBZ-Tyr-Leu	5.0 - 5.5	128
		Aminopeptidase - Leu-p-nitroaniline	7.0	
		Pro-p-nitroaniline	7.5	
(13)	Quiescent and Germinated Field Beans	Endoprotease - hydrolysis of azocasein	5.0	
		Endopeptidase - hydrolysis of BAPA	7.5	72

molecule or it is possible that small pieces are hydrolyzed from an insoluble core protein. Thus, in calculating whether an individual peptide hydrolase would be able to hydrolyze the reserve proteins as fast as the observed rates of disappearance, the calculation could be complicated if more than one peptide hydrolase were involved in the initial solubilization.

In order to further clarify the role of peptide hydrolases in storage protein hydrolysis, researchers have lately attempted to separate and purify some of the activities of crude extracts. One could test the activity of a purified protein to determine whether it is still capable of hydrolyzing the native reserve protein. It would then be possible to predict which peptide hydrolase or combination of such enzymes could function with the required speed in the hydrolysis of reserve proteins.

(4) Purification of Peptide Hydrolases

A number of workers have reported the successful partial purification of peptide hydrolase activities from germinating caryopses in cereals. Some of these reports are summarized in Table III. Both endopeptidase and exopeptidase types of activity have been isolated from quiescent and germinating barley and wheat.

For maize workers in Japan and France have recently tried to purify peptide hydrolase activities from quiescent and germinating caryopses. Moreaux (86) suggested a peptide hydrolase of molecular weight 40,000 daltons, capable of degrading haemoglobin, was present in

TABLE III

Purification of Peptide Hydrolases from Germinating Seeds

	Plant	Specificity and Assay	pH	M. W.	Ref.
(1)	Quiescent Wheat	Protease - Tyr release from haemoglobin	3.8		136
(2).	Germinated Wheat	Exopeptidases - α-amino N from haemoglobin	4.0	55,000 &	69, 108
		Carboxypeptidase - CBZ-Phe-Ala	5.7	61,000	
		Endopeptidase - hydrolysis of BAPA	8.6	59,000	68
		Endopeptidases - hydrolysis of BAPA	8.6		
		and BAEE	7.0		104, 105
(3)	Germinated Barley	Proteases - nitrogen release from haemoglobin	3.8		
		- Tyr release from casein	6.0		
		- α-amino N from gliadin	4.8		14, 15
		Endopeptidases - BAPA	8.6		
		BAEE	7.0		
		Aminopeptidases - Phe-β-napthylamide	7.2	one -	
		- Dipeptides	5.8 - 6.5	65,000	67
		Carboxypeptidase - CBZ-Phe-Ala	5.2	90,000	132

TABLE III (Continued)

	Plant	Specificity and Assay	pH	M. W.	Ref.
(4)	Germinated Maize	Endopeptidase - hydrolysis of haemoglobin	3.0	21,000	1, 2
		Endopeptidase - hydrolysis of haemoglobin		12,000 36,000 40,000	86
(5)	Germinated Sorghum	Endopeptidase - Tyr release from BSA	3.8	80,000	34, 131
(6)	Quiescent Peas	Endopeptidase - BAPA	8.3		127
		Aminopeptidase - Leu-p-nitroaniline	7.2		
(7)	Germinated Cotton	Endopeptidase - BAEE	6.6	85,000	53
(8)	Germinated Mung Bean	Endopeptidase - hydrolysis of Vicilin	5.1	23,000	141

the quiescent caryopsis. From endosperm five days post imbibition she found in addition acidic, sulphydryl sensitive proteins of 12,000 and 36,000 daltons, the former aggregating in the absence of DTT to form a fraction of 24,000 daltons. The partially purified proteins were tested to some extent for substrate specificity and were found to have measurable but low carboxypeptidase activity. She confirmed that only those synthesized after imbibition were able to degrade zein and glutelin.

Abe et al. (1, 2) purified one fraction of peptide hydrolase from germinated maize endosperm to apparent homogeneity and tested it extensively to determine substrate specificity. They found an acidic, sulphydryl sensitive enzyme of molecular weight 21,000 daltons, capable of degrading haemoglobin. These workers reported a very general specificity for their active protein, resembling pepsin rather than papain (see Table IV). The enzyme hydrolyzed the endopeptidase substrates show in Table IV, showed endopeptidase specificity with insulin B chain and hydrolyzed carboxypeptidase substrates (CBZ-dipeptides).

Thus, purification of the peptide hydrolase enzymes in maize endosperm has confirmed earlier work describing acidic endopeptidase activity in crude extracts (29, 44). Some partially purified enzymes have been shown to possess endopeptidase specificity (2, 86) and to be capable of hydrolyzing the natural storage proteins, zein and glutelin (86). However, conflicting data on molecular weights and the reporting of some carboxypeptidase activity in the isolated proteins left it unclear as to the relationship of endopeptidase and carboxypeptidase and to the relative importance of each in storage protein hydrolysis.

TABLE IV

Substrate Specificity of Purified Maize

Endopeptidase (2)

Maize peptide hydrolase P-IA is a fraction of protein from maize endosperm, purified to apparent homogeneity.

SUBSTRATE	RELATIVE ACTIVITY		
	Maize Peptide Hydrolase P-IA	Papain	Pepsin
Hemoglobin	100	100	100
CBZ-Tyr-ONP	22	100	0
BANA	0	100	0

From the observation of similar patterns of induction of these enzymes during germination, Feller et al. (29) had suggested that both these activities were involved in storage protein hydrolysis.

Endopeptidase and exopeptidase enzymes active on endosperm storage proteins and intermediate hydrolysis products might function co-operatively to produce amino acids from the proteins. The reports by Moreaux (86) and Abe et al. (1, 2) of carboxypeptidase activity (as defined by hydrolysis of CBZ-dipeptides) in their partially or fully purified endopeptidase preparations (as measured by hydrolysis of haemoglobin) could be explained by a peptide hydrolase with very wide substrate specificity or by contaminating carboxypeptidases in the purified preparations. In wheat, Preston (108), isolated enzymes, which were active on haemoglobin and which he characterized as carboxypeptidases. An important question then was whether the maize peptide hydrolase(s) degrading haemoglobin also possessed carboxypeptidase activity (as defined above). The best method for answering this question was by following the co-purification of the two activities chromatographically.

(5) Control of Reserve Protein Hydrolysis and Peptide Hydrolase Induction

The interaction of embryo and endosperm in the cereal caryopsis is important in the germination process and during early seedling growth. Early work showed that maize embryos could be cultured separately from the endosperm if sugars were added (24). Later, in maize embryo culture, it was found that in the absence of an exogenous supply of amino acids protein synthesis was much reduced for the first two

to three days of culture (93). Excised endosperms, a protein hydrolyzate released from the endosperm or a synthetic mixture of amino acids resembling the hydrolyzate in amino acid distribution could restore protein synthesis. It appears that amino acids supplied to the embryo by the endosperm inhibit amino acid synthesis in the embryo. It requires two to three days before the embryo can synthesize sufficient amounts of amino acids for its own needs (92).

The hydrolysis of the endosperm reserves may depend on the presence of the embryo. In many cultivars of barley, endosperm proteins and starch are hydrolyzed very slowly when the embryo is removed (97, 98, 99, 100). However, in maize endosperm, in most cases, reserves are hydrolyzed in the absence of the embryo (36, 45). Yet some workers (119, 138) have reported maize cultivars in which the endosperm reserves are not digested if the embryo is removed. It appears that some factor in the embryo could promote the hydrolysis of endosperm reserves in some cereals.

The major 'endosperm mobilizing factor' is now thought to be gibberellic acid (101). Gibberellic acid has at least two distinct sites of action. A great deal of work has shown in detail the effects GA has on the aleurone layer of the barley endosperm (See Table IV). However, in the embryo, GA has distinct and earlier effects. The most obvious embryo effect of GA is in breaking dormancy of many seeds (18). Naylor and Simpson (88) found that GA overcomes a metabolic block in embryos of dormant wild oats. Simpson (117) later reported

that culture of embryos from dormant wild oats required GA in the medium in addition to sugars and amino acids. Non-dormant varieties did not require GA. Drennan and Berrie (23) and Chen and Chang (16) compared the time course of α-amylase development with that of germination (defined by radical emergence) in barley and wild oats. They found that germination occurred before any increase in α-amylase activity. It appears that the first effect of GA is in the embryo and that germination is not dependent upon hydrolase development in the endosperm (16).

Other plant hormones, including abscisic acid, the cytokinins, auxins and ethylene may play a role in germination (18). Khan (65) suggested a model whereby the levels of a combination of germination promoters (gibberellins and cytokinins) and germination inhibitors determine whether germination could occur. Others (18) have argued that gibberellins and abscisic acid are the most important in this regard. Many reports have claimed that endogenous ABA levels in seeds decrease during dormancy breaking (135). Normally ABA must be applied continuously to cause dormancy (137). The biochemical mechanism by which ABA exerts its effect on the embryo is not understood. In the endosperm, some workers have shown that ABA can inhibit the development of hydrolases which are known to be necessary for early seedling growth (20, 50, 54, 137). It has been suggested that this can occur by inhibition of the translation of hydrolase mRNA (50, 54). The current theory is that ABA, acting through an RNA intermediate, interferes with translation of preformed hydrolase mRNA in the cereal endosperm.

TABLE V

Effects of GA_3 and ABA on Barley Aleurone Tissue

Time (hr)	Observation	Ref.	ABA Inhibition	Ref.
0	GA_3 added to isolated imbibed aleurones			
0.5	Changes in rate of labelling of soluble nucleotides	22		
1	Change in proteins labelled by radioactive amino acids	31	±	140
2	Increased activity of lecithin synthesis enzymes	57	+	57
3	Increase in rate of labelling of poly(A)-rich mRNA like fraction	49, 56		
	Increase in translatable α-amylase mRNA	48		
4	Stimulation of ^{14}C-choline incorporation into ER	26	+	26
	Increase in total number of ribosomes; increase in number of ribosomes as polyribosomes; stimulation of ribosome aggregation	27, 28	+	28
	Increased incorporation of ^{32}P into phospholipids	66		
	Decreased pentosan synthesis	58		

TABLE V (Continued)

Time (hr)	Observation	Ref.	ABA Inhibition	Ref.
	Proliferation of rough ER	59, 60	+	26
	β, 1-3 glucanase synthesis and release	61		
8	Incorporation of ^{3}H-uridine into a polydisperse RNA fraction	123		
	Protease and α-amylase synthesis	19, 55	+	20,55
	Synthesis of RNAase	19	+	21
12	Enhanced DNA synthesis	125		

Gibberellins can often reverse the physiological effects of ABA and vice versa (6, 20, 64, 118). If gibberellic acid, which can be isolated from barley embryos (77, 112) is added to incubating de-embryonated barley caryopses storage reserves disappear (97). It has been found that the addition of GA_3 induces the production of a number of hydrolase enzymes, including α-amylase, a protease and ribonuclease (62). Varner (21, 55) has proven that the enzymes are produced in the aleurone layer and that the appearance of these enzymes represents de novo synthesis of protein. The current model suggests that GA_3 is produced in the embryo and after imbibition it moves to the aleurone layer where new synthesis of protein is induced. The appearance of enzyme activity is of the order of days after imbibition and is inhibited by ABA (20). Recently, workers in Japan (139) have suggested that the epithelial layer of the scutellum might also produce hydrolases and secrete them into the starchy endosperm of cereals in the early stages of growth. They used histochemical techniques to show amylase synthesis in scutella epithelium of barley, rye, oats, wheat and maize and proteinase and RNAase in barley.

Although the studies on barley half seeds and aleurone layers have led to a general model for the induction of hydrolase activity in endosperm tissue of cereals during germination and early seedling growth, a number of inconsistencies have been reported. For example, a comparative study on twenty three barley varieties, one wheat and one oats variety (100) showed a similar qualitative effect on GA on some para-

meters of endosperm hydrolysis but significant quantitative differences. Naylor (87) described a variety of oats in which aleurone layers synthesized α-amylase if incubated in a GA solution or with an amino acid mixture. MacGregor found a slow induction of α-amylase in de-embryonated barley caryopses without GA (75). He further showed that GA increased α-amylase activity during germination of some barley varieties but not others (76).

In wheat, early studies showed that half seeds responded to GA with increased hydrolysis of endosperm reserves and increased production of hydrolases (113). It was observed that these enzymes were produced in the aleurone layer. Recent work (110) has attempted to clarify the role of ABA and GA_3 in the induction of hydrolytic enzymes in wheat. In the intact seed neither GA_3 nor ABA had any significant effect on development of an exoprotease activity with carboxypeptidase specificity. However, GA_3 significantly hastened the appearance and ABA significantly decreased the production of endoprotease activity. In de-embryonated seeds, GA_3 significantly increased endoprotease production by five days after soaking and caused a less dramatic increase in exoprotease.

In maize, Harvey (43) showed that de-embryonated caryopses could produce hydrolases in a suitable buffer. GA_3 caused only a small increase in production of protease and α-amylase activity. However, ABA significantly inhibited production of these enzymes and with suitable concentrations of GA_3 and ABA this inhibition could be partially reversed. Thus, it appears that in maize, endogenous levels of GA_3

may be sufficient to induce hydrolase production. This was supported by the fact that de-embryonated seeds of dwarf mutants, which lacked the ability to synthesize GA_3 showed significant increases in proteinase and α-amylase activity when incubated in 10 μM GA_3. However, in maize also, some inconsistencies have been reported. For example, Ingle and Hageman (138) saw little starch or protein hydrolysis in de-embryonated caryopses, but GA could initiate the breakdown. A recent report from Hageman's group suggested that GA induced endopeptidase but not carboxypeptidase activity (119).

Feller et al. (29) had suggested that both endopeptidase and carboxypeptidase activities were involved in reserve protein hydrolysis. If both types of activity, which increase during germination are involved in storage protein breakdown it is likely that both would show similar metabolic control.

In summary, the main points addressed in this thesis can be stated as:

1) a preliminary investigation of endopeptidase and exopeptidase activities in maize endosperm in order to determine the best parameters to use in the assay.
2) development of a method for partial purification of the endopeptidase(s) active on haemoglobin, in order to determine the number of such proteins and to allow for future studies on substrate specificity, including the ability to hydrolyze storage proteins.
3) a comparison of the effects of plant hormones, ABA and GA_3, and metabolic inhibitors, cordycepin (RNA synthesis) and cycloheximide (protein synthesis) on the induction of acid endopeptidase and

carboxypeptidase activity in de-embryonated maize caryopses, in order to determine whether the same mechanism of induction is involved.

MATERIALS AND METHODS

Plant Material

The maize hybrid W64A x W182E was used in this study. Caryopses were purchased from Wisconsin Seed Foundation, Madison, Wisconsin. Barley (Var. Bonanza, 1979) was obtained from Dr. K. Preston, Canadian Grain Research Commission, Winnipeg, Manitoba.

Preparation of Caryopses

Before sowing, caryopses were washed free of fungicide dust by three to four rinses with distilled water. For experiments requiring sterile conditions, caryopses were swirled in 1% sodium hypochlorite for ten minutes. They were then rinsed several times with sterile distilled water, washed five to six times with 0.01 M HCl and then rinsed thoroughly with sterile distilled water.

Germination of Maize Caryopses and Growth of Seedlings

After preparation of the caryopses, as outlined above, they were planted in a moist sand-vermiculite (1:1) mixture. The caryopses were germinated and seedlings grown at 28°C with a cycle of 12 hours light and 12 hours dark. At the required times, samples of at least 50 seedlings were harvested. The endosperms were separated, frozen quickly in liquid nitrogen and stored at -20°C.

Incubation of Maize Endosperms

After surface sterilization of the caryopses, as outlined above, the embryos and scutella were cut out with a razor blade under sterile conditions. In 50 ml Erlenmeyer flasks, samples of ten endosperms were incubated in 10 ml of 1 mM sodium acetate buffer, pH 5.0, containing 1 mM $CaCl_2$ and 250 ug/ml streptomycin. This solution was added through a millipore filter. The samples were shaken in darkness at 28±1°C for the required time. Aliquots of incubation media were plated on Bacto nutrient agar to test for contamination. Infected samples were not used. The endosperms were frozen in liquid nitrogen and stored at -20°C. The incubation media were assayed immediately for peptide hydrolase activities. Results from endosperms and media were summed to give total activities.

Incubation of Barley Endosperms

The embryo containing halves of barley caryopses were removed. The extreme tips of the remaining endosperm halves were also removed. The endosperm pieces were swirled for 15 minutes in 1% sodium hypochlorite, rinsed in sterile distilled water, washed for 10 minutes in 0.01 M HCl and then rinsed several times in sterile distilled water.

In 25 ml Erlenmeyer flasks, 20 endosperm pieces were incubated in 1 ml of 1 mM sodium acetate buffer, pH 5.0, containing 100 mM $CaCl_2$ and 250 ug/ml streptomycin. This solution was added through a millipore filter. The flasks were shaken in darkness at 28±1°C for three days. Then an additional 3 ml of incubation medium were added

and shaking was continued for one more day. Aliquots of incubation media were plated on nutrient agar to test for contamination. Endosperm pieces were frozen in liquid nitrogen and stored at -20°C. Media were assayed immediately for α-amylase activity, according to the method described by Varner (19).

Extraction and Assay of Peptide Hydrolase Activities

(a) Acid Endopeptidase

Frozen endosperms were homogenized either with a mortar and pestle, on ice, or at 4°C in an Omnimix. The buffer was 0.2 M sodium acetate, pH 3.8, containing 5 mM β-mercaptoethanol. Two ml of buffer was used for each gram of tissue. The homogenate was filtered through Miracloth and centrifuged at 20,000 x g for 15 minutes at 0°C. The supernatant was assayed for activity. The standard assay contained 50 mg haemoglobin, 1.0 ml of 0.05 M sodium acetate buffer, pH 3.8, containing 2.5 mM EDTA; 0.3 ml of extraction buffer and 0.2 ml of extract. Before addition of the enzyme the reaction mixture was equilibrated to the assay temperature, 40°C. The reaction was started by adding the enzyme and stopped after 30 minutes by adding 2.5 ml of cold 5% TCA. The blank, contained no enzyme and was incubated along with the test mixture. For the blank, TCA was added immediately after addition of the enzyme. After leaving the samples at 4°C for 15 minutes, the precipitate was pelleted by spinning in a clinical centrifuge at top speed for 10 minutes. Activity was measured as the increase in absorbance at 280 nm of the supernatant. This was calibrated against absorbance of tryptophan at 280 nm (44).

(b) Carboxypeptidase and Aminopeptidase

Frozen endosperms were homogenized, as above, in 0.05 M sodium phosphate buffer, pH 6.7. The supernatant from a centrifugation at 20,000 x g for 15 minutes could be used directly for the aminopeptidase assay.

For the carboxypeptidase assay, the supernatant was dialyzed overnight against several changes of 0.1 M sodium acetate, pH 5.2. The substrate was prepared by dissolving 5 mg of N-carbobenzoxy-L-phenylalanyl-L-alanine in 0.1 ml of DMSO and diluting to 5.0 ml with 0.1 M sodium acetate, pH 5.2. The reaction was started by adding 50 µl of extract to 0.5 ml of substrate at 37°C. After 60 minutes, the reaction was stopped by adding 1.0 ml of 0.83% w/v ninhydrin in methyl cellosolve, capping with a marble and transferring to a boiling water bath for 15 minutes. For blanks, ninhydrin was added before the enzyme and the sample was then incubated in boiling water. The samples were cooled in running water and diluted with 60% ethanol to 5.0 ml. Activity was measured as the increase in absorbance at 570 nm. Alanine was used for the standard curve (29).

For aminopeptidase, the substrate was prepared by dissolving 5 mg of L-leucine-p-nitroanilide in 0.2 ml of DMSO and diluting to 10.0 ml with 0.05 M sodium phosphate, pH 6.7. The reaction was started by adding 50 µl of extract to 1.0 ml of substrate at 37°C. The reaction was stopped after 60 minutes by adding 1.0 ml of 1 N acetic acid. For blank determination, acetic acid was added before the enzyme. Activity was recorded as the increase in absorbance at 410 nm and

calibrated against a standard curve of p-nitroaniline (29).

In addition, measurements were made of endopeptidase activity at pH 6.0, using azocaseinase substrate (111) and of activity with haemoglobin at pH 4.0 by an automated method (106). This latter assay measured carboxypeptidase activity in wheat following germination (108).

Partial Purification of Acid Endopeptidase

This procedure was performed on an extract prepared from endosperms harvested six days after sowing (see extraction procedure). All purification steps were carried at 4°C.

(a) Phenylalanine Substituted Sepharose

The extract was dialyzed against 0.025 M sodium acetate pH 4.0 and 20 ml was then loaded onto a column of phenylalanine substituted sepharose (0.8 x 10 cm) prepared according to Preston (107) and equilibrated with the same buffer. The activity was eluted with a convex gradient 0.05 - 0.25 M sodium acetate, pH 4.0.

(b) DEAE Cellulose

To the extract, $(NH_4)_2SO_4$ was added slowly, with constant stirring until 60% saturation (0.42 g/ml) was reached. After incubation on ice for one hour, the sample was centrifuged at 20,000 x g for 15 minutes. The pellet was taken up in one tenth of the original volume, with 0.2 M sodium acetate, pH 3.8 containing 5 mM β-mercaptoethanol and loaded onto a Sephadex G-100 column (1.8 x 80 cm), equilibrated with the same buffer. The most active fractions, as judged by assay for acidic endopeptidase were pooled and dialyzed overnight against three changes of 0.025 M Tris buffer, pH 8.1, 5 mM

β-mercaptoethanol. They were then loaded onto a DEAE cellulose column (1.5 - 20 cm), equilibrated with the same buffer. The column was washed with 50 ml of 0.025 M Tris buffer, pH 8.1, 5 mM β-mercaptoethanol and the protein then eluted with a linear gradient of 0.0 - 0.4 M NaCl in the column buffer. The most active fractions were pooled and dialyzed against the original extraction buffer. Protein concentrations were determined by the Folin-Lowry method (73) using BSA as standard.

