HYDROLYSIS OF STORAGE PROTEINS IN BARLEY ENDOSPERMS: ROLE OF GIBBERELLIC ACID

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

McMaster University

March, 1986

HYDROLYSIS OF STORAGE PROTEINS IN BARLEY ENDOSPERMS

DOCTOR OF PHILOSOPHY (1986) McMASTER UNIVERSITY
(Department of Biology) Hamilton, Ontario

TITLE: Hydrolysis of Storage Proteins in Barley Endosperms: Role of Gibberellic Acid

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NUMBER OF PAGES: xviii, 189

ABSTRACT

The storage proteins in barley endosperms, hordeins, are insoluble in aqueous buffers. During early seedling growth, they are hydrolyzed to small peptides and amino acids. The overall hydrolysis of endosperm reserves is dependent on enzymes, such as alpha-amylases, carboxypeptidases, and endoproteases, which are induced in response to gibberellic acid. Soluble products released from the hydrolysis of hordeins were analyzed to determine: a) the mode of hydrolysis of these proteins; b) the role of gibberellic acid in the initial hydrolysis of hordein proteins; and finally c) whether the initial events in the hydrolysis are mediated by a specific protease. Soluble proteins were prepared from dry endosperms (controls) and embryo-less endosperm pieces incubated for 24 and 72 hours in a buffered medium in the presence and absence of gibberellic acid. The hordein-related polypeptides in the soluble fraction were identified by using IgG's prepared against urea-denatured hordeins. In the control samples, several bands were detected on western immunoblots. Additional bands in the size range of 25-30 kilodaltons and 40 kilodaltons appeared in the absence of gibberellic acid. In the presence of gibberellic acid, however, fragments in the size range of less than 15 kilodaltons were more

dominant. Furthermore, the levels of small peptides (less than 30-35 amino acid residues in length) and free amino acids increased when samples were incubated for longer times in the presence of gibberellic acid. The results are consistent with the idea that a gibberellic acid-independent endoprotease(s) is involved in the initial stages of hordein hydrolysis. Further hydrolysis is, however, dependent on the presence of proteases induced by gibberellic acid. The results are discussed in relation to both the mode of hydrolysis and the specificity of initial events of hordein hydrolysis.

PREFACE

The experiments described in this thesis were carried out in the Department of Biology, McMaster University, from September 1980 to August I985. This thesis consists entirely of my work, except where others are specifically mentioned.

ACKNOWLEDGEMENTS

I express my deep thanks and appreciation to my supervisor, Prof. Ann Oaks, for her expert guidance, close supervision, and patience through the course of this study. I also thank Dr. T. T. Chen (Biology Department), for his constant encouragement, useful suggestions, and constructive criticisms. Fruitful discussions with Prof. J. E. Varner (Biology Department, Washington Univ., St. Louis, U.S.A.), are gratefully acknowledged. Comments from Dr. G. E. Gerber (Biochemistry Department), are also acknowledged.

I thank my colleagues for their help and for maintaining a healthy research atmosphere in the lab. In particular, the generous help from Mr. Mike Winspear, Ms. Ingrid Boesel, Mrs. Leslie Cass, and Mrs. Valerie Goodfellow is greatly acknowledged. I also thank Dr. Michele Poulle and Dr. Akira Suzuki, from whom I learned immunological techniques. I would also like to acknowledge two other colleagues, Mr. Mike Coulthart and Mr. Lou Agellon, for their help.

Special thanks are extended to Mrs. Rekha Singh and Dr. Rama S. Singh, for their unreserved help and constant moral support.

Last but not least, I express my deep gratitude and thanks to my mentor and former teacher, Prof. Satish. C. Maheshwari (Botany Department, Delhi Univ., Delhi, India), who inspired me to choose a road in pursuit of knowledge.

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ABBREVIATIONS

A	absorbance
ABA	abscisic acid
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
BAla	beta-alanine
bp	base-pairs
BSA	bovine serum albumin
C	celsius
C-TERMINAL	carboxy-terminal
cDNA	complementary deoxyribonucleic acid
cv.	cultivar
CysA	cysteic acid
DEAE-cellulose	diethylaminoethyl-cellulose
DMSO	dimethyl sulfoxide
µE	microeinstein
EDTA	ethylenediaminetetraacetate
g	gram
GAba	gamma-aminobutyric acid
GA_3	gibberellic acid

GAR-HRP	goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
HPLC	high performance liquid chromatography
IEF	isoelectrofocusing
IgG	immunoglobulin G
Ile	isoleucine
kDa	kilodaltons
Leu	leucine
Lys	lysine
M	molar ($mol.l^{-1}$)
m^2	square meter
mA	milliampere
Met	methionine
min	minute(s)
ml	milliliter
mM	millimolar
µM	micromolar
mRNA	messenger ribonucleic acid
N	normal (concentration)
N-TERMINAL	amino-terminal
nm	nanometer
OPA	ortho-phthaldialdehyde

Phe	phenylalanine
pI	isoelectric pH
poly-(A^+)	polyadenylated
poly (U)-SEPHAROSE	poly-uridine sepharose
Pro	proline
S	Svedberg unit
s	second(s)
(-SH)	sulfhydryl group
Ser	serine
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
Trp	tryptophan
Tyr	tyrosine
TEMED	N,N,N',N'-tetramethylethylenediamine
THF	tetrahydrofuran
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
Val	valine

To my loving mother and father.