(c) CM-Cellulose

The extract was initially made 60% in $(NH_4)_2SO_4$ (0.42 g/ml) as in the previously outlined purification. After centriguation at 20,000 x g for 15 minutes, the pellet was reconstituted in one tenth of the original volume of extraction buffer and loaded onto a column of Sephadex G-50 (3 x 40 cm), equilibrated with the same buffer. The most active acidic endopeptidase fractions were pooled and dialyzed overnight against three changes of 0.010 M sodium acetate, pH 3.8, 5 mM β-mercaptoethanol. The dialyzed protein solution was loaded onto a CM-cellulose column (1.5 x 15 cm), equilibrated with the same buffer. The column was washed with 50 ml of 0.010 M sodium acetate, pH 3.8, 5 mM β-mercaptoethanol and the bound protein then eluted with a linear gradient of 0.0 - 0.4 M NaCl dissolved in the column buffer. The most active fractions were pooled. Protein was assayed using the Folin-Lowry method (73) with BSA as standard.

(d) Estimation of Molecular Weight by Sephadex Chromatography

The molecular weight of the acid endopeptidase was estimated

in the pelleted protein which was precipitated by 60% (0.42 g/ml) $(NH_4)_2SO_4$. The reconstituted pellets were loaded onto a (1.8 x 80 cm) column of Sephadex G-100 which was equilibrated with either 0.025 M Tris buffer, pH 8.1 or 0.2 M sodium acetate - 5 mM β-mercaptoethanol, pH 3.8. The molecular weight estimation was also performed with the most pure fraction of acid endopeptidase, peak 'A' from a CM-cellulose column (see Figure 12). In this case an aliquot was loaded onto a (0.9 x 58 cm) column of Sephadex G-200 which had been equilibrated with 0.2 M sodium acetate - 5 mM β-mercaptoethanol, pH 3.8.

(e) SDS Disc Gel Electrophoresis

The fractions of partially purified acid endopeptidase were checked for purity by SDS disc gel electrophoresis. An aliquot of the sample, containing 10 µg of protein was dialyzed against distilled water and then concentrated by lyophilization to 50 µl. An aliquot of the crude extract, containing 25 µg of protein was also used. The samples were made 0.1% in SDS and placed in boiling water for 5 minutes. The separating gel contained 15% acrylamide, pH 8.9; the stacking gel 2.5% acrylamide, pH 6.7 (32). The tank buffer was 0.005 M Tris-glycine, pH 8.3. The electrophoresis was carried out at 4°C; one mamp/tube for thirty minutes followed by two mamp/tube for two hours. Protein was stained with Coomassie Blue R stain.

RESULTS

I. Characterization of Peptide Hydrolase Activities

A. Extraction Procedure

Several buffers of different pH were used for extraction of peptide hydrolase enzymes. An acid pH was found to be better than a neutral pH for the acid endopeptidase, as seen in Table VI. For carboxypeptidase and aminopeptidase there did not seem to be a significant difference between the various extraction conditions. Since there had been suggestions in the literature that carboxypeptidase could hydrolyze terminal residues of the endopeptidase substrate, haemoglobin, and it was known that autolysis by a proteinase would give activity in the carboxypeptidase reaction it was decided to extract the acid endopeptidase at pH 3.8 and the peptidases at pH 6.7, thus minimizing interference.

B. Stability

Enzyme extracts of endosperm tissue were tested for peptide hydrolase activities and then stored before reassaying. The results, shown in Table VII, were used to minimize loss of activity in storage and to predict changes in activity during prolonged experiments. The acid endopeptidase is stable for a number of hours on ice, is less stable when stored at room temperature and loses significant activity when frozen. Freezing of carboxypeptidase and prolonged storage of

TABLE VI

Extraction of Peptide Hydrolases

Endosperms were extracted with the buffers shown and the enzyme activities then determined under standard condition as described in Materials and Methods.

Extraction Buffer	Enzyme Activity	
	Acid Endopeptidase µg Trp equiv/min/g tissue	
0.2 M Na acetate, pH 3.8 5 mM β-mercaptoethanol	(1) 15.1	(2) 27.5
0.2 M Na phosphate, pH 6.7 5 mM β-mercaptoethanol	9.5	12.0
	Carboxypeptidase µg Ala/min/g tissue	
0.2 M Na acetate, pH 3.8 5 mM β-mercaptoethanol	27	
0.1 M Na acetate, pH 5.2	25	
0.05 M Na phosphate, pH 6.7	29	
	Aminopeptidase µg p-nitroaniline/min/g tissue	
0.05 M Na acetate, pH 4.4	8.4	
0.05 M Na phosphate, pH 6.7	6.8	

TABLE VII

Stability of Peptide Hydrolases

An enzyme extract was stored under the conditions described for the time indicated. At the end of that period the extract was assayed in the routine manner. Units are standard.

Acidic Endopeptidase: µg Trp equivalents/min/g fresh weight
Carboxypeptidase: µg Ala released/min/g fresh weight
Aninopeptidase: µg p-nitroaniline released/min/g fresh weight

Sample Time (hr)	Room Temperature	On Ice	Frozen (-20°C)
		Acidic Endopeptidase	
0		41.3±0.8 (100)	
3	38.1±1.2 (93)	41.9±2.9 (102)	
6	37.5±1.3 (91)	43.5±2.1 (106)	
9	34.3±1.1 (83)	37.5±1.3 (91)	
24	27.3±0.4 (66)		28.2±1.7 (69)
96	22.3±3.7 (54)		
168	13.0±2.7 (32)		30.1±0.5 (73)
		Carboxypeptidase	
0		43.4±3.6	
6	41.3±1.5 (95)	35.7±3.0 (82)	23.7±2.6 (54)
24	42.3±0.7 (97)	39.2±1.7 (90)	33.7±2.1 (78)
		Aminopeptidase	
0		9.9±0.3 (100)	
6	8.8±0.1 (89)	9.3±0.0 (94)	9.3±0.6 (94)
24	12.0±0.7 (121)	9.6±0.5 (97)	9.6±0.2 (97)
0			9.8±0.1 (100)
5 months			5.4±0.6 (55)

Mean ± Standard Deviation

Figures in brackets represent percentages of control.

aminopeptidase at -20°C cause significant loss of activity but the peptidases are stable on ice up to 24 hours. Routinely, enzyme extracts were kept on ice and assayed within two hours of extraction.

C. Products of Acid Endopeptidase Activity

A number of proteins were tested as substrates for protease at pH 3.8 (Table VIII). Haemoglobin showed the highest activity under the testing conditions, when activity was measured by the increase in TCA soluble material absorbing at 280 nm. Acid pre-treatment of the substrates increased activity. All substrates were hydrolyzed predominantly by endopeptidase activity at this pH, as indicated by the ratio of total nitrogen to α-amino nitrogen for the products. For an exopeptidase the ratio would be between one and two (23). The results in Table VIII show ratios of eight or greater.

D. Products of the Carboxypeptidase Assay

It can be seen from Figure 2 (A) that when a crude extract is used to measure carboxypeptidase activity an increase is seen in ninhydrin reactive material during the assay time, even when no carboxypeptidase substrate is present. Thus, it appears that autolysis may occur at this assay pH. The autolytic activity was confirmed with a number of extracts. Dialysis of the extract results in elimination of the measured autolysis. Dialysis could be removing small autolytic substrates (less than 8000 daltons) or necessary co-factors for autolytic enzymes. The product of the carboxypeptidase

TABLE VIII

Characterization of Products from Acid Endopeptidase Activity

Assays were conducted according to the standard procedure except that 10 mg BSA, 25 mg gliadin, or 25 mg haemoglobin were used in each assay. The total nitrogen in the TCA soluble products was measured with Nessler's reagent following Kjeldahl digestion and calibrated against NH_4Cl. The α-amino nitrogen in the TCA soluble products was measured with ninhydrin using alanine as standard. Units of enzyme activity are standard.

A - Protein incubated in 0.1 N HCl, 1 hr at room temperature

B - Protein incubated in 1 N HCl, 1 hr at 50°C

Substrate	Enzyme Activity µg Trp equivalents released/min/g fresh weight	Total N µg N/assay	α-amino N µg N/assay	Total N / α-amino N
BSA				
Native	8.1	125	6.0	21
A	6.0	160	12.5	13
B	11.7	370	21.0	18
Gliadin				
Native	6.3	255	6.5	39
A	7.7	215	5.5	39
B	13.0	390	12.0	33
Haemoglobin				
Native	11.3	55	6.5	9
A	18.1	135	11.5	12
B	34.5	400	49.5	8

Figure 2

Characteristics Of Carboxypeptidase Reaction

(A) Dialysis of Extract

Endosperms, 6 days post sowing, were extracted and assayed for carboxypeptidase activity with increasing amounts of substrate. A second sample of endosperms was extracted and dialyzed against assay buffer before assaying. The assay conditions were standard (see Materials and Methods). Activity was measured as the increase in ninhydrin reactive material during a one hour incubation and is expressed as the equivalent amount of Ala released/min/g fresh weight.

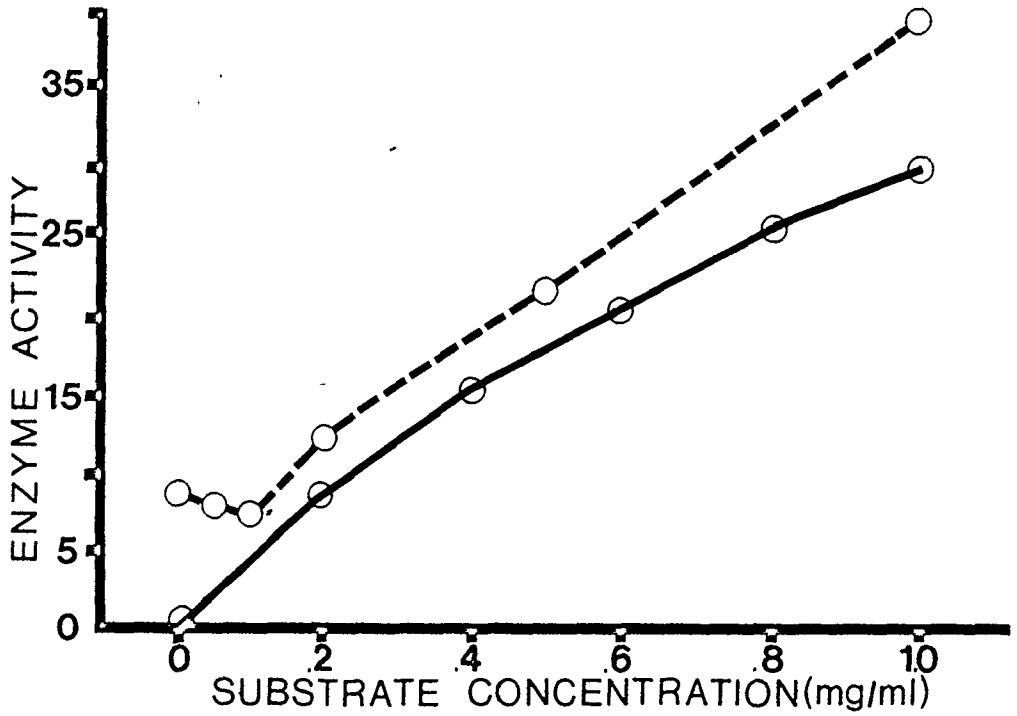
Before dialysis (○- - -○)

After dialysis (○———○)

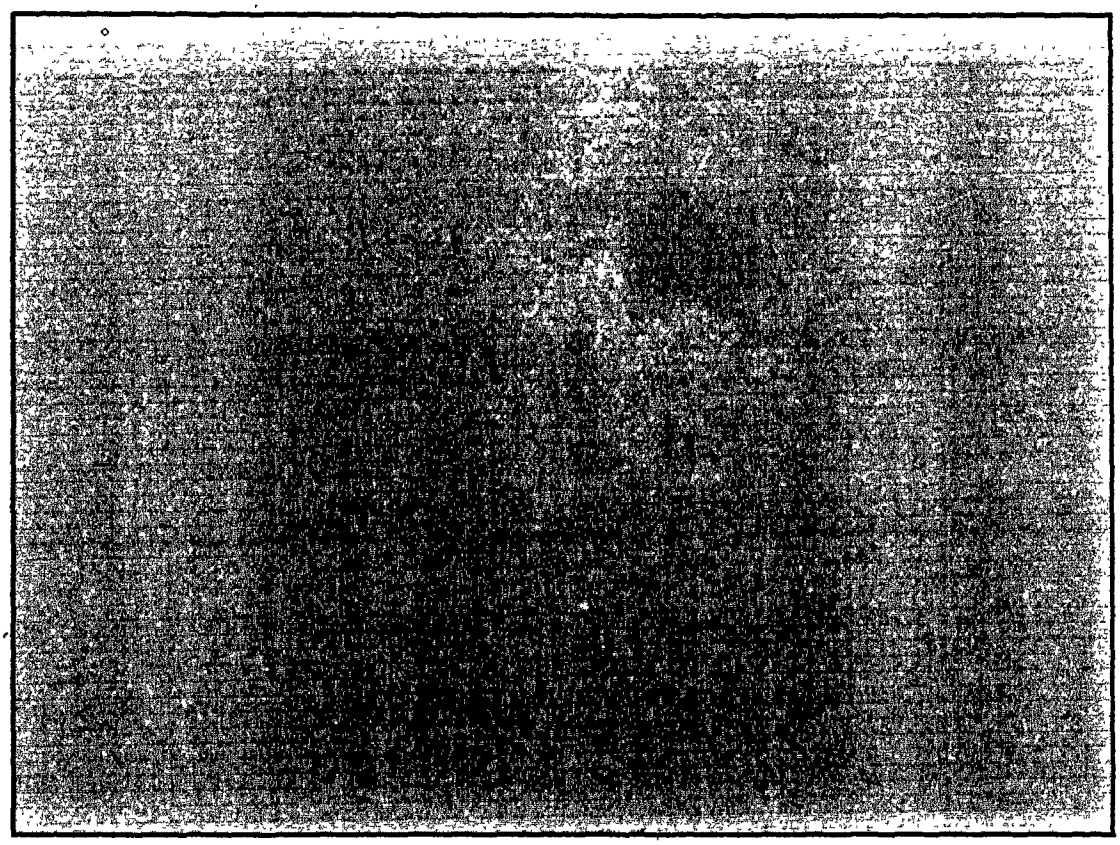
(B) Characterization of Products of Carboxypeptidase

Carboxypeptidase was assayed under standard conditions. After 1 or 2 hours the reaction was terminated by boiling. An aliquot of the assay mixture was spotted on Whatman Chromatography paper and the chromatogram run in butanol-acetic acid-water (120/30/50). The chromatogram was sprayed with 0.2% (w/v) ninhydrin in acetone and developed at 90°C.

A



B



Ala 0 hr 1 hr 2 hr

reaction was examined by paper chromatography. A ninhydrin-positive spot, with an Rf similar to alanine, was observed after a 1 or 2 hr incubation period [Figure 2 (B)], showing that alanine is cleaved from the substrate N-carbobenzoxy-phenylalanyl-alanine. The products of aminopeptidase reaction were not confirmed as the production of a yellow colour in the assay mixture was judged to be sufficiently characteristic of p-nitroanaline production.

E. Dependence upon pH

The optimum assay pH for the acid endopeptidase is between 3.4 and 3.6 [Figure 3 (A)]. However, activity was found over a wide range of pH. Harvey found the pH optimum to be approximately 3.8 (23). For carboxypeptidase, the optimum occurred between pH 4.4 and 5.0 [Figure 3 (B)]. Below pH 4.0 substrate solubilization was difficult. Aminopeptidase activity was evident over a wide range of pH, with a broad peak from 6.5 to 8.0 [Figure 3 (C)]. Above pH 8.0 complete solubilization of the substrate was a problem. Thus, the overlapping pH ranges for the different peptide hydrolase activities might allow interference by one activity in another assay. Aminopeptidase is unlikely to act on a carboxypeptidase substrate and vice versa. However, an endopeptidase which also possessed carboxypeptidase specificity might act on the carboxypeptidase substrate and it is possible that the carboxypeptidase could hydrolyze C-terminal amino acids from haemoglobin at pH 3.8. These aspects were examined in more detail with a partially purified endopeptidase.

Figure 3

Variation of Peptide Hydrolase Activities with pH

(A) Acid Endopeptidase (□——□)

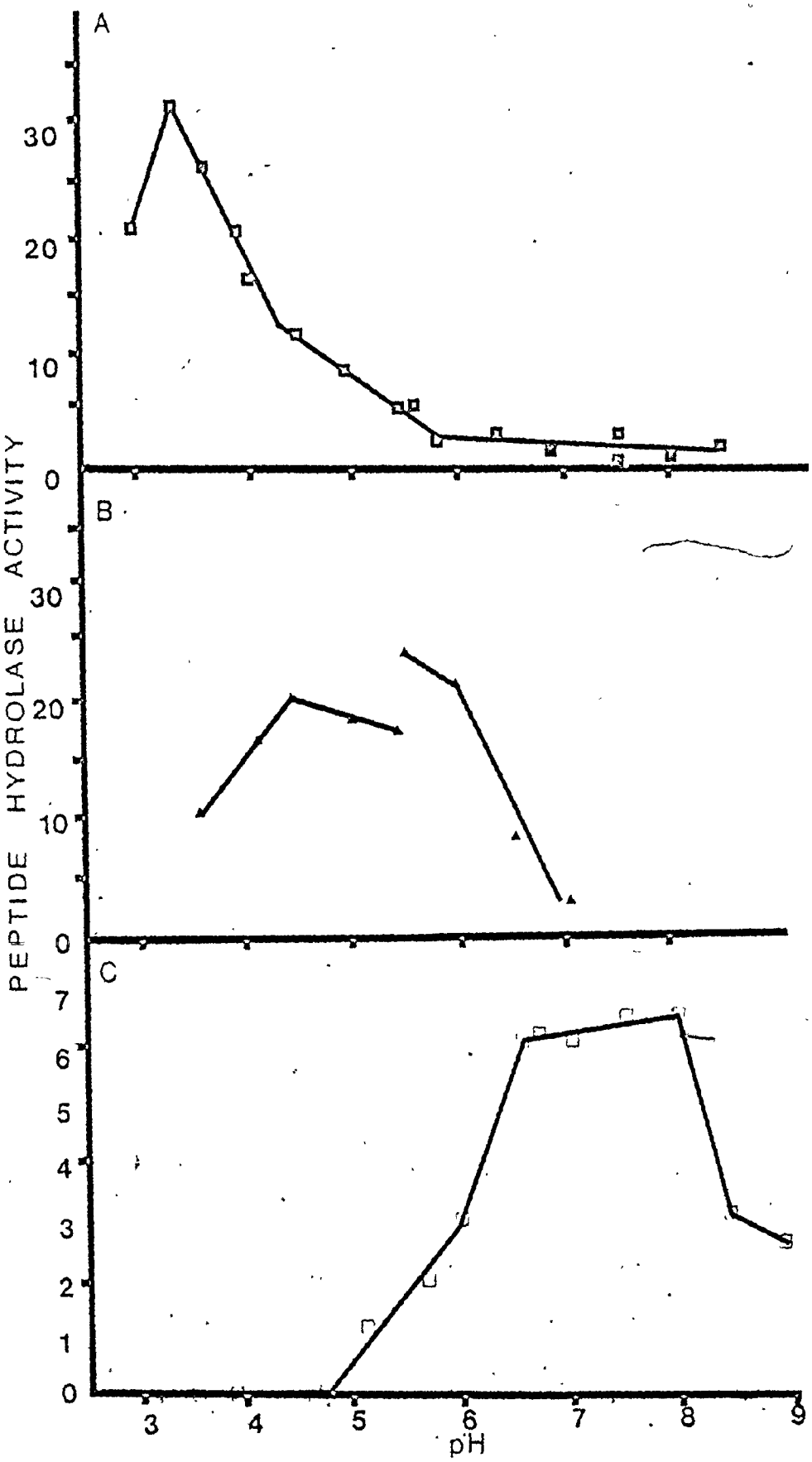
Endosperms from caryopses, 6 days post-imbibition, were homogenized in 0.2 M sodium acetate buffer, pH 3.8, containing 5 mM β-mercaptoethanol. Activity was measured with 5% haemoglobin substrate (1.0 ml), enzyme extract (0.1 ml) and 0.05 M buffers (1.4 ml) in the following pH ranges: pH 3.0-5.5, acetate; pH 5.6-7.5 phosphate; pH 7.5-8.5, Tris. The pH values were measured on the final assay mixture. Activity is expressed as µg Trp released in the assay per minute per gram of tissue.

(B) Carboxypeptidase (△——△)

Endosperms from caryopses, 6 days post-imbibition were homogenized in 0.05 M sodium phosphate, pH 6.7, and dialyzed overnight against 0.05 M sodium acetate pH 5.2. The substrate was dissolved in 0.05 M buffers in the following pH ranges: pH 3.0-5.6 acetate; pH 6.0-7.5 phosphate. After termination of the reaction, the pH was adjusted to 5.2 and α-amino nitrogen was determined. Activity is expressed as mg Ala released/min/gram tissue.

(C) Aminopeptidase (□——□)

Endosperms from caryopses, 2 days post-imbibition were homogenized in 0.05 M sodium phosphate, pH 6.7. The substrate was dissolved in 0.05 M buffers in the following pH ranges: pH 3.0-5.6 acetate; pH 6.0-7.5 phosphate; pH 8.0-9.0 Tris. Activity is expressed as µg p-nitroaniline released in the assay per minute per gram tissue.


A
B
C
30
20
10
0
30
20
10
0
7
6
5
4
3
2
1
0
3
4
5
6
7
8
9
pH
PEPTIDE HYDROLASE ACTIVITY

F. Substrate Concentration

Above ten mg haemoglobin in each assay, acid endopeptidase activity increases only slightly [Figure 4 (A)]. Thus, in the standard assay, use of fifty mg means that the substrate is not limiting in the reaction. For carboxypeptidase, measured activity is still increasing at the standard concentration of 1.0 mg/ml [Figure 4 (B)]. However, at higher concentrations the substrate was incompletely soluble. Thus, the carboxypeptidase assay is subject to error from variation in quantity of substrate added. For aminopeptidase, above the standard concentration of 0.5 mg/ml there is some increase in activity [Figure 4 (C)]. Above 1.0 mg/ml the substrate was incompletely soluble.

G. Quantity of Enzyme

In all cases it appears that the measured enzyme activity is linear over a range of enzyme quantity added [Figures 5 (A), (B), and (C)]. However, there appears to be a constant error throughout, as indicated by the change in slope beyond the lowest added volume of enzyme. This could be due to faulty pipettes, poor pipetting technique or inaccuracies at small volumes.

H. Temperature

All enzymes show high temperature optima [Figures 6 (A), (B), and (C)]. Aminopeptidase optimum was observed at 45°C, carboxypeptidase at 50°C and acid endopeptidase at 60°C. The enzyme assays, as outlined on pages 30-31 were routinely run at below optimum temperature.

Figure 4

Variation of Peptide Hydrolase Activities with

Substrate Concentration

(A) Acid Endopeptidase (□——□)

Endosperms from caryopses, 6 days post-imbibition, were homogenized in 0.2 M sodium acetate, pH 3.8, containing 5 mM β-mercaptoethanol. Varying amounts of haemoglobin were added to each assay. Activity is expressed as µg Trp equivalents released per minute per gram tissue.

(B) Carboxypeptidase (△——△)

Endosperms from caryopses, 6 days post-imbibition were homogenized in 0.05 M sodium phosphate, pH 6.7 and dialyzed overnight against 0.1 M sodium acetate pH 5.2. Activity is expressed as µg Ala released per minute per gram tissue.

(C) Aminopeptidase (□——□)

Endosperms from caryopses, 2 days post-imbibition were homogenized in 0.05 M sodium phosphate, pH 6.7. Activity is expressed as µg p-nitroaniline released per minute per gram tissue.

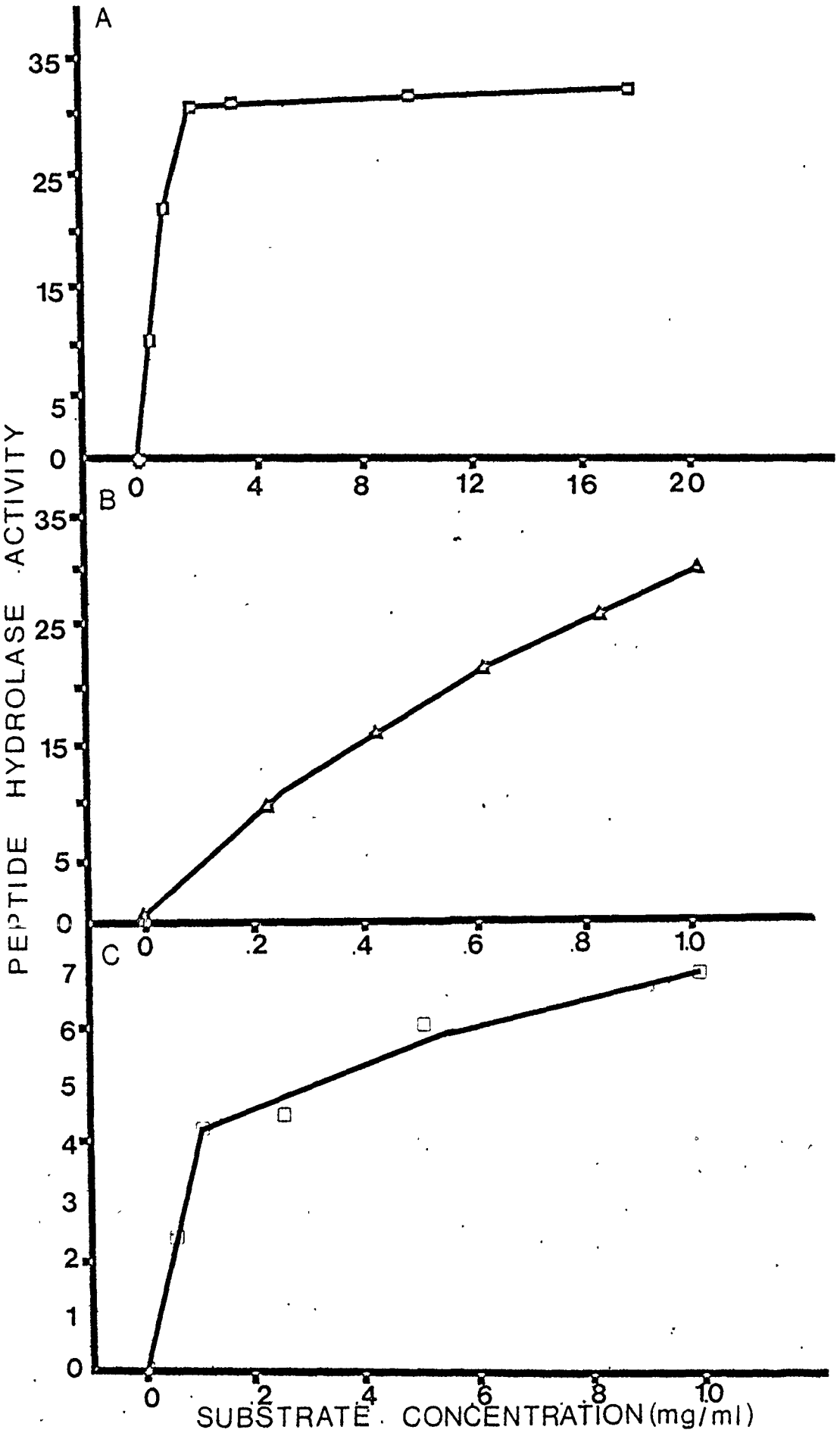

A
35
25
15
5
0
0
4
8
12
16
20
B
35
25
15
5
0
0
.2
.4
.6
.8
1.0
C
7
6
5
4
3
2
1
0
0
2
4
6
8
1.0
PEPTIDE HYDROLASE ACTIVITY
SUBSTRATE CONCENTRATION (mg/ml)

Figure 5

Variation of Peptide Hydrolase Activities with Amount of Enzymes

(A) Acid Endopeptidase (□——□)

Endosperms from caryopses, 6 days post-imbibition were homogenized in 0.2 M sodium acetate pH 3.8 containing 5 mM β-mercaptoethanol. The assay mixture contained 5% haemoglobin in water (1.0 ml); 0.05 M sodium acetate pH 3.8 (1.0 ml). The various volumes of extract shown and the appropriate volume of extraction buffer to total 2.5 ml. Activity is expressed as µg Trp equivalents released per minute.

(B) Carboxypeptidase (▲——▲)

Endosperms from caryopses, 6 days post-imbibition were homogenized in 0.05 M sodium phosphate pH 6.5 and the extract dialyzed overnight against 0.1 M sodium acetate pH 5.2. To the substrate solution the appropriate volume of extract was added in addition to sufficient 0.1 M sodium acetate buffer pH 5.2, to total 0.55 ml. Activity is expressed as µg Ala released per minute.

(C) Aminopeptidase (□——□)

Endosperms from caryopses, 2 days post-imbibition were homogenized in 0.05 M sodium phosphate, pH 6.7. The appropriate volume of extract was added to the substrate solution plus extraction buffer to total 1.05 ml. Activity is expressed as µg p-nitroaniline released per minute.

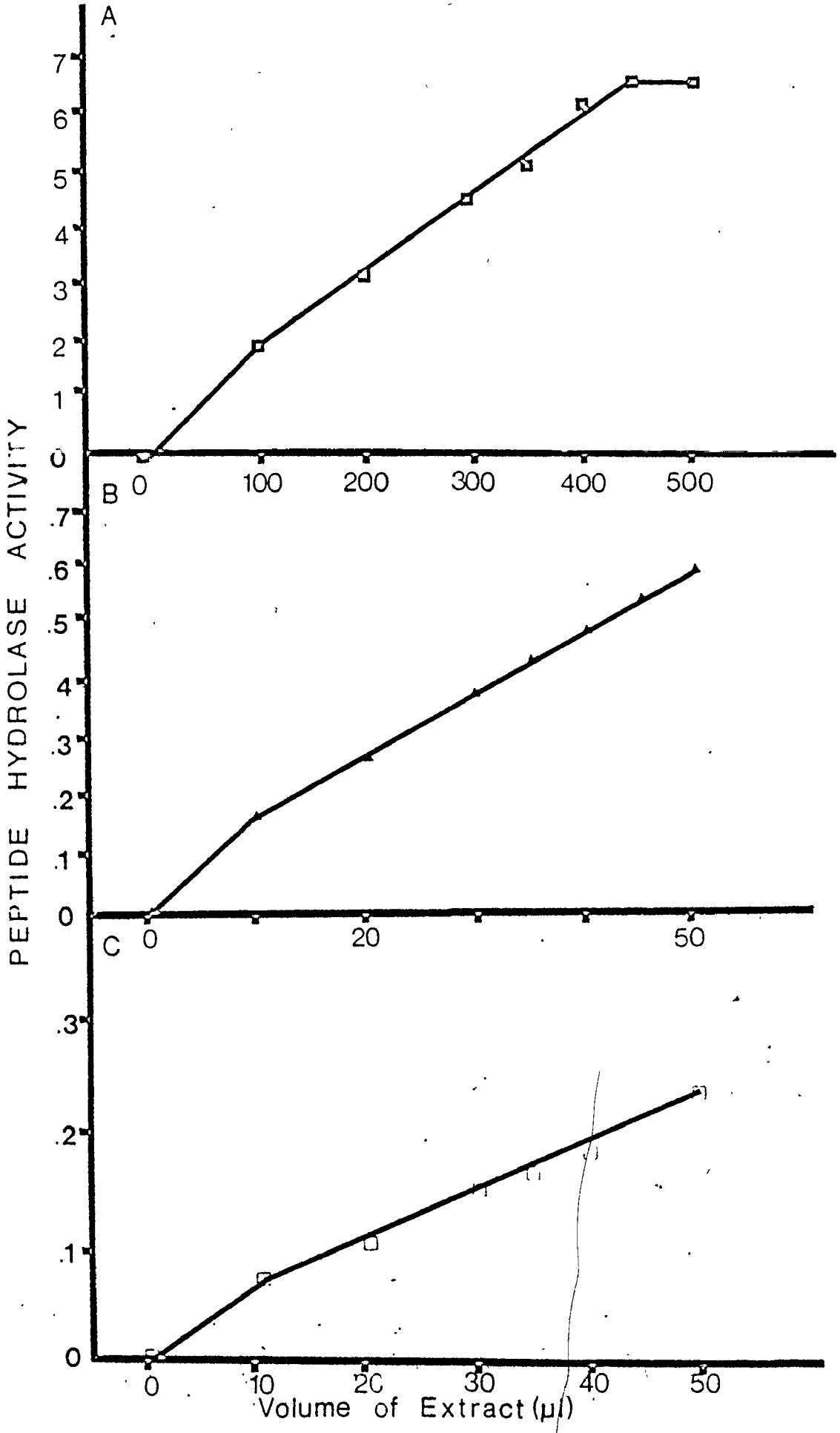

A
7
6
5
4
3
2
1
0
B 0
100
200
300
400
500
.7
.6
.5
.4
.3
.2
.1
0
C 0
20
50
.3
.2
.1
0
0
10
20
30
40
50
PEPTIDE HYDROLASE ACTIVITY
Volume of Extract (µl)

Figure 6

Variation of Peptide Hydrolase Activities with Temperature

(A) Acid Endopeptidase (□——□)

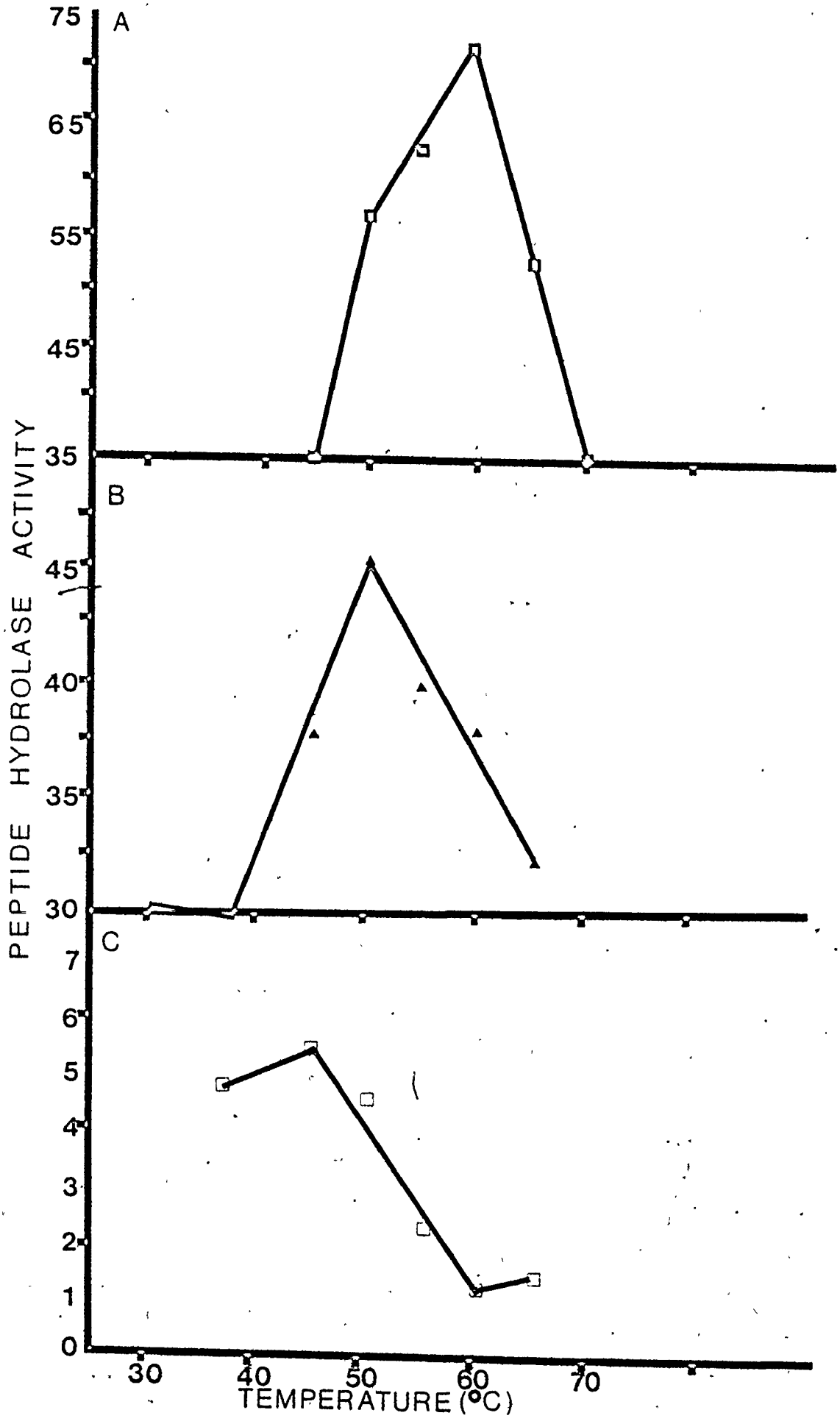
Endosperms from caryopses, 6 days post-imbibition were homogenized in 0.2 M sodium acetate, pH 3.8, containing 5 mM β-mercaptoethanol. The extract was assayed under standard conditions of time and quantity of enzyme (see Materials and Methods). However, the temperature was varied from 40-70°C. Activity is expressed as µg Trp equivalents released per minute per gram fresh weight.

(B) Carboxypeptidase (▲——▲)

Endosperms from caryopses, 6 days post-imbibition were homogenized in 0.05 M sodium phosphate, pH 6.7 and the extract dialyzed overnight against 0.1 M sodium acetate pH 5.2. The extract was assayed under standard conditions of time and quantity of enzyme (see Materials and Methods). However the temperature was varied from 30-70°C. Activity is expressed as µg Ala released per minute per gram tissue.

(C) Aminopeptidase (□——□)

Endosperms from caryopses, 2 days post-imbibition were homogenized in 0.05 M sodium phosphate, pH 6.7. The extract was assayed under standard conditions of time and quantity of enzyme (see Materials and Methods). However, the temperature was varied from 37-70°C. Activity is expressed as µg p-nitroanline released/gram fresh weight.


A
B
C
75
65
55
45
35
45
40
35
30
7
6
5
4
3
2
1
0
PEPTIDE HYDROLASE ACTIVITY
30
40
50
60
70
TEMPERATURE (°C)

I. Time Dependence

Acid endopeptidase activity is linear for at least thirty minutes [Figure 7 (A)]. Beyond this time there appears to be a slowing of activity, possibly due to enzyme denaturation, substrate depletion or a combination of these factors. Carboxypeptidase activity is linear after an initial lag period [Figure 7 (B)]. This may suggest activation of the enzyme is needed. Aminopeptidase activity is linear over the time period investigated [Figure 7 (C)].

J. Enzyme Inhibitors

Active site reagents were incubated with the enzyme preparations in an effort to inhibit the activities (Table IX). However, no significant differential effects were seen. All activities showed the greatest inhibition by the sulphydryl reagent para-chloromercuribenzoate (PCMB), aminopeptidase being the most sensitive, followed by carboxypeptidase and acid endopeptidase. At 5 mM phenylmethylsulphonylfluoride (PMSF) the acid endopeptidase activity was inhibited by 36% and the aminopeptidase activity by 25%. The inhibitor solvent decreased the carboxypeptidase activity by 30%, obscurring any effect of PMSF. 5 mM EDTA had no significant effect on any of the assays.

Figure 7

Formation of Products in Peptide Hydrolase Reactions with Time

(A) Acid Endopeptidase (□——□)

Endosperms from caryopses, 6 days post-imbibition were homogenized in 0.2 M sodium acetate, pH 3.8, containing 5 mM β-mercaptoethanol. The extract was assayed under standard conditions of temperature and quantity of enzyme (see page). However, the reaction was terminated at various times after initiation. Activity is expressed as µg Trp equivalents released/gram fresh weight.

(B) Carboxypeptidase (▲——▲)

Endosperms from caryopses, 6 days post-imbibition were homogenized in 0.05 M sodium phosphate, pH 6.7 and the extract dialyzed overnight against 0.1 sodium acetate, pH 5.2. The extract was assayed under standard conditions of temperature and quantity of enzyme (see page 31). However, the reaction was stopped at various times after initiation. Activity is expressed as µg Ala released/gram fresh weight.

(C) Aminopeptidase (□——□)

Endosperms from caryopses, 2 days post-imbibition were homogenized in 0.05 M sodium phosphate, pH 6.7. The extract was assayed under standard conditions of temperature and quantity of enzyme (see page). The reaction was stopped at various times after initiation. Activity is expressed as µg p-nitroaniline released/gram fresh weight.

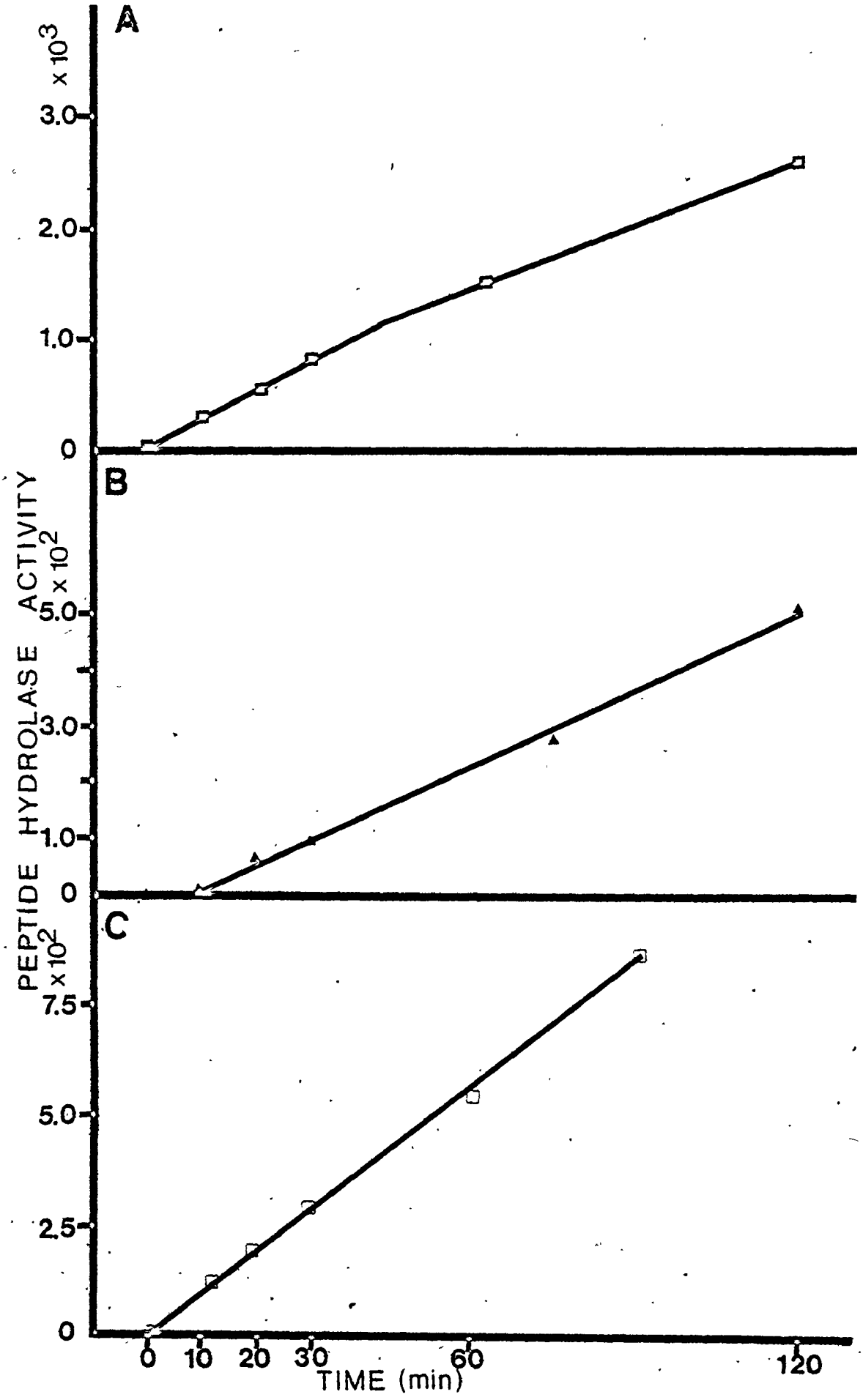

A
B
C
PEPTIDE HYDROLASE ACTIVITY
x 10^3
3.0
2.0
1.0
0
x 10^2
5.0
3.0
1.0
0
x 10^2
7.5
5.0
2.5
0
0
10
20
30
60
120
TIME (min)

TABLE IX

Effects of Inhibitors on Proteolytic Activities

Inhibitors were dissolved in the appropriate solvent and added to enzyme extracts to give the final concentrations which are shown. Extracts were incubated on ice for three hours with the inhibitors before assaying. Units are the standard terms given earlier.