INTRODUCTION

PART I. REVIEW

Seeds are quite remarkable for the following reasons: a) they are metabolically inactive in their dry state and yet living, as they can resume normal growth upon hydration, and b) they contain large reserves of food material such as carbohydrates, proteins, and lipids, which are efficiently degraded during the early stages of seedling growth. Seed germination, therefore, offers a relatively easy system to study the regulation of synthetic and/or degradative processes, which are initiated by simple manipulation of environmental conditions such as the addition of water.

1. Hydrolysis of Storage Reserves in the Cereal Endosperms:

Large reserves of carbohydrates and proteins are laid down and stored in the cereal endosperms during seed development (9). Although the 'molecular signals' that turn on the genes involved in the synthesis of these reserves are not known, it is generally accepted that the relative levels of plant growth regulators, such as gibberellic acid (GA_3) and abscisic acid (ABA) are critical. During the active phase of synthesis, for example, ABA levels are high and GA_3 levels low (9). With the onset of germination, the metabolic

activity in the endosperm switches to the degradative phase, and at this stage, GA_3 is released from the embryonal part and possibly from the terminal region of the seed. The otherwise insoluble starch and protein reserves in the endosperms are rapidly hydrolyzed to sugars and amino acids.

1.1. Role of Hormones: Starch, the major energy source, is degraded by α- and β- amylases during the early stages of seedling growth. Using the technique of isoelectrofocusing (IEF), MacGregor (69) reported the presence of as many as 18 isozymes of α-amylase in barley kernels. The control of synthesis of α-amylase is fairly well understood (57, 82, 84, 94). Varner and co-workers established the requirement of GA_3 for the induction of α-amylase and other hydrolases in embryo-less endosperm pieces and the aleurone layers of barley cv. 'Himalaya' (21, 24, 25, 49, 52, 116). Aleurone layers, which consist of living cells surrounding the starchy endosperms, were shown to be the site of synthesis for the hydrolases (24, 25). In the presence of $CaCl_2$, these enzymes were released into the bathing media. Addition of ABA along with GA_3 resulted in the cessation of the development of α-amylase (82). ABA thus appears to antagonize the GA_3-enhanced processes (52). Since, the effect of ABA was observed even after the synthesis of α-amylase mRNA, Ho and Varner concluded that ABA acts at a post-transcriptional level. Recently, Mozer provided

evidence in support of the possibility that ABA exercised its effect at the translational level (82), since ABA did not suppress the amount of translatable mRNAs but affected the efficiency of their translation (50).

In recent years, a great deal of work has been undertaken to understand the molecular mechanism of GA_3 induction of α-amylase in aleurone cells. Sensitivity to cycloheximide and other inhibitors, and density labelling experiments in the late sixties, indicated that the production of α-amylase involved new protein synthesis (25). Higgins and co-workers isolated poly-(A^+) mRNAs from GA_3-treated aleurone layers. When translated *in vitro* they produced several proteins, of which the major one was α-amylase (50). Later, the same group as well as several others showed a close parallel between the accumulation of α-amylase protein *in situ* and the levels of translatable α-amylase mRNA (21, 49, 82, 84). This message constituted 15-25% of the total translatable messages after presentation of GA_3 to the aleurone layers. The increase in the levels of α-amylase mRNAs has recently been shown to be due to an increased rate of transcription of the α-amylase gene (21, 84, 94, 95). The workers made use of a cDNA probe to identify the newly synthesized mRNAs in GA_3- treated aleurone layers. Another possible level of control of α-amylase synthesis by GA_3 is at translation (82). Mozer showed that the GA_3 additions resulted in preferential translation of

certain messages, e.g. α-amylase (49, 84). Interestingly, for reasons not quite obvious, the continued synthesis of α-amylase protein required the presence of GA_3 even after the completion of α-amylase mRNA synthesis in aleurone layers (82). This may be due to either preferential stabilization/translation of α-amylase mRNAs, and/or inhibition of α-amylase breakdown by controlling the levels of an α-amylase inhibitor. Support for the latter view has come from recent work of Mundy, who has shown that the levels of ABA and GA_3 controlled the synthesis of an α-amylase inhibitor (83, 118).