Acidic Endopeptidase: µg Trp equivalents released/min/g fresh weight
Carboxypeptidase: µg Ala released/min/g fresh weight
Aminopeptidase: µg p-nitroaniline released/min/g fresh weight

Sample	Acid Endopeptidase	Carboxypeptidase	Aminopeptidase
Control	34±5 (100)	27±2 (100)	5.7±.2 (100)
PMSF			
Isopropanol	34 (100)	19±1 (70)	5.3±.3 (94)
1 mM PMSF	28±3 (84)	21±2 (76)	5.3±.2 (94)
5 mM PMSF	21±3 (64)	17±2 (63)	4.3±.2 (76)+
PCMB			
1 mM PCMB	25±3 (76)	19±1 (71)	1.3±.2 (24)x
5 mM PCMB	9±1 (28) +	3±0 (11)+	0.0±.0 (0)x
EDTA			
1 mM EDTA	28±3 (84)	24±1 (88)	5.8±.3 (103)
5 mM EDTA	34±3 (100)	23±1 (83)	5.3±.0 (94)

+ $p < 0.05$

x $p < 0.01$ compared to Control, according to Student's t-test

Mean ± Standard Deviation

Figures in brackets represent percentages of control.

II. Partial Purification of Acid Endopeptidase

The initial report of a successful purification of an endopeptidase fraction from maize endosperm found a low molecular weight protein (21,000 daltons) with a low isoelectric point (1). Thus, gel permeation and ion-exchange chromatography were useful methods in the purification.

A. Ammonium Sulphate Precipitation

The majority of the acid endopeptidase activity is precipitated by 20-60% ammonium sulphate (81% of the recovered activity) although a substantial fraction (16% of the recovered activity is precipitated by 20% (0.14 g/ml) ammonium sulphate (Table X). In the test shown only 56% of the activity was recovered but later in routine work recovery was always higher (75-95%). In the routine procedure which was developed, the crude extract was made 60% (0.42 g/ml) in ammonium sulphate and the pellet was reconstituted for further purification work (see Materials and Methods for procedure).

B. Chromatography on Sephadex

Three pore sizes of Sephadex were used in initial purification of the acid endopeptidase. Typical results of these trials are shown in Figure 8. In all cases there appears to be more than one peak of activity. All types of Sephadex gave only a small degree of purification as judged by increase in specific activity: Sephadex G-50 - 1.3 fold; Sephadex G-100 - 0.5-1.3 fold; and Sephadex G-200 - 0.6-0.7 fold. It appears that all acid endopeptidase protein is smaller than

TABLE X

Fractionation of Acid Endopeptidase Activity

by Ammonium Sulphate

Endosperms from caryopses six days post-imbibition were homogenized by the standard procedure (see Materials and Methods). The crude extract was assayed. The extract was made progressively more concentrated in $(NH_4)_2SO_4$. For each fraction, the pellet was reconstituted in a known volume of 0.2 M sodium acetate and assayed for activity. The final supernatant was dialyzed against 0.2 M sodium acetate and assayed. Activity is expressed as µg Trp equivalents released/min/total volume.

Sample	Activity	Percentage
Crude Extract	99.2	100.0
0-20% $(NH_4)_2SO_4$ Pellet	8.6	8.8
20-40% $(NH_4)_2SO_4$ Pellet	22.6	22.8
40-60% $(NH_4)_2SO_4$ Pellet	22.6	22.8
60-80% $(NH_4)_2SO_4$ Pellet	0.8	0.8
80% $(NH_4)_2SO_4$ Supernatant	1.0	1.0
Total Recovered	55.6	56.0

Figure 8

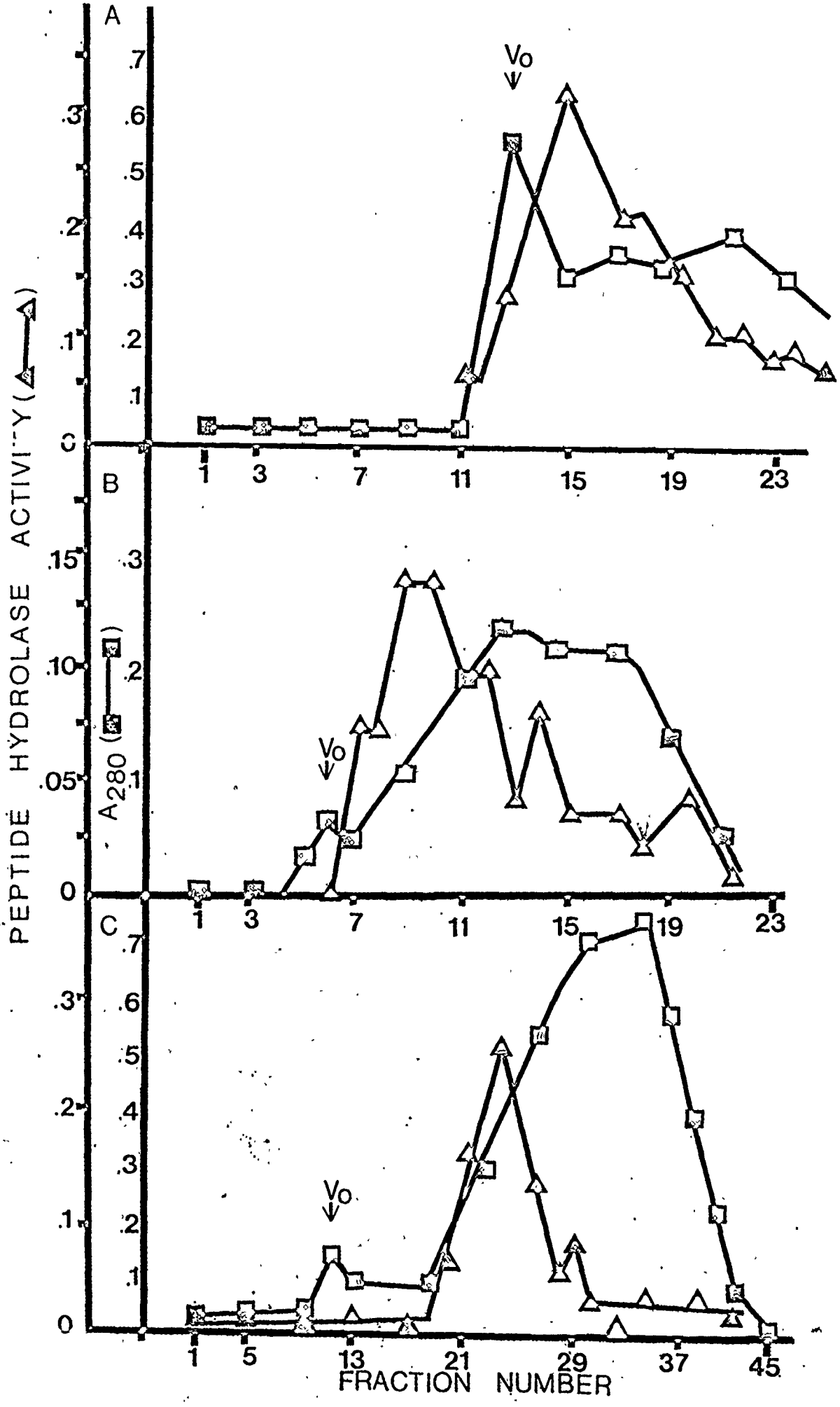
Chromatography of Acid Endopeptidase on Sephadex

Endosperms from caryopses harvested six days post-imbibition were homogenized by the standard procedure. After precipitation with 60% ammonium sulphate the pelleted protein was redissolved in extraction buffer and loaded onto Sephadex columns, which were equilibrated with extraction buffer. Fractions were assayed for absorbance at 280 nm and for acid endopeptidase activity. Activity is reported as change in absorbance at 280 nm.

(A) Sephadex G-50

(B) Sephadex G-100

(C) Sephadex G-200


A
B
C
Vo
PEPTIDE HYDROLASE ACTIVITY (Δ——Δ)
A280 (——)
FRACTION NUMBER
0
.1
.2
.3
.4
.5
.6
.7
.05
.10
.15
1
3
5
7
11
13
15
19
21
23
29
37
45

the exclusion limit of Sephadex G-50 (approximately 30,000 daltons). In all cases there is a large amount of low molecular weight material which absorbs light at 280 nm. This material could be from the hydrolysis of the storage proteins.

C. Chromatography on DEAE Cellulose

After preliminary treatment with 60% $(NH_4)_2SO_4$ (0.42 g/ml) and Sephadex G-100 chromatography, the endopeptidase extract (pH 3.8) was dialyzed against the ion-exchange column buffer (pH 8.1). This resulted in a large loss of activity (60%). Only part of the activity was recovered upon redialysis with extraction buffer, after DEAE cellulose chromatography. Thus, treatment with DEAE cellulose, although appearing to separate the acid endopeptidase into a number of activity peaks (see Figure 10) did not result in an increase in specific activity (see Table XI).

D. Chromatography on CM-cellulose

The initial attempt was with 60% $(NH_4)_2SO_4$ followed by Sephadex G-200 chromatography and CM-cellulose (Figure 11). However, this resulted in little purification (Table XII). With Sephadex G-50, proteins of molecular weight greater than approximately 30,000 daltons are excluded. It appears from Figure 11 that almost all acid endopeptidase activity is associated with proteins which are smaller than 30,000 daltons whereas carboxypeptidase activity is associated with larger proteins. When the pooled fractions were dialyzed into the ion exchange buffer, applied to the CM-cellulose and a linear

TABLE XI

Recovery of Acid Endopeptidase on Sephadex and DEAE Cellulose

Endosperms from caryopses, harvested six days post-imbibition were extracted and purified as described in Materials and Methods.

Sample	Total Activity (µg Trp/min/ total volume	Total Protein (mg)	Specific Activity	Recovery	Purification
Crude	480	23.1	20.8	100	1.00
60% $(NH_4)_2SO_4$	426	13.5	31.6	89	1.52
Sephadex G-100					
onto column	404	12.8	31.6	84	1.52
recovered	407	10.2	40.1	85	1.93
DEAE cellulose					
onto column	337	8.4	40	70	1.9
recovered	21	.7	32	4	1.5

Figure 9

Chromatography of Acid Endopeptidase on Sephadex and DEAE Cellulose

(A) Sephadex G-100

Fractions were assayed for acid endopeptidase and absorbance at 280 nm. Activity is expressed as the change in absorbance at 280 nm of the TCA supernatant during the assay.

(B) DEAE Cellulose

The column was eluted with a linear gradient of sodium chloride. Fractions were assayed for acid endopeptidase and absorbance at 280 nm. Activity is expressed as the change in absorbance at 280 nm of the TCA supernatant during the assay.

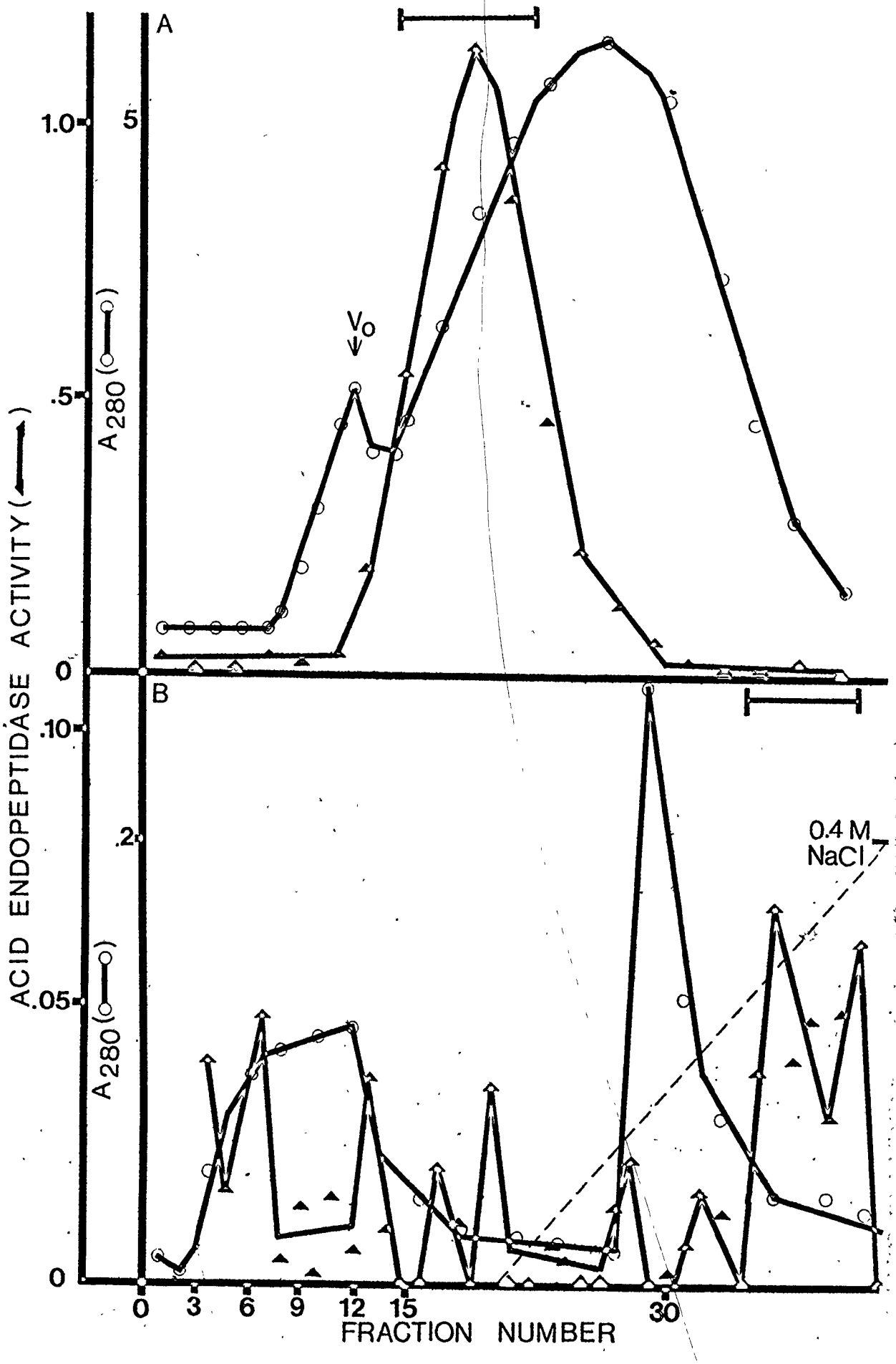

A
B
1.0
.5
0
5
A_{280} (—○—)
V_o
.10
.05
.2
0
ACID ENDOPEPTIDASE ACTIVITY (—▲—)
0.4 M NaCl
0 3 6 9 12 15 30
FRACTION NUMBER

TABLE XII

Recovery of Acid Endopeptidase on G-200 Sephadex and CM-Cellulose

Endosperms from caryopses, harvested six days post-imbibition were extracted and purified as described in Materials and Methods.

Sample	Total Activity (µg Trp/min/ total volume	Total Protein (mg)	Specific Activity	Recovery	Purification
Crude	460	17.36	26.5	100	1.00
60% $(NH_4)_2SO_4$	359	8.79	40.8	78	1.55
Sephadex G-200					
onto column	336	8.24	40.8	73	1.55
recovered	215	7.89	27.2	47	1.03
CM-Cellulose					
onto column	191	7.00	27.2	41	1.03
recovered - T	11	0.03	36.8	2	1.4
A	55	1.30	42.4	12	1.6

Figure 10

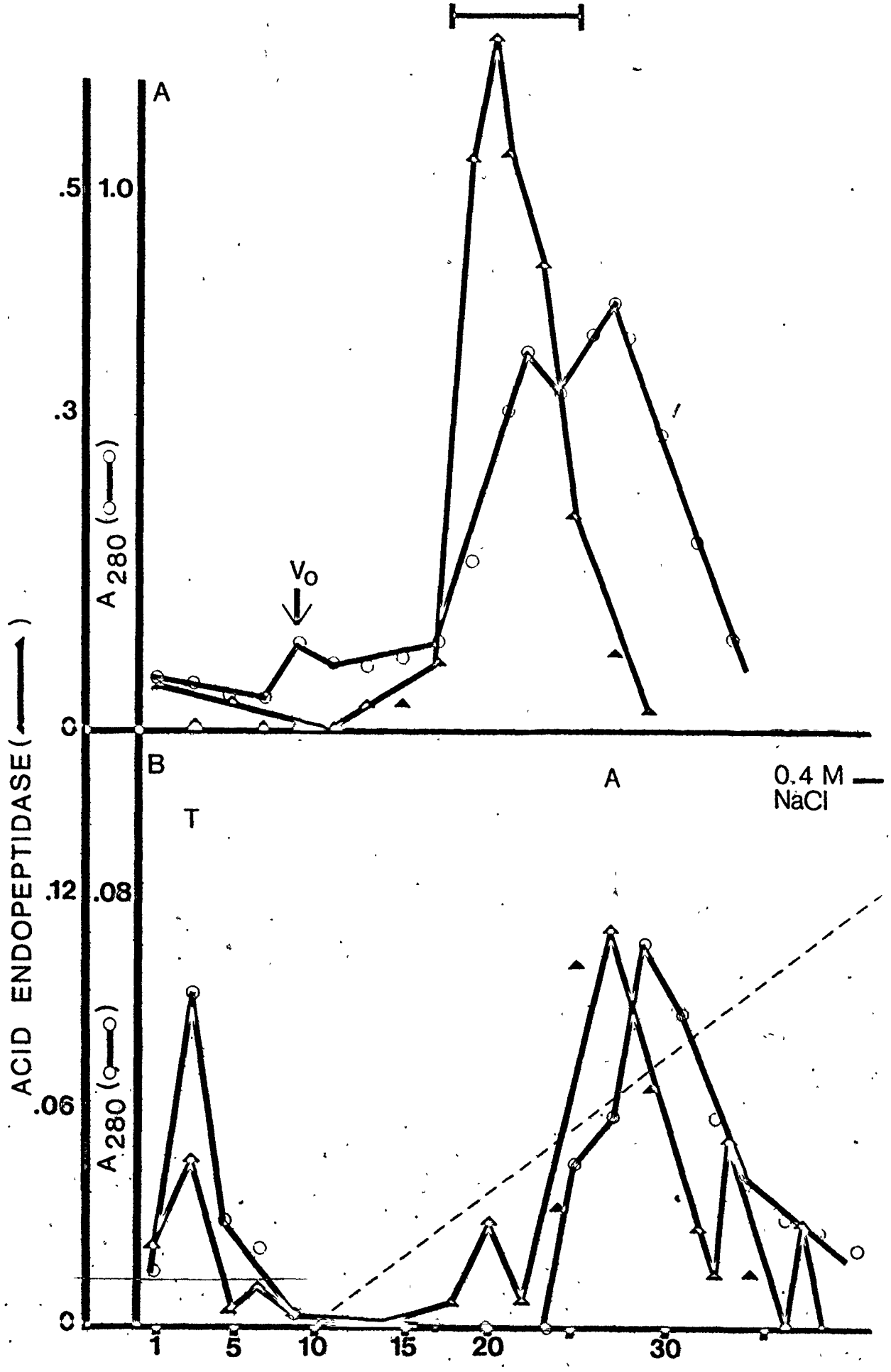
Chromatography of Acid Endopeptidase on Sephadex G-200 and CM-Cellulose

(A) Sephadex G-200

Fractions were assayed for acid endopeptidase activity and absorbance at 280 nm. Activity is expressed as the change in absorbance at 280 nm in the TCA supernatant during the assay.

(B) CM-Cellulose

The column was eluted with a linear gradient of sodium chloride (0-0.4 M). Fractions were assayed for acid endopeptidase and absorbance at 280 nm. Activity is expressed as the change in absorbance at 280 nm in the TCA supernatant during the assay.


A
.5
1.0
.3
0
A280 (—○—)
Vo
B
T
A
0.4 M —
NaCl
ACID ENDOPEPTIDASE (—▲—)
.12
.08
.06
0
A280 (—○—)
1
5
10
15
20
30

salt gradient (0-0.4 M NaCl) used for elution, the results shown in Figure 12 were obtained. A number of peaks are evident. These have been separated into the through fraction (T) and fractions A, B, and C of the bound protein. The most pure fraction was peak 'A'. The protein level in peak 'T' was too low for detection. It can be seen in Table XIII that there was a substantial increase in specific activity with CM-cellulose column but also a substantial loss of active protein. In addition there was little detectable carboxypeptidase activity associated with any of the peaks. The total carboxypeptidase activity recovered was 2-3% of the original activity compared to approximately 17% for acid endopeptidase.

E. Molecular Weight Estimation

Figure 13 shows a molecular weight estimation for a fraction pelleted by 60% (0.42 g/ml $(NH_4)_2SO_4$ and for CM-cellulose bound fraction 'A'. The peak for the crude extract corresponds to a molecular weight of 30,000 daltons and the peak from fraction 'A' corresponds to an protein of 21,000 daltons.

F. SDS-Disc Gel Electrophoresis

Certain fractions were subjected to SDS polyacrylamide gel electrophoresis. At least four distinct bands staining with Coomassie were seen in the crude extract (Figure 14). For both the CM-cellulose through fraction 'T' and bound fraction 'A', there was one main protein band with fainter bands on each side of the main band. These protein bands were not shown to co-migrate with haemoglobinases. Therefore, the results simply confirm that some of the proteins present in the crude extract are not present in fractions 'T' and 'A' and do not prove pure en-

TABLE XIII

Co-Purification of Acid Endopeptidase

and Carboxypeptidase

Endosperms, for caryopses, harvested six day post-imbibition were extracted and purified according to the procedure outlined in Materials and Methods.