In recent years, it has been established that α-amylase is comprised of two groups of proteins, A and B (55, 94). These two groups differ in the following features: a) pI, group A between 4.6 and 5.2, and group B between 5.9 and 6.6; b) peptide maps; and c) their loci, which are present on different chromosomes. Rogers and co-workers have isolated cDNA clones for both types of α-amylase from a cDNA library. The cDNA library was prepared from mRNAs induced in the presence of GA_3 in the aleurone layers (94, 95, 96). Their studies showed that gene families were regulated differently by GA_3. For example, the mRNAs for group A were present in the aleurone layers in the absence of GA_3, and in its presence, they increased by 20-fold. On the other hand, mRNAs for group B were barely detectable in the aleurone

layers in the absence of GA_3, and in its presence they increased by 100-fold (94). cDNA clones for both groups A and B have been completely sequenced and were found to share regions of partial homology. The sequence analysis also suggested the existence of a possible hairpin loop structure in the 5' region of the mRNA.

It is instructive to note that cycloheximide additions to GA_3-treated aleurone layers blocked the synthesis of the α-amylase message, suggesting that a protein factor 'x' was required for either stabilization of the message or efficient expression of the α-amylase gene (84). The identity of factor 'x' remains to be established.

Despite the great wealth of information made available in recent years, it is still not known how GA_3 results in increased rates of transcription of genes coding for the α-amylase protein. The growth regulator may either interact with the promoter of the α-amylase gene or may result in the synthesis of a factor, which in turn leads to increased transcription and translation of the α-amylase gene and α-amylase mRNA, respectively.

1.2. Hydrolysis of Cereal Proteins: Work on the cereal proteases is fragmentary and as a result the mechanism of the initial hydrolysis of cereal storage proteins is not known. It remains to be established whether protease activities measured with non-plant proteins or artificial

peptides as substrates could mediate the early solubilization step(s). Despite these uncertainties, there is a general agreement in the literature that initially the storage proteins are cleaved into a few polypeptide fragments as a result of one or more specific cuts possibly by a 'specific' protease(s) (8, 75, 97). Presumably, the products released at early times are soluble and could serve as substrates for the proteolytic activities, e.g. endo- and exoproteases, appearing a few days after imbibition. These enzyme activities probably have broader substrate specificities. The final products of the overall hydrolysis are small peptides and amino acids.

2. Storage Proteins of Cereals:

The storage proteins of cereal grains were classified on the basis of their solubility into albumins (water-soluble), globulins (saline-soluble), prolamins (alcohol-soluble), and glutelins (alkali-soluble), by Osborne (88). In seeds belonging to different families of major cultivated crops, a particular class of protein is stored. In cereals, for example, the prolamins are stored, whereas in legume seeds, the bulk of the storage proteins are globulins. Some exceptions however, were noted, e.g. nearly 80% of the storage proteins in oats and rice is generally regarded as globulins because these proteins are soluble in saline buffers (91) and show structural similarities with globulins.

During the development and maturation of the cereal kernel, prolamins are actively synthesized and deposited in the protein bodies. The protein bodies are ovoid organelles, a few microns in diameter, which are embedded between starch granules in the starchy endosperm (54). The zeins in corn are stored exclusively within the protein bodies (15, 72). In barley, the hordeins too are initially stored in these organelles during the development of endosperm. At maturity, however, the protein bodies disappear and clusters of proteins are present among the starch granules (18, 54, 72).

2.1. Structure of Prolamins: The prolamins are hydrophobic in nature and their amino acid composition have several unique features (Table 1). For example, they are rich in proline and glutamine/glutamate and poor in lysine and tryptophan (11, 53, 74). Since these proteins are insoluble in water, they have generally been extracted with propanol, and studied in detail only in the presence of SDS or urea. As a result, the component polypeptides rather than the protein itself have been investigated. The different techniques adopted to analyze these polypeptides are based on electrophoresis and include starch gel, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), urea-PAGE, IEF, and two-dimensional procedures combining SDS-PAGE with either IEF or urea-PAGE (74, 103, 104, 105, 106, 107).

Table 1. Amino Acid Composition of Total Hordeins in Barley Endosperms

Amino Acid	mole % (Amino Acid Residue/100 Residues) Miflin (74)	Holder (53)	Brandt (11)
Methionine	0.2	1.1	0.9
Cysteine	1.7	2.3	1.6
Aspartic acid*	1.0	1.7	1.6
Glutamic acid**	41.2	34.9	34.7
Threonine	1.0	2.0	2.0
Serine	4.6	4.1	4.2
Proline	31.9	21.5	23.0
Glycine	0.3	2.5	2.5
Alanine	0.7	2.6	2.4
Valine	1.0	4.8	4.6
Isoleucine	2.6	3.9	3.8
Leucine	3.6	7.2	6.9
Tyrosine	2.3	2.3	2.4
Phenylalanine	8.8	5.6	5.9
Histidine	1.1	1.3	1.0
Lysine	0.2	0.7	0.5
Arginine	0.8	1.5	2.0