Sample	Total Activity (µg Trp/min/ total volume	Total Protein (mg)	Specific Activity	Recovery	Purification
A. ACID ENDOPEPTIDASE					
Crude	654	24.8	26.4	100	1.00
60% $(NH_4)_2SO_4$	512	8.9	57.5	78	2.18
Sephadex G-50					
put on	494	8.6	57.5	75	2.18
recovered	526	--	--	--	--
collected	220	2.8	78.6	34	2.98
CM-Cellulose					
put on	210	2.7	78.6	32	2.98
collected through - T	26	0.8	34	4	1.3
A	34	0.1	340	5	12.8
B	20	0.1	200	3	7.6
C	34	0.3	113	5	4.3

TABLE XIII (Continued)

Sample	Total Activity (µg Ala/min/ total volume	Total Protein (mg)	Specific Activity	Recovery	Purification
B. CARBOXYPEPTIDASE					
Crude	1360	24.8	54.8	100	1.00
60% $(NH_4)_2SO_4$	1269	8.9	142.6	93	2.60
Sephadex G-50					
put on	1223	8.6	142.6	90	2.60
recovered	1079	--	--	79	--
collected	205	2.8	73.2	15	1.33
CM-Cellulose					
put on	196	2.7	73.2	14	1.33
collected - T	13	0.8	16	1	0.3
A	18	0.2	90	1	1.6
B	0	0.1	0	0	0.0
C	6	0.3	20	0	0.4

Figure 11

Co-Chromatography of Acid Endopeptidase and Carboxypeptidase on Sephadex G-50

Fractions were assayed for acid endopeptidase and carboxypeptidase activity and the absorbance at 280 nm was measured. Activity of acid endopeptidase is expressed as the change in absorbance at 280 nm in the TCA supernatant over the assay period. Carboxypeptidase activity is expressed as the change in absorbance at 570 nm over the assay period.

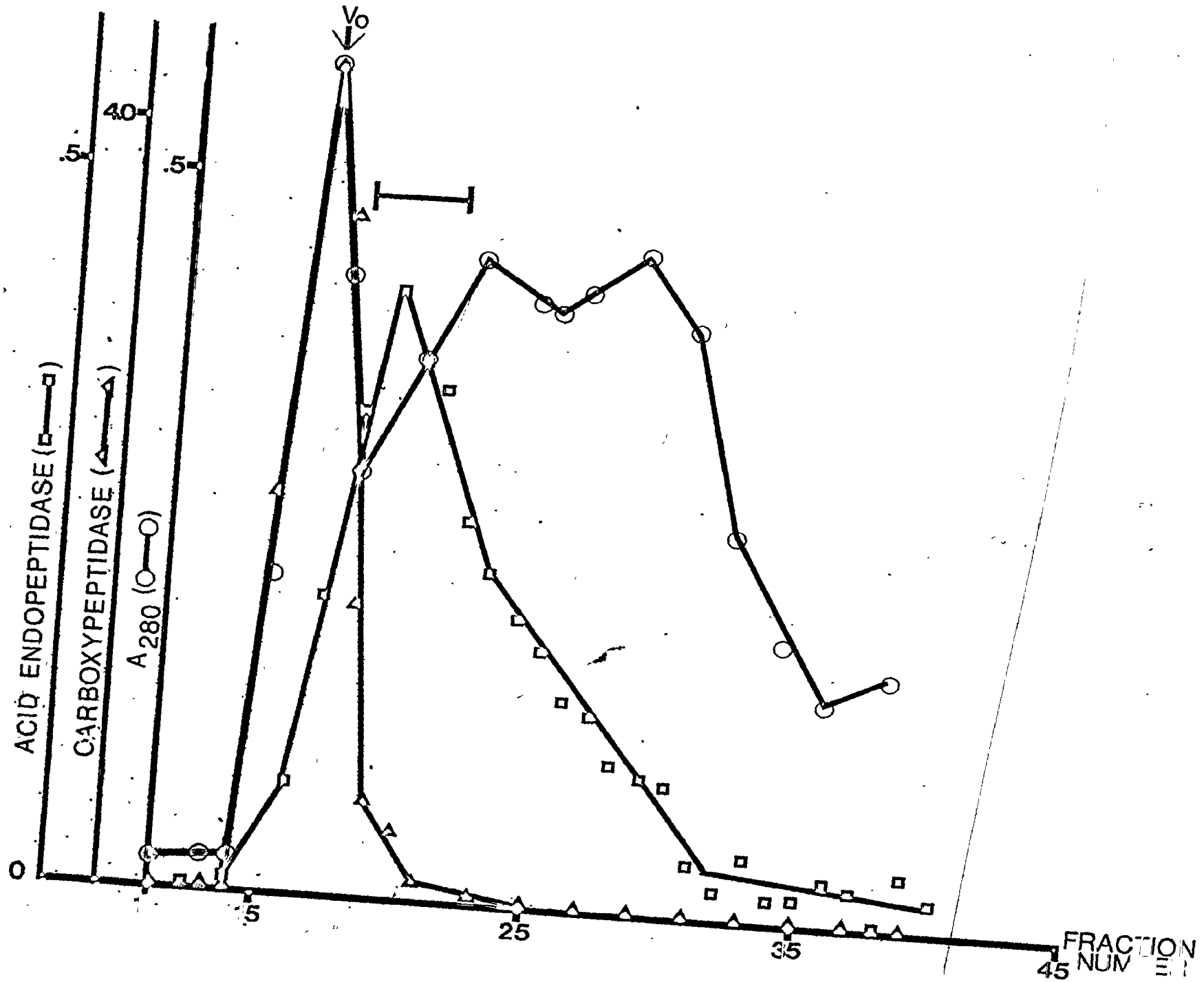

Vo
.5
40
.5
ACID ENDOPEPTIDASE (□—□)
CARBOXYPEPTIDASE (△—△)
A280 (○—○)
0
5
25
35
45
FRACTION NUMBER

Figure 12

Co-Chromatography of Acid Endopeptidase and Carboxypeptidase on CM-Cellulose

The column was eluted with a linear gradient of sodium chloride (0-0.4 M). Fractions were assayed for acid endopeptidase and carboxypeptidase activity and absorbance at 280 nm was recorded. Acid endopeptidase activity is expressed as the change in absorbance at 280 nm in the TCA supernatant over the assay period. Carboxypeptidase activity is expressed as the change in absorbance at 570 nm over the assay period.

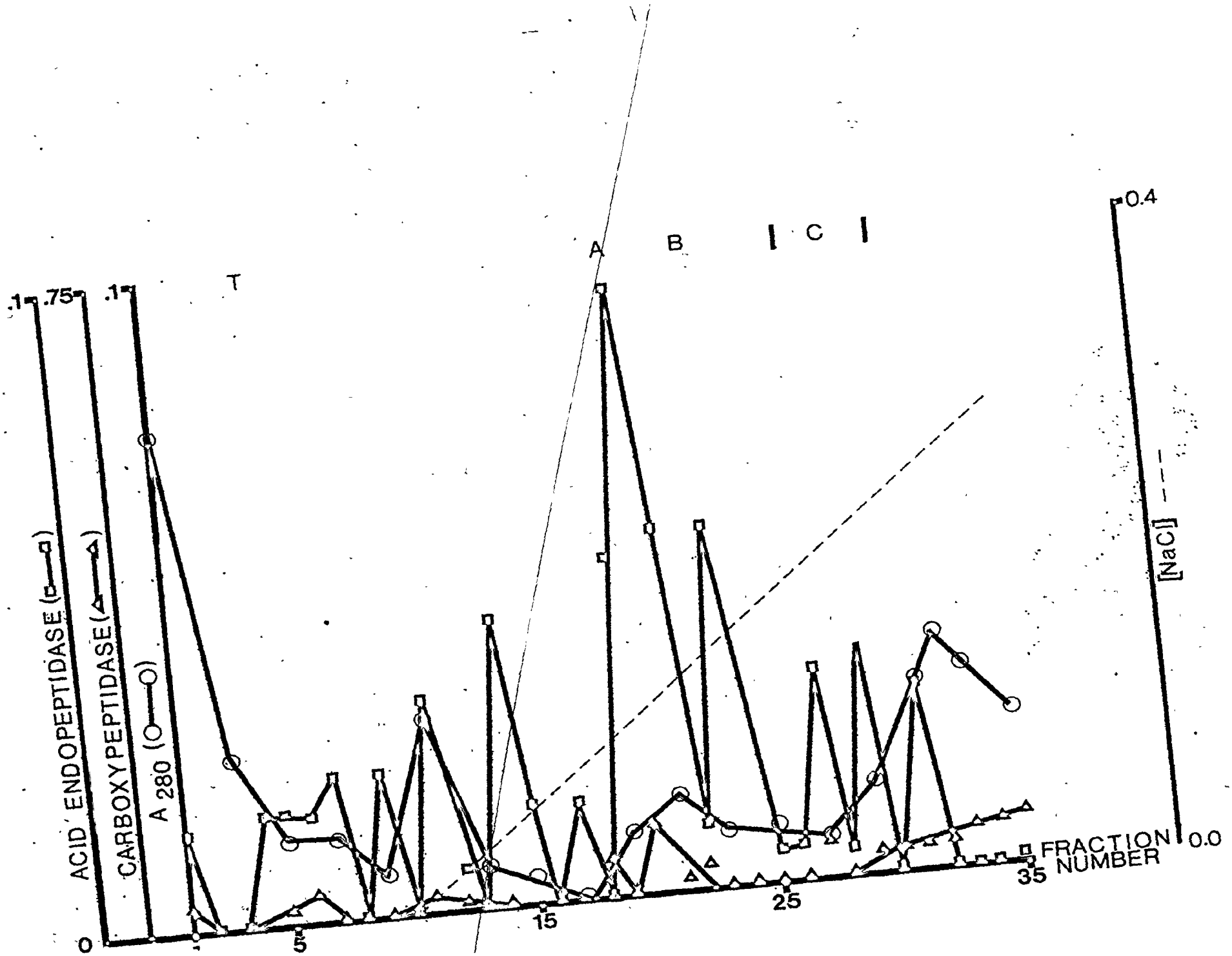

A
B
C
T
.1
.75
.1
ACID ENDOPEPTIDASE (□—□)
CARBOXYPEPTIDASE (△—△)
A 280 (○—○)
0
5
15
25
35
FRACTION NUMBER
0.4
0.0
[NaCl] — — —

Figure 13

Molecular Weight Determination of Acid Endopeptidase

(A) Ammonium Sulfate Precipitate

Endosperms from caryopses harvested six days post-imbibition were extracted and incubated with 60% (0.42 g/ml) $(NH_4)_2SO_4$ as described in Materials and Methods. The pellet was reconstituted with 0.025 M Tris buffer, pH 8.1 (a) or 0.2 M Na acetate pH 3.8 - 5 mM β-mercaptoethanol pH 3.8; (b) and loaded onto a (1.88 x 80 cm) column of Sephadex G-100, which had been equilibrated with the same buffer. The void volume (V_o) and the elution volume V_e were taken as the mid-points of the initial peak in absorbance at 280 nm and the highest peak of acid endopeptidase activity respectively. The column had previously been calibrated with standard proteins of known molecular weight.

(B) CM-Cellulose Bound Fraction

Endosperms from caryopses harvested six days post-imbibition were homogenized in buffer and the acid endopeptidase activity partially purified according to the procedure described in Materials and Methods. An aliquot of the partially purified fraction labelled 'A' in Figure 13 was loaded onto a (0.9 x 58 cm) column of Sephadex G-200 equilibrated with 0.2 M Na acetate - 5 mM β-mercaptoethanol pH 3.8. The column had previously been calibrated

Figure 13 (continued)

with standard proteins of known molecular weight. Fractions were assayed for absorbance at 280 nm and acid endopeptidase activity.

Standards
- a – cyt c - cytochrome c
- b – Trp In - trypsin inhibitor
- c – OA - ovalbumin
- d – BSA - bovine serum albumin
- e – ADH - alcohol dehydrogenase

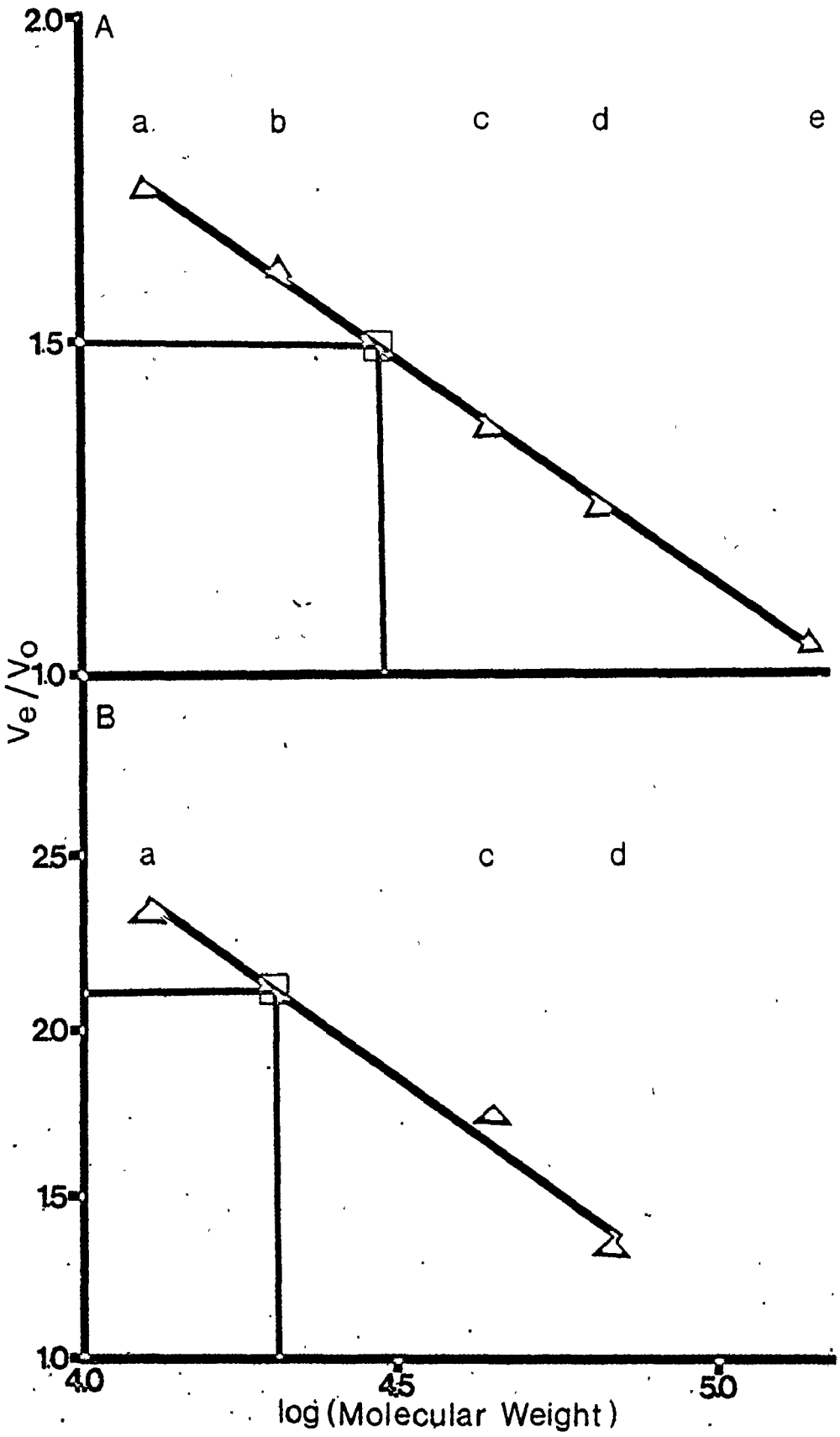

2.0
A
a
b
c
d
e
1.5
1.0
Ve/Vo
B
25
a
c
d
20
15
1.0
4.0
4.5
5.0
log (Molecular Weight)

Figure 14

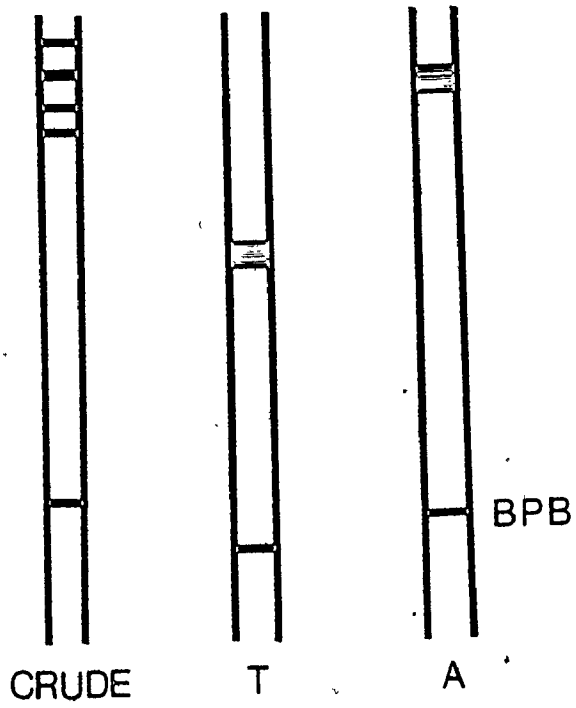
SDS-Gel Electrophoresis of Acid Endopeptidase

After denaturation of samples and reaction with sodium dodecyl sulfate (see Materials and Methods), aliquots (50 ul) of crude and partially purified extracts of endosperm tissue, 6 days post-imbibition were mixed with 20 ul of glycerol and loaded on 15% polyacrylamide gels (32). Ten ml of partially purified extracts were dialyzed against water and concentrated by lyophilization. The gels were run at 1 mamp/tube for 30 minutes and 2 mamp/tube for 2 hours. Gels were fixed in 50% TCA, stained in Coomassie R and destained in Methanol:Acetic Acid:H_2O (5:7:88).

T - through fraction

A - major acid endopeptidase fraction

BPB - bromophenol blue


BPB
CRUDE
T
A

zymes. For bound fraction 'A' the protein band corresponds with one in the crude extract. However, for the through fraction the protein band has migrated much farther than any visible band in the crude.

G. Chromatography of Peptide Hydrolases on Phenylalanine Substituted Sepharose

Preston had reported that purified wheat carboxypeptidases were the active proteins in his haemoglobinase assay (108). Therefore, he has used this assay to measure 'exopeptidase' activity. However, in maize, Harvey (44) had reported that an endopeptidase enzyme was the important component in the hydrolysis of haemoglobin. The two assays are similar but not identical. Preston (106) measures, at pH 4.0, the release from haemoglobin of dialyzable products which react with glycyl-glycine to give a fluorescent compound. Harvey (44) measures, at pH 3.8, the TCA soluble products which absorb radiation at 280 nm (principally Trp and Tyr containing peptides or the amino acids themselves). Theoretically, either assay could measure endopeptidase or exopeptidase activity.

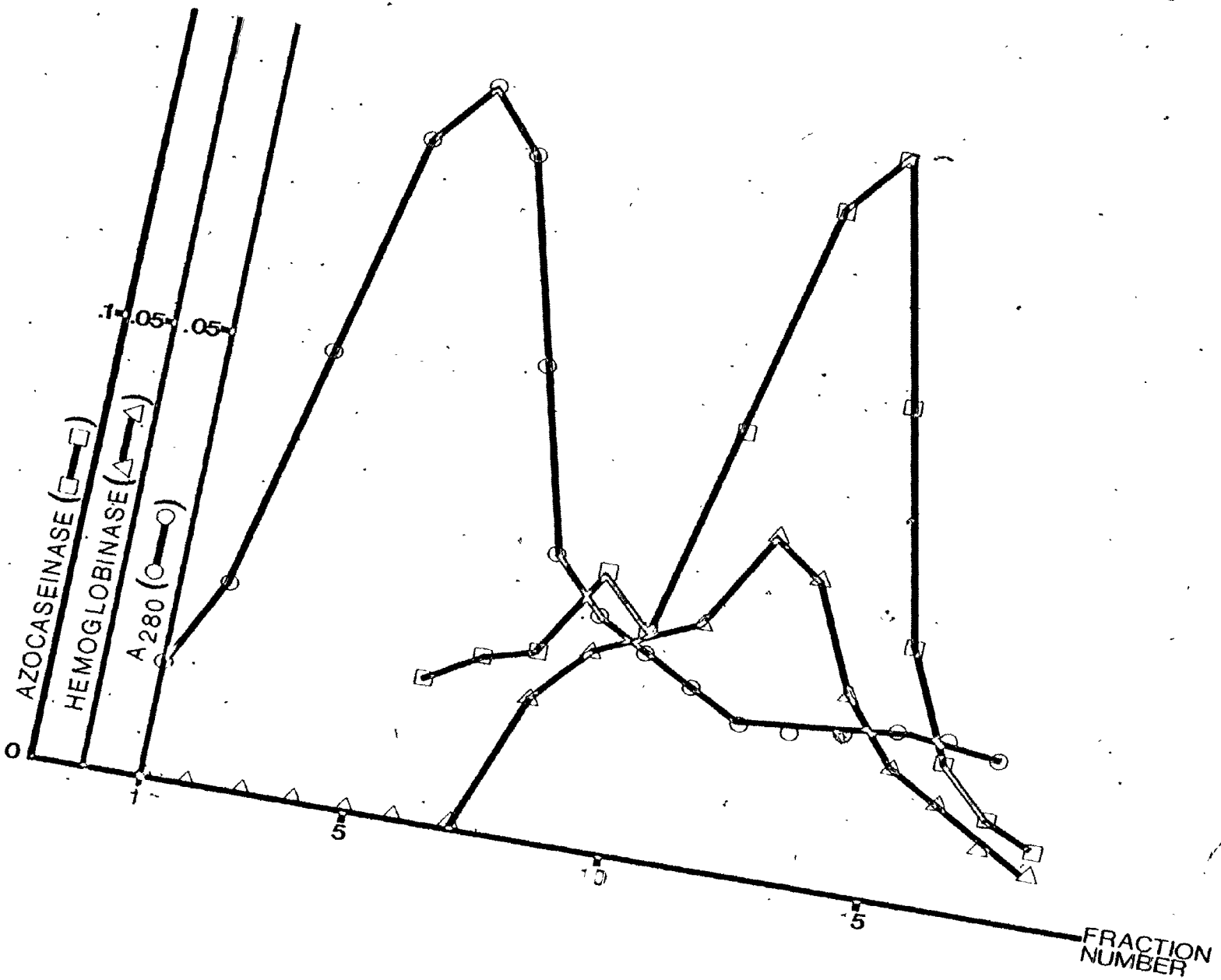
The earlier purification work in this thesis (Table XIII) has shown that there is little carboxypeptidase activity (as measured by hydrolysis of CBZ-Phe-Ala) associated with the haemoglobin degrading peaks, as measured by Harvey's method. Figure 15 shows similar elution patterns on phenylalanine substituted Sepharose of an endopeptidase (pH 6.0) and the reported 'exopeptidase' activity (pH 4.0 - haemoglobinase) in maize endosperm. In wheat endosperm, the

Figure 15

Chromatography of Peptide Hydrolases on Phe-Substituted Sepharose

Endosperms from caryopses, harvested 6 days post-imbibition were lyophilized. The lyophilized endosperms were ground in a coffee grinder. Five grams of powder was stirred with 25 ml of 0.1 N sodium acetate, pH 4.0 for one hour. The suspension was centrifuged for 20 minutes at 35,000 x g. The pellet was resuspended in extraction buffer and submitted to a second identical extraction. The supernatants were combined. The extract was dialyzed against 0.025 M sodium acetate, pH 4.0.

Twenty ml of extract was loaded onto a column of phenylalanine substituted sepharose (0.8 x 10 cm), prepared as described by Preston and equilibrated with 0.025 M sodium acetate, pH 4.0. Protein was eluted with a convex gradient of 0.05-0.25 M sodium acetate, pH 4.0. Fractions were measured for absorbance at 280 nm, Azocaseinase (111) and Haemoglobinase (106).


AZOCASEINASE (□—□)
HEMOGLOBINASE (△—△)
A280 (○—○)
.1
.05
.05
0
1
5
10
15
FRACTION NUMBER

carboxypeptidases which were active on haemoglobin also degraded CBZ-Phe-Ala (43).

Definitive work to determine whether any of the haemoglobin degrading fractions in maize are carboxypeptidases will be done on purified fractions. By examining the products from polypeptides and proteins with known amino acid sequence one will be able to determine the specificity. Already workers have reported that some fractions of haemoglobin degrading enzymes from maize are endopeptidases which can also degrade the carboxypeptidase substrates CBZ-dipeptides (41, 2, 86).

III. Hormonal Control of Peptide Hydrolase Activities

Peptide hydrolase activities were measured in endosperms at different stages of the life cycle. As seen in Figure 16, the exopeptidase activities are high at the two time points during development of the endosperm tissue. However, the acid endopeptidase activity is quite low.

In the post germination period the aminopeptidase declines from a relatively high value in the mature caryopsis to almost no activity. The acid endopeptidase and carboxypeptidase activities show a different pattern of change. From a low value in the mature caryopsis the latter activities rise to a peak about five days post germination.

The development of the acid endopeptidase in cultured endosperms was looked at in order to compare the development with the intact plant and to determine a time point at which the effect of inhibitors could be measured. Comparative data are shown in Figure 16 and Figure 17. The pattern of development of the acid endopeptidase was very similar to that for the intact caryopsis (Figure 16 (A); Figure 17). Harvey had reported earlier that levels in de-embryonated caryopsis were nearly equal to intact caryopsis (46). In my experiments the peak activity was seen five to six days post-imbibition in each case. The absolute level of activity, however, was significantly reduced in the de-embryonated caryopsis.

When excised endosperms were cultured in the presence of the plant hormone ABA (2 μM) significantly lower levels of both acid endopeptidase and carboxypeptidase were found six days later (Table XIV). A higher level of ABA (10 μM) resulted in significant inhibition of aminopeptidase development as well. The plant hormone GA_3 (30 or 100 μM) had no effect by itself upon development of the peptide hydrolase activity. However, 30 μM GA_3 was able to partially reverse the inhibition of acid endopeptidase and carboxypeptidase development invoked by 2 μM ABA.

Cordycepin, an RNA synthesis inhibitor had no effect upon development of the peptide hydrolase activities, wherease incubation with cycloheximide, a protein synthesis inhibitor, resulted in a

large decrease in the development of the activities. Cordycepin also did not influence the inhibition of peptide hydrolase development caused by ABA. If ABA works through a poly A RNA intermediate, as has been suggested (20, 50) cordycepin might interfere with the hormone action. This has been observed with α-amylase in barley (50). The fact that cordycepin inhibits the development of α-amylase in barley and maize shows that it is having an effect on the system.

The effects of the hormones ABA and GA on haemoglobinase, as measured by Preston (106), were also observed (Table XV). Preston had found that this assay measured a carboxypeptidase activity in wheat (108). However, the induction of haemoglobinase activity in de-embryonated maize caryopses seems to show a pattern, intermediate between acid endopeptidase and carboxypeptidase. The percentage increase in activity of Hbase is greater than carboxypeptidase but less than acid endopeptidase. The percentage inhibition of Hbase by 10 μM ABA is greater than carboxypeptidase but less than acid endopeptidase.

Figure 16

Peptide Hydrolase Activities at Various Stages of Life Cycle of Zea mays

Caryopses of the maize hybrid W64A x W182E were prepared, plated and grown, as described in Materials and Methods. At various times after imbibition the seedlings were harvested and endosperms stored as described in Materials and Methods. The endosperms were homogenized and assayed for peptide hydrolase activities as described in Materials and Methods.

(A) Acid Endopeptidase (□——□)

Activity is expressed as µg Trp equivalents release/min/g fresh weight.

(B) Carboxypeptidase (△——△)

Activity is expressed as µg Ala released/min/g fresh weight.

(C) Aminopeptidase (□——□)

Activity is expressed as µg p-nitroaniline released/min/g fresh weight.

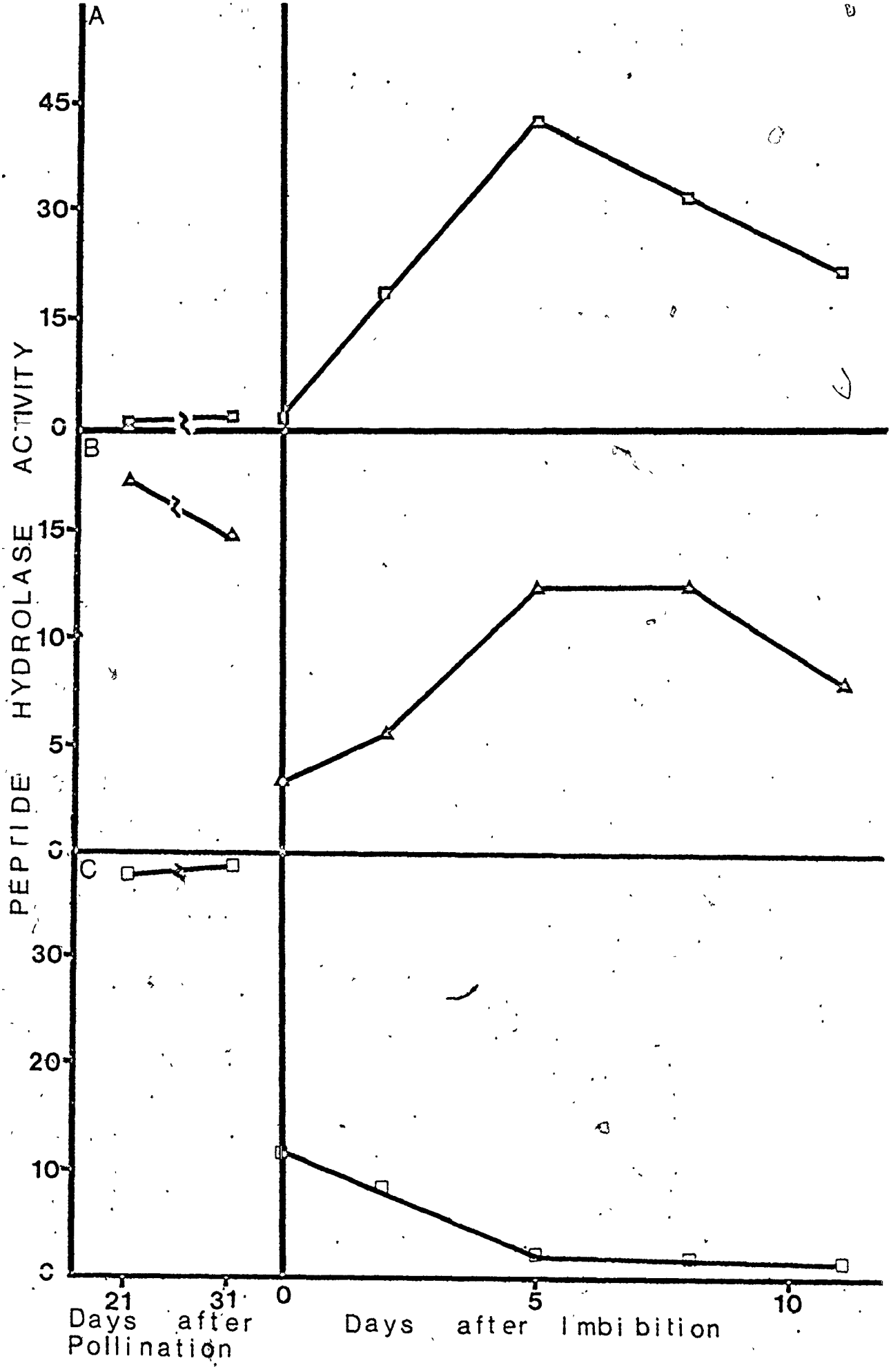

A
B
C
45
30
15
0
15
10
5
0
30
20
10
0
PEPTIDE HYDROLASE ACTIVITY
21
31
0
5
10
Days after Pollination
Days after Imbibition

Figure 17

Development of Acid Endopeptidase Activity

in Excised Endosperms of Zea mays

Endosperms of maize caryopses were prepared and incubated as described in Materials and Methods. Endosperms were harvested at various times after imbibition and assayed for acid endopeptidase as described earlier. Activity is expressed as µg Trp equivalents released/min/g fresh weight.

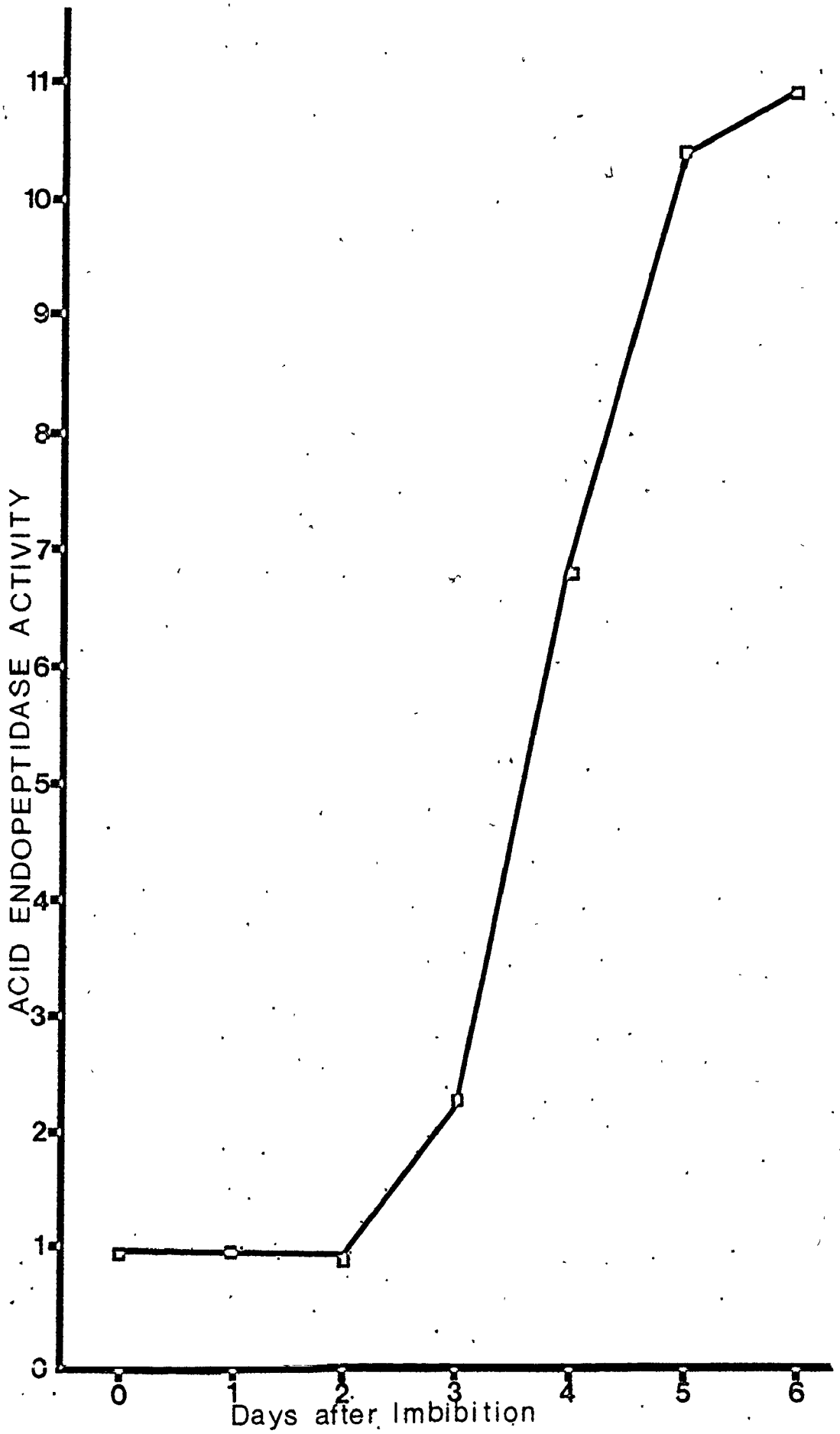

ACID ENDOPEPTIDASE ACTIVITY
0
1
2
3
4
5
6
7
8
9
10
11
0
1
2
3
4
5
6
Days after Imbibition

TABLE XIV

Effect of Abscisic Acid, Gibberellic Acid (GA_3), Cordycepin and Cycloheximide on Development of Hydrolase Activities in De-embryonated Caryopses

Caryopses were surfaced sterilized and the embryo and suctellum removed as outlined in the Methods. Endosperms were cultured at 28°C for 6 days in the appropriate medium. Units of the assays are:

Acid Endopeptidase: µg Trp equivalents released/min/g fresh weight
Carboxypeptidase: µg Ala released/min/g fresh weight
Aminopeptidase: µg p-nitroaniline released/min/g fresh weight
α-amylase: mg starch degraded/min/g fresh weight

Sample W64A x 182E	Acid Endopeptidase	Carboxypeptidase	Aminopeptidase	Amylase
0 Day	0.9	3.3	10.7	0.082
4 Day				
Control	1.5			2.82±0.12
100 µM Cordycepin	1.7			0.87±0.07
6 Day				
Control	6.9±0.5 (100)	11.0±0.3 (100)	15.9±0.5 (100)	
2 µM Abscisic Acid	2.8±0.6 (41) X	7.1±0.8 (65) x	15.4±0.8 (97)	
30 µM GA_3	7.3±1.7 (106)	10.6±1.5 (96)	13.3±1.6 (84)	
ABA and GA_3	5.8±1.3 (84) +	10.3±1.8 (94) +	12.4±0.7 (78)	

TABLE XIV (Continued)

Sample W64A x 182E	Acid Endopeptidase	Carboxypeptidase	Aminopeptidase	Amylase
Control	12.5±2.5 (100)	20.7±2.9 (100)	15.0±0.5 (100)	
10 µM Abscisic Acid	2.2±0.5 (18) X	7.8±0.9 (38) x	8.1±1.0 (54) X	
100 µM GA_3	10.7±2.5 (86)	19.1±1.3 (92)	13.0±1.3 (87)	
ABA and GA_3	1.7±0.5 (14) X	9.5±0.8 (46) x	9.2±0.1 (61) X	
Control	8.5±1.6 (100)	10.9±2.0 (100)	14.1±1.4 (100)	
2 µM Abscisic Acid	2.2±0.5 (26) X	6.8±1.2 (62) x	11.4±0.6 (81) x	
10 µM Cordycepin	9.5±0.2 (112)	11.9±4.3 (109)	13.6±0.5 (96)	
ABA and Cordycepin	2.3±1.0 (27) X	7.3±2.1 (67)	10.3±0.5 (73) x	
Control	12.5±2.5 (100)			
10 µM Abscisic Acid	2.2±0.5 (18) X			
100 µM Cordycepin	11.0±0.7 (89)			
ABA and Cordycepin	2.2±0.3 (18)			
Control	11.1±1.4 (100)	6.5±2.4 (100)	15.3±1.3 (100)	
5 µg/ml Cycloheximide	0.0±2.1 (0) X	1.3±0.4 (20) x	6.1±0.5 (40) X	
Maize from Dr. R.H. Hageman - 0 Day				3.6
3 Day - Control				43.3±1.2
- 30 µM GA_3				63.1±1.2

TABLE XIV (Continued)

Sample W64A x 182E	Acid Endopeptidase	Carboxypeptidase	Aminopeptidase	Amylase
Barley (Bonanza var. 1979) - 0 Day				1.2
1 Day - Control				11.4
- 30 µM GA_3				26.9
- 30 µM GA_3 and 100 µM Cordycepin				10.5

X - P < 0.01 and x - P < 0.05 compared to Control
++- P < 0.01 and + - P < 0.05 compared to plus ABA
Mean ± Standard Deviation
according to Student's t-test

Figures in brackets represent percentages of control.

TABLE XV

Comparison of Effects of Plant Hormones on Development of Acid Endopeptidase, Carboxypeptidase and Haemoglobinase

Endosperms were incubated under the conditions described in Table XIV. Some endosperm samples were analyzed for acid endopeptidase and carboxypeptidase activity. Other endosperms were analyzed for ability to hydrolyze haemoglobin according to the automated assay of Dr. K. Preston (106).

	Acid Endopeptidase µg Trp equiv/min/g	Hbase µmoles g/yg/y/g	Carboxypeptidase
0 Day	0.9 (13)	0.8 (22)	3.3 (30)
6 Day			
Control	6.9 (100)	3.6 (100)	11.0 (100)
+2 µM ABA	2.8 (40)	2.6 (72)	7.1 (65)
+2 µM ABA & 30 µM GA_3	5.8 (84)	3.3 (92)	10.3 (94)
Control	12.5 (100)	5.9 (100)	20.7 (100)
10 mM ABA	2.2 (18)	1.5 (25)	7.8 (38)
+100 µM GA_3	10.7 (86)	5.5 (93)	19.1 (92)
+ ABA & GA_3	1.7 (14)	2.9 (49)	9.5 (46)

Figures in brackets represent percentages of control.

DISCUSSION

Peptide Hydrolases of Germination and Early Seedling Growth

The importance of the peptide hydrolases in the hydrolysis of reserve proteins in cereal caryopses and specifically in maize has been outlined earlier. Some of the characteristics of these enzymes can be compared with other published results. For example, the acid endopeptidase activity showed maximum activity at pH 3.4-3.7, an optimum temperature of 55 - 60°C and is significantly inhibited by PCMB. Harvey (44) described an endopeptidase extracted from the maize hybrid Wf9 x 38-11 which hydrolyzed haemoglobin at pH optimum 3.8 and temperature optimum 46°C. The differences in results might be due to different cultivars of seed. Feller (29) also described an endopeptidase fraction, active on casein, with a wide pH optimum from 3.8-5.4. This activity measured at pH 3.8 or 5.4 showed significant inhibition by PMSF (1 mM), and by PHMB (1 mM) if no β-mercaptoethanol was added to the extract. Addition of β-mercaptoethanol reversed the inhibition by PHMB. PMSF (1 mM or 5 mM) seemed to inhibit the acid endopeptidase slightly in the present study, although the result was not statistically significant, according to Student's t-test. However, whereas Feller's study had used endosperm tissue from caryopses which were harvested eight days after imbibition the present study used six day tissue. Feller's use of a longer

incubation with the inhibitors may also have made the effects of PMSF more visible. Abe et al. (1) reported inhibition of a purified acid endopeptidase fraction by PCMB (1 mM) and iodoacetate (1 mM).

With carboxypeptidase, Feller, also saw a very significant inhibition by PMSF as well as slight inhibition by PHMB. Again, in the present study, there was only a slight effect by PMSF (not statistically significant) and at higher concentrations (5 mM) a significant inhibition by PCMB. For aminopeptidase activity, Feller saw significant inhibition by sulphydryl inhibitors PHMB and NEM. Inhibition by PCMB was evident in the present study.

The observation of inhibition by more than one active site reagent might suggest that more than one enzyme is functioning in the acid endopeptidase and carboxypeptidase assays. If only one enzyme is active, it is possible that the active site can be blocked by reaction with more than one amino acid in the enzyme.

The development of the acid endopeptidase activity is shown in Figure 16. The activity increases many fold during early seedling growth and reaches a peak on a fresh weight basis five to eight days after sowing. These results are similar to those of Harvey (43) and Lenz (70). They found that the peak in activity came eight days post imbibition. Feller (29) saw similar rises in caseolytic activity with peaks at about five days for the pH 3.8 assay and six days for the pH 5.4 assay. Carboxypeptidase showed a

parallel increase in both the present study and the work of Feller (29). The peak of activity for carboxypeptidase appeared slightly after the peak for acid endopeptidases in both cases. The general decline seen in aminopeptidase is a contrast and may suggest a different controlling mechanism. Lenz (70) saw a similar decline in another neutral peptidase (BAPNAase). These results suggest that acid endopeptidase and carboxypeptidase activity are important in hydrolysis of reserve proteins, whereas the neutral peptidases may play a different role, perhaps in the initiation of reserve protein hydrolysis as Lenz suggests (70).

The properties of peptidases have been most thoroughly studied in barley. Early work by Burger and co-workers (14, 15) with partially purified endopeptidases showed them to be inactive on protein substrates. Inhibitors were not tested. The peptidases were assayed at neutral-basic pH. One peptidase, a neutral BAEEase, was inactivated above pH 8.0. However, this enzyme proved to be more temperature stable than the others which were quickly inactivated at 45°C or above. This group also looked at protein degrading enzymes describing five peaks of activity on Sephadex G-100 and CM-cellulose (15). The proteins were active on haemoglobin, casein and gliadin. Bhatty (10) described the appearance of BAPAase and a haemoglobin degrading hydrolase in the germination period. Both were present to some extent in the dry caryopsis and increased to about two to three times that level by five days. Mikola's group reported a partially purified carboxypeptidase with a pH optimum at 5.2 and inhibited by

both DFP and PCMB (132). Later work, assaying the same activity in crude extracts but under similar conditions showed inhibition by PMSF (115).

In germinated wheat, Mountfield (85) described a protease which hydrolyzed edestin at about pH 4.0 and which was inactivated quickly above 55°C. He also reported the presence of neutral dipeptidases. Prentice et al. (103, 104) extended this work, showing a number of endopeptidase activities which increased with germination time and which were active at neutral to basic pH. Further observation has elucidated enzymes with carboxypeptidase activity in germinating wheat (69, 108). These enzymes showed maximum activity with haemoglobin at pH 4.2 and were inhibited by DFP.

Recently, workers have looked at changes in the SDS-polyacrylamide pattern of storage proteins during germination. In field beans (72) Lichtenfeld et al. investigated the changes in legumin and vicilin. In pumpkin seeds, Hara and Matsubara (40) observed that extracts from dry seeds contained an enzyme activity which could hydrolyze the α or β subunits of the pumpkin seed globulin (63,000 and 56,000 daltons respectively) to an subunit labelled F α, β (43,000 daltons). A second activity was not present in the dry seed but was present by the first day after imbibition. The activity showed some reactivity with the native globulin but was more active in hydrolyzing F α, β to smaller peptides. It increased in activity to day 4. The technique of observing changes in electrophoretic patterns

of native substrate proteins could be used with purified proteolytic enzymes (eg., from maize endosperm), as in mung bean (141).

Purification of Peptide Hydrolases

Purification of peptide hydrolases has generally supported the observation in crude extracts that there are numerous proteins of this type in the tissues of germinating seeds. For example, in this study of the endosperm of the corn caryopsis, at least three major haemoglobin hydrolyzing activities were recovered from CM-cellulose. Other recent reports from maize (1, 86) also suggest at least as many peptide hydrolases capable of hydrolyzing haemoglobin. For example, Abe et al. (1) found that two activity peaks were separated on preparative polyacrylamide gel electrophoresis. Moreaux (86) also reported three peptide hydrolases, capable of hydrolyzing haemoglobin, in germinated maize endosperm.

The purification results shown in Table XIII and Figures 11 and 12 illustrate that the major proteases degrading haemoglobin do not co-purify with protein possessing carboxypeptidase specificity as defined by the hydrolysis of CBZ-Phe-Ala. However, the partially purified protease in fraction 'A' may possess the ability to hydrolyze the carboxypeptidase substrate. Abe et al. (2) reported that their haemoglobin degrading endopeptidase, purified to apparent homogeneity, could hydrolyze a variety of CBZ-dipeptides: Those containing aromatic amino acids were hydrolyzed most quickly. Moreaux (86) attributed activity with CBZ-dipeptides in her partially

purified haemoglobin degrading enzymes to contaminating carboxypeptidases.

Figure 15 shows that in endosperm from maize seedlings, six days post-imbibition, haemoglobin degrading enzymes, as measured by Preston's method (106) co-chromatograph on a Phenylalanine-substituted Sepharose column (107), with enzymes active on azocasein (thought to be specifically endopeptidases). Abe et al. reported that purified maize protease could hydrolyze endopeptidase substrates including insulin B chain (2) while Moreaux reported that haemoglobin degrading enzymes could also hydrolyze zein and glutelin (86). The evidence from Abe's group, Moreaux's group and our work makes it clear that the haemoglobin degrading enzymes in maize endosperm, at six days post-imbibition are not specifically carboxypeptidases as Preston (108) has reported in wheat. As already mentioned, carboxypeptidases appear to be important during germination in the endosperm of barley (132) and in cotton cotyledons (53).

The enzymes which have been partially purified appear to be relatively samll proteins. The average molecular weight in the post ammonium sulphate extract was estimated at 30,000 daltons and the most purified fraction 'A' at 21,000 daltons. These estimations are close to those reported by Abe et al. (1) who isolated an enzyme with molecular weight of 21,000. Moreaux (86) also reported an aggregated form of 24,000 daltons (from two 12,000 dalton subunits) as well as fractions which contained proteins with molecular weights of 36,000 and 40,000 daltons.

The fact that the major haemoglobin degrading fractions are separated from activity on CBZ-Phe-Ala by Sephadex G-50 proves that these two activities are not part of the same enzyme function and are thus suitable to use in comparing the control of endopeptidase and carboxypeptidase activities in the endosperm of germinating maize caryopses.

Control of Peptide Hydrolase Production

(a) RNA Synthesis

RNA synthesis is detected within hours of imbibition in both the cereal embryo (96) and the aleurone layer of the cereal endosperm (see Table V in the Introduction) even though rRNA, t RNA and mRNA species have been isolated from dry seeds. In fact, increased activity of template mRNA for production of α-amylase, one of the hydrolase enzymes, has been observed in the barley aleurone layer (48), following stimulation by the hormone gibberellic acid. This could be due to some effect of GA in decreasing the breakdown of mRNA or in activating the mRNA for translation. However, the current interpretation of the results suggest an increased synthesis. In cotton cotyledons mRNA for post-germination carboxypeptidase is preformed during embryogenesis (51) but apparently needs to be activated by polyadenylation (133). In studying the appearance of any new enzyme activity during germination of a seed, one can then ask whether it is mediated by new mRNA synthesis or translation of stored mRNA.

In cereals, most work on the metabolism of the endosperm following imbibition has been conducted on barley aleurone tissue (see Table V in the Introduction). Incubation with RNA synthesis inhibitors, 6-methylpurine, actinomycin D or the polyadenylation inhibitor (mRNA synthesis inhibitor), cordycepin can indicate whether RNA synthesis is needed to induce production of peptide hydrolase activities. In barley aleurone layers (55) 6-methylpurine and actinomycin D inhibited the development of a protease activity. In germinating wheat (110) incubation with actinomycin D or cordycepin inhibited development of endo- and exo-proteolytic activity in the endosperm only slightly. In de-embryonated maize, Harvey (46) showed inhibition of development of acid endopeptidase by 6-methylpurine and actinomycin D. Thus, it appears that RNA synthesis may be needed for development of certain peptide hydrolase activities in cereal endosperms. Although general synthesis of RNA may be needed for production of some peptide hydrolases, it is not yet proven that template mRNA must be synthesized de novo for those peptide hydrolases.

In the present work cordycepin did not significantly inhibit the development of peptide hydrolases in de-embryonated maize, although cordycepin did inhibit development of α-amylase in barley and maize (see Table XIV). Cordycepin (3-deoxyadenosine) is an RNA chain terminator, and is most effective in halting mRNA processing since it blocks activation of the mRNA by polyadenylation. The present result is in contradiction to the inhibition of the

development of acid endopeptidase and α-amylase seen by Harvey with the general RNA synthesis inhibitors actinomycin D and 6-methylpurine in maize (46). As mentioned, cordycepin has a very slight inhibitory effect on peptide hydrolase development in wheat (110) and can inhibit development of α-amylase in barley aleurone layers if it is added within twelve hours of imbibition (50). The present result with cordycepin in maize may indicate the mRNA for acid endopeptidase and carboxypeptidase is preformed. Although Harvey (46) showed that general RNA synthesis is necessary for development of maize α-amylase and acid endopeptidase, it is possible that mRNA synthesis is not necessary.

(b) Protein Synthesis

Protein synthesis begins early in germinating seeds, being translated from preformed mRNA (25). In cotton embryos, Hammett and Katterman (39) have suggested that preformed mRNA molecules do not need to be polyadenylated before being translated. Similarly, in wheat embryos polyribosomes can form when polyadenylation is blocked with cordycepin (120). However, in cotton cotyledons, stored mRNA for carboxypeptidase apparently needs to be polyadenylated (133). In wheat embryos there is a large increase in protein synthesis in the first five hours after imbibition. However, there is no growth of the embryo during this initial period (79). Presumably, early protein synthesis helps prepare the embryo for growth.

In barley endosperm tissue, Filner and Varner (30) have established de novo synthesis of protein and specifically the hydrolase proteins α-amylase and a proteinase during germination. In wheat caryopses, Preston (110) saw drastic decreases in the production of endosperm endo- and exo-protease activity and almost no hydrolysis of reserve protein when seeds were germinated in the presence of cycloheximide. In embryoless wheat seeds there was no effect on Hbase (exopeptidase) but development of azocaseinase (endopeptidase) activity was blocked. Harvey (43) found that addition of cycloheximide (5 µg/ml) at any time during incubation of embryoless maize caryopses caused the cessation of peptide hydrolase or amylase production and decreased endosperm and starch breakdown. This indicates de novo synthesis of the peptide hydrolase enzyme.

In the present work, the effect of addition of cycloheximide (5 µg/ml) to embryoless caryopses was seen to be an inhibition of development for both acid endopeptidase and carboxypeptidase, indicating that general protein synthesis was needed for development of these activities.

Thus, in cereal endosperms, the evidence suggests that protein synthesis is needed for the development of peptide hydrolase activities (30, 55, 110) and in barley it has been proven, using radioactive tracers and density labelling that a peptide hydrolase enzyme (55) as well as α-amylase (30) are actually synthesized de novo.

(c) Role of ABA

Plant hormones can regulate the appearance of peptide hydrolase activities by acting at the level of transcription or translation. If RNA synthesis is required, the hormone might interfere by binding to the DNA template or by interfering with the processing of the mRNA. At the level of translation, the hormones might bind to the mRNA template or interfere with the processing of a zymogen for peptide hydrolase activity.

Abscisic acid was shown to inhibit both acid endopeptidase and carboxypeptidase development during incubation of de-embryonated maize caryopses, while only a very high concentration (10 μM) stopped the small increase observed in aminopeptidase in de-embryonated caryopses (Table XIV). This confirms the observation made earlier by Harvey (46) for acid endopeptidase and the observation of a similar inhibition for carboxypeptidase supports the hypothesis that both of these activities are under similar hormonal control. The degree of inhibition was greatest for acid endopeptidase. Many groups have demonstrated an ABA inhibition of hydrolase development and some have shown that ABA can cause embryo dormancy in certain species (6, 80). Thus, some observers have claimed a universal role for ABA in inducing seed dormancy or preventing germination (4).

Abscisic acid reverses many of the effects of GA in the barley aleurone layer (see Table V), including synthesis of α-amylase and protease. Ihle and Dure (52, 54) originally showed that ABA prevents translation of the mRNA for carboxypeptidase in cotton-seed. The mRNA is known to be transcribed during embryogenesis (51). In barley aleurone tissue, Varner has postulated that ABA functions to prevent hydrolase synthesis by interfering with the translation of mRNA (50).

The present results confirm the observation that cordycepin can inhibit the appearance of α-amylase in barley and show this to be true in maize also. Yet in maize cordycepin does not prevent the appearance of the peptide hydrolase activities, suggesting that the mRNA for these enzymes is already present in maize. ABA does inhibit development of the peptide hydrolase activities. It may then function at the level of translation. If cordycepin is added twelve hours after imbibition of barley aleurone layers, the ABA inhibition of GA stimulated amylase production can be reversed (50), leading Ho and Varner to suggest that cordycepin acts via an RNA intermediate. However, cordycepin did not inhibit the ABA effect in maize (see Table XIV).

(d) Role of Gibberellic Acid

This hormone did not significantly increase levels of the peptide hydrolase activities in cultured de-embryonated caryopses. This is in agreement with results seen by Harvey (46) in maize but

is contrary to results seen in many seeds and particularily cereals (55, 62, 110). However, GA_3 was able to partially reverse the inhibition of acid endopeptidase and carboxypeptidase development caused by ABA. Thus, as in many other systems GA_3, does show an antagonistic effect with ABA (126). The similar responses of acid endopeptidase and carboxypeptidase (Table XIV) support the hypothesis that both these enzymes are under the same hormonal regulation.

A number of workers have suggested de novo synthesis of GA_3 during germination (62). This can be tested by incubation of germinating seed with compounds which can interfere with gibberellin biosynthesis, such as AMO-1618, CCC or Phosphon D. Harvey (46) found a partial inhibition of protease and amylase production by these compounds in de-embryonated maize caryopses at high concentration. However, since it was not possible to reverse the effect by addition of exogenous GA_3 she concluded that the effect was a non-specific one. It was concluded that GA was present in the dry endosperm and was not synthesized in endosperm pieces following imbibition.

The mode of action of GA_3 in affecting germination, as for ABA, has not been well characterized. Although some workers have hypothesized that the induction of hydrolase production by GA_3 is the primary event which controls germination of the embryo (4, 33) a number of effects previous to hydrolase production are observed,

eg., in barley aleurone tissue (Table V). In some seeds, concentrations of GA_3 which induce some hydrolase production do not induce germination (17). As already mentioned, germination preceeds development of α-amylase activity in rye and wild oats (16, 23). Thus, it appears that the initial effect of GA is on embryo dormancy itself (17). A second direct effect of GA_3 could be on the induction of the hydrolases. This latter effect would be essential for the continued growth of the seedling.

The mechanism by which GA_3 stimulates production of hydrolases is not well understood. In barley aleurone tissue, GA_3 does not affect synthesis of total RNA (56), but it does cause an increase in a poly A rich mRNA which has been identified as the mRNA for α-amylase (48, 49, 56). It was unclear whether increased activation, turnover or synthesis of mRNA was responsible, but the researchers suggested that increased synthesis was most probable. Workers have shown that RNA synthesis is necessary for the GA effect (21, 37). It is not clear whether the RNA synthesis inhibitors interfere with the GA stimulated production of mRNA specific for peptide hydrolases or some other secondary but necessary RNA synthesis. The current model in barley aleurone cells is that GA stimulates increased synthesis of mRNA for the hydrolases, leading to increased production of the proteins (48, 49). ABA would function by inducing the production of an RNA intermediate which could

interfere with translation of the GA stimulated message.

In the present work with maize, GA_3 had no significant effect upon induction of peptide hydrolase activities, although it did reverse ABA inhibition. Harvey (43) has postulated that high endogenous levels of GA in the mature maize caryopsis obscure any effect by added GA and that the mechanism of induction is similar to barley.

MODEL OF RESERVE PROTEIN HYDROLYSIS IN MAIZE ENDOSPERM

From previous work in maize (1, 2, 29, 43, 44, 45, 46, 70, 82, 86) and from results reported in this thesis it is possible to present an updated model of reserve protein hydrolysis of peptide hydrolases in maize endosperm (see Figure 18). The results are consistent with the following sequence of events.

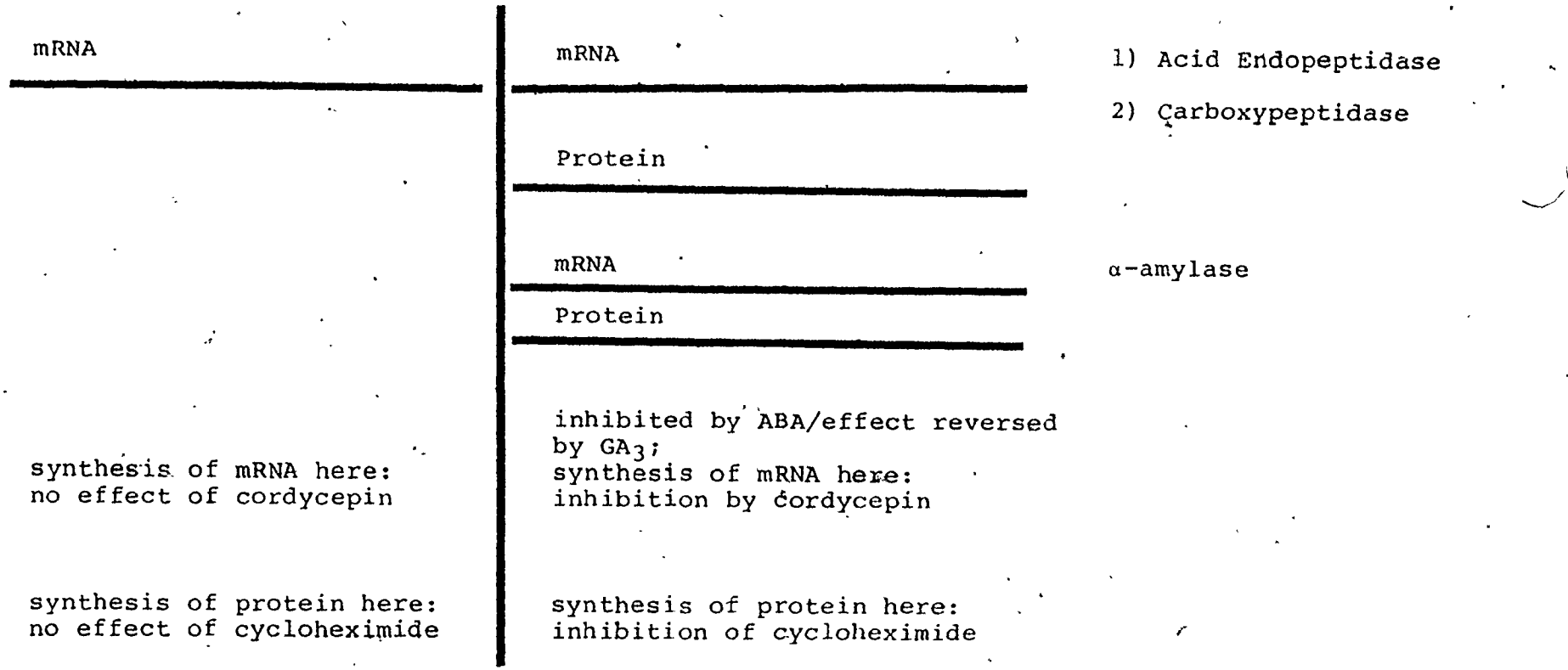
1. Imbibition of dry maize caryopsis results in activation of protease activity (possibly BAPNase or Leu-aminopeptidase) which denatures or solubilizes native storage proteins (zein and possibly glutelin). This can occur because of a favourable ABA:GA ratio .
2. Starting on Day 2, de novo α-amylase synthesis results in initiation of starch hydrolysis.
3. Starting on Day 3, production of acid proteases and peptidases (acid endopeptidase and carboxypeptidase) begins hydrolysis of zein and glutelin.
4. Increase in α-amylase and peptide hydrolase activities from Day 4-8 results in hydrolysis of starch and storage proteins (zein and glutelin). Sugars and amino acids are transported to the embryo or used in the endosperm.
5. Amylase and peptide hydrolase activities decline as storage reserves are depleted.

Future research should initially concentrate on determining which proteolytic enzymes are capable of hydrolyzing the native or denatured natural reserve proteins of maize endosperm, zein and glutelin. To determine that it would be necessary to purify the protein to homogeneity and then to show that the protein can hydrolyze the reserve proteins. This could be done for the denatured reserve proteins by suspending them in an agar gel and using the assay described by Harvey (40). It might be possible to show hydrolysis of native reserve proteins by incubating excised endosperms with the purified enzyme and cycloheximide and then measuring the disappearance of zein and glutelin. Alternatively, one could observe the change in the SDS polyacrylamide electrophoresis pattern of zein or glutelin after incubation with a purified enzyme. The appropriate active site reagent should be shown to inhibit both the proteolytic enzyme assay used to detect the enzyme (eg., haemoglobin hydrolysis) as well as the hydrolysis of the reserve proteins.

Figure 18

Model for Induction of Hydrolase Enzymes

in Maize Endosperm


mRNA
mRNA
Protein
mRNA
Protein
1) Acid Endopeptidase
2) Carboxypeptidase
α-amylase
inhibited by ABA/effect reversed by GA_3;
synthesis of mRNA here: inhibition by cordycepin
synthesis of mRNA here: no effect of cordycepin
synthesis of protein here: inhibition of cycloheximide
synthesis of protein here: no effect of cycloheximide
Imbibition

SUMMARY

From work outlined in this thesis it is seen that there are a number of protein fractions with acid endopeptidase activity (Figures 11, 12) and that it is possible to use haemoglobin and Z-CBZ-Phe-Ala to measure distinct acid endopeptidase and carboxypeptidase activities respectively (Table XIII). Both acidic endopeptidase and carboxypeptidase show parallel patterns of development (Figure 16) and appear to be under similar metabolic and hormonal control (Table XIV). General protein synthesis is required for the appearance of the two activities. ABA inhibits development of the activities and GA can overcome this inhibition. Aminopeptidase follows a very different pattern of development (Figure 16). The properties and development of aminopeptidase are more similar to BAPNAase, reported by Lenz (62).

REFERENCES

1. Abe, M., S. Arai and M. Fujimaki, 1977. Purification and characterization of a protease occurring in the endosperm of germinating corn. Agric. Biol. Chem. 41: 893-899.
2. Abe, M., S. Arai and M. Fujimaki, 1978. Substrate specificity of a sulfhydryl protease purified from germinating corn. Agric. Biol. Chem. 42: 1813-1817.
3. Altschul, A., L. Yatsu, R.L. Ory and E.M. Engleman, 1966. Seed proteins. Ann. Rev. Plant Physiol. 17: 113-136.
4. Amen, R.D., 1968. A model of seed dormancy. Bot. Rev. 34: 1-31.
5. Ashton, F.M., 1976. Mobilization of storage proteins of seeds. Ann. Rev. Plant Physiol. 27: 95-117.
6. Aspinall, D., L.G. Paleg and F.T. Addicott, 1967. Abscisin II and some hormone-regulated plant responses. Aust. J. Biol. Sci. 20: 869-882.
7. Barrett, A.J. and J.T. Dingle, 1971. Tissue proteinases. North Holland Publishing Co., Amsterdam or American Elsevier Publishing Co., New York.
8. Berlyn, G.P., 1972. In Seed Biology, T.T. Kozlowski ed, Academic Press, New York. pp. 223-312.

9. Bewley, J.D. and M. Black, 1978. Physiology and biochemistry of seeds. Vol. 1, Springer-Verlag; Berlin.

10. Bhatty, R.S., 1969. Note on the development of proteolytic enzymes in germinating barley. Cereal Chem. 46: 74-77.

11. Blackburn, S., 1976. Enzyme structure and function. Marcel Dekker Inc., New York.

12. Boyer, P.D., 1971. The Enzymes. Vol. III, Hydrolysis of peptide bonds, Academic Press, New York.

13. Burger, W.C. and H.W. Siegelman, 1966. Location of a protease and its inhibitor in the barley kernel. Physiol. Plant. 19: 1089-1093.

14. Burger, W.C., N. Prentice, J. Kastenschmidt and M. Moeller, 1968. Partial purification and characterization of barley peptide hydrolases. Phytochem. 7: 1261-1270.

15. Burger, W.C., N. Prentice, M. Moeller and G.S. Robbins, 1970. Stabilization, partial purification and characterization of peptidyl hydrolases from germinating barley. Phytochem. 9: 49-58.

16. Chen, S.S.C. and J.L.L. Chang, 1972. Does gibberellic acid stimulate seed germination via amylase synthesis. Plant Physiol. 49: 441-442.

17. Chen, S.S.C. and W.M. Park, 1973. Early actions of gibberellic acid on the embryo and on the endosperm of Avena fatua seeds. Plant Physiol. 52: 174-176.

18. Chen, S.S.C. and J.E. Varner, 1973. Hormones and seed dormancy. Seed Sci. and Technol. 1: 325-338.

19. Chrispeels, M.J. and J.E. Varner, 1965. Gibberellic-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol. 42: 398-406.

20. Chrispeels, M.J. and J.E. Varner, 1966. Inhibition of gibberellic acid induced formation of α-amylase by abscisin II. Nature (London 212: 1066-1067.

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

22. Collins, G.G., C.F. Jenner and L.G. Paleg, 1972. The levels of soluble nucleotides in wheat aleurone tissue. Plant Physiol. 49: 398-403.

23. Drennan, D.S.H. and A.M. Berrie, 1962. Physiological studies of germination in the Genus Avena. I. The development of amylase activity. New Phytol. 61: 1-9.

24. Dure, L.S., 1960. Gross nutritional contribution of maize endosperm and scutellum to germination and growth of maize axis. Plant Physiol. 35: 919-925.

25. Dure, L.S., 1977. In the Physiology and Biochemistry of Seed Dormancy and Germiantion. A.A. Khan ed., North Holland Publishing Company, New York, pp. 335-345.

26. Evins, W.H. and J.E. Varner, 1971. Hormone controlled synthesis of endoplasmic reticulum in barley aleurone cells. P.N.A.S., U.S.A. 68: 1631-1633.

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

28. Evins, W.H. and J.E. Varner, 1972. Hormonal control of polyribosome formation in barley aleurone layers. Plant Physiol. 49: 348-352.

29. Feller, U., T.-S. T. Soong and R.H. Hageman, 1978. Patterns of proteolytic enzyme activities in different tissues of corn (Zea mays L.). Planta 140: 155-162.

30. Filner, P. and J.E. Varner, 1967. A test for de novo synthesis of enzymes: density labelling with H_2O^{18} of barley α-amylase induced by gibberellic acid. P.N.A.S., U.S.A. 58: 1520-1526.

31. Flint, D.H. and J.E. Varner, 1973. Early effects of GA and ABA on protein synthesis in barley aleurone cells. Plant Physiol. 51: supp. p. 5.

32. Gabriel, O., 1971. In Methods in Enzymology, Vol. XXII, W.G. Jakoby ed., Academic Press, New York, pp. 576-578.

33. Galston, A. and P.J. Davies, 1969. Hormonal regulation in higher plants. Science 163: 1288-1297.

34. Garg, G.K. and T.K. Virupaksha, 1970. Acid protease from germinating sorghum. I. Purification and characterization of the enzyme. Eur. J. Biochem. 17: 4-12.

35. Gitler, G., 1964. Protein digestion and absorption by non-uminants in Mammalian Protein Metabolism. Ed. H.N. Munro and J.B. Allison, Academic Press, New York, pp. 35-69.

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

37. Goodwin, P.B. and D.J. Carr, 1972. Actinomycin D and the hormonal induction of amylase synthesis in barley aleurone layers. Planta 106: 1-9.

38. Grant, D.G. and C.C. Wang, 1972. Dialyzable components resulting from proteolytic activities in extracts of wheat flour. Cereal Chem. 49: 201-208.

39. Hammett, J.R. and F.R. Katterman, 1975. Storage and metabolism of poly (adenylic acid) mRNA in germinating cotton seeds. Biochemistry 14: 4375-4379.

40. Hara, I. and H. Matsubara, 1980. Pumpkin (Cucurbita sp.) seed globulin. V. Proteolytic activities involved in globulin degradation in ungerminated seeds. Plant and Cell Physiol. 21: 217-232.

41. Hara, I. and H. Matsubara, 1980. Pumpkin (Cucurbita sp.) seed globulin. VI. Proteolytic activities appearing in germinating cotyledons. Plant and Cell Physiol. 21: 233-245.

42. Hara, I. and H. Matsubara, 1980. Pumpkin (Cucurbita sp.) seed globulin, VII. Immunofluorescent study on protein bodies in ungerminated and germinating cotyledon cells. Plant and Cell Physiol. 21: 2-7-254.

43. Harvey, B.M.R., 1973. Hydrolysis of endosperm proteins in germinating maize. Ph.D. Thesis, McMaster University.

44. Harvey, B.M.R. and A. Oaks, 1974. Characteristics of an acid protease from maize endosperm. Plant Physiol. 53: 449-452.

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

46. Harvey, B.M.R. and A. Oaks, 1974. The role of gibberellic acid in the hydrolysis of endosperm reserves in Zea mays. Planta 121: 67-74.

47. Hayward, H.E., 1938. The Structure of Economic Plants. MacMillan Publishing Co., pp. 111-140.

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

49. Ho, D.H. and J.E. Varner, 1974. Hormonal control of messenger RNA metabolism in barley aleurone layers. P.N.A.S., U.S.A. 71: 4783-4786.

50. Ho, D.T.-H. and J.E. Varner, 1976. Responses of barley aleurone layers to abscisic acid. Plant Physiol. 57: 175-178.

51. Ihle, J.N. and L.S. Dure III, 1969. Synthesis of a protease in germinating cotton cotyledons catalyzed by mRNA synthesized during embryogenesis. Biochem. Biophys. Res. Comm. 36: 705-710.

52. Ihle, J.N. and L.S. Dure III, 1970. Hormonal regulation of translation inhibition requiring RNA synthesis. Biochem. Biophys. Res. Comm. 38: 995-1001.

53. Ihle, J.N. and L.S. Dure III, 1972. The developmental biochemistry of cottenseed embryogenesis and germination. I. Purification and Properties of a carboxypeptidase from germinating cotyledons. J. Biol. Chem. 247: 5034-5040.

54. Ihle, J.N. and L.S. Dure III, 1972. The developmental biochemistry of cottonseed embryogenesis and germination. III. Regulation of the enzymes utilized in germination. J. Biol. Chem. 247: 5048-5055.

55. Jacobsen, J.V. and J.E. Varner, 1967. Gibberellic acid induced synthesis of a protease by isolated aleurone layers of barley. Plant Physiol. 42: 1596-1600.

56. Jacobsen, J.V. and J.A. Zwar, 1974. Gibberellic acid causes increases in synthesis of RNA which contains poly A in barley aleurone tissue. P.N.A.S., U.S.A. 71: 3290-3293.

57. Johnson, K.D. and H. Kende, 1971. Hormonal control of lecithin synthesis in barley aleurone cells: regulation of the CDP-choline pathway by gibberellin. P.N.A.S., U.S.A. 68: 2674-2677.

58. Johnson, K.D. and M.J. Chrispeels, 1973. Regulation of Pentosan biosynthesis in barley aleurone tissue by gibberellic acid, Planta 111: 353-364.

59. Jones, R.L., 1969. Gibberellic acid and the fine structure of barley aleurone cells. I. Changes during the lag phase of α-amylase synthesis. Planta 87: 119-132.

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

61. Jones, R.L., 1971. Gibberellic acid enhanced release of β-1, 3-glucanase from barley aleurone cells. Plant Physiol. 47: 412-416.

62. Jones, R.L. and J.L. Stoddart, 1977. In the Physiology and Biochemistry of Seed Dormancy and Germination. A.A. Khan ed., North Holland Publishing Co., New York, pp. 77-109.

63. Kaminski, E. and W. Bushuk, 1969. Wheat proteases. I. Separation and detection by starch-gel electrophoresis. Cereal Chem. 46: 317-324.

64. Khan, A.A. and C.E. Heit, 1969. Selective effects of Hormones on nucleic acid metabolism during germination of pear embryos. Biochem. J. 113: 707-712.

65. Khan, A.A. and E.C. Waters, 1969. On the hormonal control of post-harvest dormancy and germination in barley seeds. Life Sciences 8-II: 729-736.

66. Kochler, D.E. and J.E. Varner, 1973. Hormonal control of orthophosphate incorporation into phospholipids of barley aleurone layers. Plant Physiol. 52: 208-214.

67. Kolehmainen, L. and J. Mikola, 1971. Partial purification and enzymatic properties of an aminopeptidase from barley. Arch. Biochem. Biophys. 145: 633-642.

68. Kruger, J.E., 1971. Purification and some properties of malted wheat BAPAse. Cereal Chem. 48: 512-522.

69. Kruger, J.E. and K. Preston, 1977. The distribution of carboxypeptidases in anatomical tissues of developing and germinating wheat kernels. Cereal Chem. 54: 167-174.

70. Lenz, D.E., 1978. Hydrolysis of maize endosperm. M.Sc. Thesis, McMaster University.

71. Leonard, W.H. and J.H. Martin, 1963. Cereal Crops. MacMillan Publishing Co. Inc., New York, pp. 146-172.

72. Lichtenfeld, C., R. Manteuffel, K. Muntz, D. Neuman, G. Scholz, and E. Weber, 1979. Protein degradation and proteolytic activities in germinating field beans (Vicia faba, var. minor). Biochem: Physiol. Pflazen 174: 255-274.

73. Lowry, O.H., N.J. Rosenburg, A.L. Farr and R.J. Randall, 1951. Protein measurement with folin-phenol reagent. J. Biol. Chem. 193: 265-275.

74. Luers, H., 1936. Changes in enzymes during malting. Cereal Chem. 13: 153-171.

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

76. MacGregor, A.W., 1978. Changes in α-amylase enzymes during germination. J. Am. Soc. Brew. Chem. 36: 1-12.

77. MacLeod, A.M. and G.H. Palmer, 1967. Gibberellin from barley embryos. Nature 216: 1342-1343.

78. Mahaboob Basha, S.M. and J.P. Cherry, 1978. Proteolytic enzyme activity and storage protein degradation in cotyledons of germinating peanut (Arachis hypogaea) seeds. J. Agric. Food Chem. 26: 229-234.

79. Marcus, A., J. Feeley and T. Volcani, 1966. Protein synthesis in imbibed seeds. III. Kinetics of amino acid incorporation, ribosome activation and polysome formation. Plant Physiol. 41: 1167-1172.

80. Mayer, A.M. and A. Poljakoff-Mayber, 1975. The Germination of Seeds. Pergamon Press, New York.

81. McDonald, C.E. and L.L. Chen, 1964. Properties of wheat flour proteinases. Cereal Chem. 41: 433-455.

82. Melville, J.C. and J.G. Scandalios, 1972. Maize endopeptidase: genetic control, chemical characterization and relationship to endogenous trypsin inhibitors. Biochem. Genetics 7: 15-33.

83. Mertz, E.T., L.S. Bates and O.E. Nelson, 1964. Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science 145: 279-280.

84. Mikola, J. and L. Kolehmainen, 1972. Localization and activity of various peptidases in germinating barley. Planta 104: 167-177.

85. Mountfield, J.D., 1936. The proteolytic enzymes of sprouted wheat. Biochem. J. 30: 549-557.

86. Moreaux, T., 1979. Protein breakdown and protease properties of germinating maize endosperm. Phytochem. 18: 1173-1177.

87. Naylor, J.M., 1966. Dormancy studies in seeds of Avena fatua. V. On the response of aleurone cells to gibberellic acid. Can. J. Bot. 44: 19-32.

88. Naylor, J.M. and G.M. Simpson, 1961. Dormancy studies in seeds of Avena fatua. II. A gibberellin-sensitive inhibitory mechanism in the embryo. Can. J. Bot. 39: 281-295.

89. Nelson, O.E., E.T. Mertz and L.S. Bates, 1965. Second mutant gene affecting the pattern of maize endosperm proteins. Science 150: 1469-1470.

90. Nielsen, H.C., J.W. Paulis, C. James and J.S. Wall, 1970. Extraction and structure studies on corn glutelin proteins. Cereal Chem. 47: 501-512.

91. Nowak, J. and T. Mierzwinska, 1978. Activity of proteolytic enzymes in rye seeds of different ages. Z. fur Pflanzenphys. 86: 15-22.

92. Oaks, A., 1965. The synthesis of leucine in maize embryos. Biochim. Biophys. Acta 111: 79-89.

93. Oaks, A. and H. Beevers, 1964. The requirements for organic nitrogen in Zea mays embryos. Plant Physiol. 39: 37-43.

94. Ory, R.L. and K.W. Hennigsen, 1969. Enzymes associated with protein bodies isolated from ungerminated barley seeds. Plant Physiol. 44: 1488-1498.

95. Osborne, T.B., 1897. The amount and properties of the proteins of the maize kernel. J. Am. Chem. Soc. 19: 525-534.

96. Osborne, D.J., 1977. Nucleic acids and seed germination. In The Physiology and Biochemistry of Seed Dormancy and Germination. A.A. Khan, ed., North Holland Publishing Co., New York, pp. 319-333.

97. Paleg, L.G., 1960. Physiological effects of gibberellic acid. I. On carbohydrate metabolism and amylase activity in barley endosperm. Plant Physiol. 35: 293-299.

98. Paleg, L.G., 1960. Physiological effects of gibberellic acid. II. On starch hydrolyzing enzymes of barley endosperm. Plant Physiol. 35: 902-906.

99. Paleg, L.G., 1961. Physiological effects of gibberellic acid. III. Observation on its mode of action on barley endosperm. Plant Physiol. 36: 829-837.

100. Paleg, L.G., 1962. Physiological effects of gibberellic acid. V. Endosperm responses of barley, wheat and oats. Plant Physiol. 37: 798-803.

101. Paleg, L.G., 1965. Physiological effects of gibberellins. Ann. Rev. Plant Physiol. 16: 291-322.

102. Pett, L.B., 1935. Distribution of enzymes in wheat seeds. Biochem. J. 29: 1898-1904.

103. Prentice, N., W.C. Burger, J. Kastenschmidtand and J.D. Huddle, 1967. The distribution of acidic and neutral poptidases in barley and wheat kernels. Physiol. Plantarum 20: 361-367.

104. Prentice, N., W.C. Burger and M. Moeller, 1968. Partial purification and characterization of peptide hydrolases from germinated wheat. Phytochem. 7: 1899-1905.

105. Prentice, N., W.C. Burger, M. Moeller and J. Kastenschmidt, 1969. Characterization of an acid-stable peptide hydrolase and of peptide hydrolase A in germinated wheat. Phytochem. 8: 1897-1900.

106. Preston, K., 1975. An automated assay for proteolytic activity in cereals. Cereal Chem. 52: 451-458.

107. Preston, K.R., 1978. Note on the separation and partial purification of wheat proteases by affinity chromatography. Cereal Chem. 55: 793-798.

108. Preston, K.R. and J.E. Kruger, 1976. Purification and properties of two proteolytic enzymes with carboxypeptidase activity in germinated wheat. Plant Physiol. 58: 516-520.

109. Preston, K.R. and J.E. Kruger, 1976. Location and activity of proteolytic enzymes in developing wheat kernels. Can. J. Plant Sci. 56: 217-223.

110. Preston, K.R. and J.E. Kruger, 1979. Physiological control of exo- and endoproteolytic activities in germinating wheat and their relationship to storage protein hydrolysis. Plant Physiol. 64: 450-454.

111. Preston, K.R., J.E. Dexter and J.E. Kruger, 1978. Relationship of exoproteolytic and endoproteolytic activity to storage protein hydrolysis in germinating durum and hard red spring wheat. Cereal Chem. 55: 877-888.

112. Radley, M., 1959. Occurrence of gibberellin-like substances in barley and malt. Chem. and Ind., pp. 877-878.

113. Rowsell, E.V. and L.S. Goad, 1964. Some effects of gibberellic acid on wheat endosperm. Biochem. J. 90: 11.

114. Ryan, C.A., 1973. Proteolytic enzymes and their inhibitors in plants. Ann. Rev. Plant Physiol. 24: 173-196.

115. Schroeder, R.L. and W.C. Burger, 1978. Development and localization of carboxypeptidase activity in embryo-less barley half-kernels. Plant Physiol. 62: 458-462.

116. Segal, H.L. and D.J. Doyle, 1978. Protein Turnover and Lysosome Function, Academic Press, New York.

117. Simpson, G.M., 1965. Dormancy studies in Avena fatua. IV. The role of gibberellin in embryo dormancy. Can. J. Bot. 43: 793-816.

118. Sondheimer, E. and E.C. Galston, 1966. Effects of abscisin II and other plant growth substances in germination of seeds with stratification requirements. Plant Physiol. 41: 1397-1398.

119. Soong, T.-S.T., F.E. Below and R.H. Hageman, 1979. Synthesis and release of proteolytic enzymes in scutella of germinating corn. Plant Physiol. 63: supp. 69.

120. Spiegel, S. and A. Marcus, 1975. Polyribosome formation in early wheat embryo germination independent of either transcription or polyadenylation. Nature, London 256: 228-230.

121. Sundblom, N.O. and J. Mikola, 1972. On the nature of proteinases secreted by the aleurone layer of the barley grain. Physiol. Plantarum 27: 281-284.

122. Sutcliffe, J.F. and Q.A. Baset, 1973. Control of Hydrolysis of reserve materials in the endosperm of germinating oats. Plant Sci. Letters 1: 15-20.

123. Swar, J.A. and J.V. Jacobsen, 1972. A correlation between an mRNA fraction selectively labelled in the presence of gibberellic acid and amylase synthesis in barley aleurone layers. Plant Physiol. 49: 1000-1004.

124. Sylvester-Bradley, R. and B.F. Folkes, 1976. Cereal grains; their protein components and nutritional quality. Sci. Prog. Oxford 63: 241-263.

125. Taiz, L. and J.E. Starks, 1977. Gibberellin acid enhancement of DNA turnover in barley aleurone cells. Plant Physiol. 60: 182-189.

126. Tao, K.-L. and A.A. Khan, 1977. Hormonal regulation of nucleic acids and proteins in germination. In The Biochemistry and Physiology of Seed Dormancy and Germination. A.A. Khan, ed., North Holland Publishing Co., New York, pp. 413-433.

127. Tomomatsu, A., N. Iwatsuki and T. Asahi, 1978. Purification and properties of two enzymes hydrolyzing synthetic substrates N-α-Benzoyl-D, L-arginine paranitroaniline and leucine paranitroaniline from pea seeds. Agric. Biol. Chem. 42: 315-322.

128. Tully, R.E. and H. Beevers, 1978. Proteases and peptidases of castor bean endosperm-enzyme characterization and changes during germination. Plant Physiol. 62: 746-750.

129. Turner, J.E., J.A. Boundy and R.J. Dimler, 1965. Zein: A heterogenous protein containing disulfide-linked aggregates. Cereal Chem. 42: 452-460.

130. Van Huy Steer, B., 1978. Survey of major proteases in germinating peanut seeds by affinity chromatography. Z. für Pflanzen Physiol. 89: 51-58.

131. Virupaksha, T.K. and K. Wallenfels, 1974. Affinity chromatography of sorghum acid protease. FEBS Letters 40: 287-289.

132. Visuri, M., J. Mikola and T.M. Enari, 1963. Isolation, partial purification and characterization of carboxypeptidase from barley. Eur. J. Biochem. 7: 193-199.

133. Walbot, V., A. Capedevila and L.S. Dure, 1974. Action of 3' d-Adenosine (Cordycepin) and 3' d-Cytidine on the translation of stored mRNA of cotton cotyledons. Biochem. Biophys. Res. Comm. 60: 103-110.

134. Walsh, K., 1975. Unifying concepts among proteases. In Proteases and Biological Control, E. Reich, D.B. Rifkin and E. Shaw, ed., Cold Spring Harbour Laboratory.

135. Walton, D.C., 1977. Abscisic acid and seed germination. In The Physiology and Biochemistry of Seed Dormancy and Germination, A.A. Khan, ed., North Holland Publishing Co., New York, pp. 145-156.

136. Wang, C.C. and D.R. Grant, 1969. The proteolytic enzymes in wheat flour. Cereal Chem. 46: 537-544.

137. Yomo, H. and J.E. Varner, 1973. Control of the formation of amylases and proteases in the cotyledons of germinating peas. Plant Physiol. 51: 708-713.

Supplementary

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

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

140. Mozer, T.J., 1980. Control of Protein Synthesis in Barley Aleurone Layers by the Plant Hormones Gibberellic Acid and Abscisic Acid. Cell 20: 479-485.

141. Baumgartner, B. and M.J. Chrispeels, 1977. Purification and Characterization of Vicilin Peptidohydrolase, the Major Endopeptidase in the Cotyledons of Mung Bean Seedlings. Eur. J. Biochem. 77: 223-33.