* -- values include asparagine

** -- values include glutamine

Zeins, the major prolamin in maize, consist of two major components, one of 21 kDa and the other of 19 kDa (3, 15, 17, 109). On the other hand, hordeins, the major fraction of prolamins in barley, can be separated into a number of discrete bands. These have been pooled into 3 groups, e.g., A (15-20 kDa) with several bands, B (60-70 kDa) with 2 major and 2-4 minor bands, and C (90-100 kDa) with 3 major and up to 4 minor bands (91, 100, 101, 104, 108).

2.2. Immunochemistry of Prolamins: In recent years, the prolamins have been analyzed using immunochemical techniques (28, 29, 66). Insolubility of these proteins in aqueous buffers has necessitated the use of 3-6 M urea to dissolve the prolamin polypeptides. In general, since the prolamins are poor immunogens, fairly high quantities of hordeins (2.5-10 mg) and zeins (2.5 mg) were injected into the rabbits (28, 91) in order to elicit a good antibody response. Cross-reactivity of the crude sera against the respective immunogens have generally been tested with the double immunodiffusion assay (29) and immunoelectrophoretic methods (28, 66).

The prolamins from corn, Sorghum, and millet do not cross-react with antibodies raised against group C polypeptides of hordeins. These antibodies, however, do cross-react with the prolamins of not only barley, but also of rye and wheat (28, 91). The immunochemical differences

among the prolamins of different cereals confirm the grouping of corn, Sorghum, and millet into sub-family Panicoidae, and barley, rice, and wheat into sub-family Triticeae (91).

It should be mentioned that the cross-reactivity of antibodies raised against zeins and hordeins has not been tested against the soluble albumin and globulin proteins extracted from the kernels of corn and barley. Since a rather arbitrary criterion, i.e. solubility, was originally adopted to classify cereal proteins (88), the result of such an experiment might be important in determining how similar or dissimilar the two types of proteins really are.

2.3. Molecular Biology of Prolamin Synthesis: The zeins from corn consist of two major polypeptides of 19kDa and 21 kDa. However, two-dimensional analysis of the zeins show that there are about 25 components (54, 65, 109), suggesting that these are quite polymorphic proteins. A synchronous synthesis of these polypeptides begins 15 days after pollination (54). Initially, the polypeptides are synthesized on membrane-bound polysomes, after which they are deposited in the protein bodies. The zein messages were isolated from membrane-bound polysomes. When the zein mRNAs were translated in vitro using a rabbit reticulocyte system, ethanol-extractable prolamins, identical in electrophoretic and immunochemical features with those translated in situ, were synthesized (15, 17, 64, 65). The zein messages have normal features of eukaryotic mRNAs, e.g., a) the presence of

a 7' methylated guanosine residue (cap) at the 5'-end, and b) a poly-(A^+) tail at the 3'-end (17, 109). They have an average length of 1.1-1.2 x 10^3 bases (16, 17, 64). The zein genes lack introns. The genomic DNA from corn embryos was restricted using a number of restriction enzymes and probed with labelled zein cDNA in Southern hybridization experiments to estimate the number of zein genes. As many as 100 genes scattered throughout the genome were detected (16, 37, 90, 119). Extensive work has been done in an effort to elucidate the structure and expression of zein genes. A complete sequence of the 19 kDa zein gene has been reported recently (90). The predicted sequence of the zein polypeptide from this gene shows a conserved peptide of 20 amino acids, which is repeated 9 times. The repeating sequence is as shown below:

Gln-Gln-Leu-Leu-Pro-Phe-Asn/Ser-Gln-Leu-Ala-Ala-Leu/
Pro-Asn-Ser-Ala-Ala-Tyr-Leu-Gln-Gln

The cloned zein genes have been expressed using *in vitro* transcription systems such as HeLa cell extracts or extracts of germinal oocytes from *Xenopus* (64). Two correct start sites, the promoters P1 and P2, were identified. While the P2 promoter is situated just before the coding sequence, the other promoter P1 lies about 1000 bp upstream (64). Recently, a heterologous *in vivo* expression system for zeins

has also been developed. The 19 and 21 kDa zein genes were integrated into chromosome II of the yeast Saccharomyces cerevisiae. It was shown that both P1 and P2 promoters were recognized accurately to produce two messages of the correct sizes (63).

Hordeins, like the zeins, are also highly polymorphic proteins. These polypeptides are actively synthesized between 15 and 35 days after pollination (54, 108). There are two major groups of hordein polypeptides, one sulphur-rich (B-hordeins), and the other sulphur-poor (C-hordeins). The two groups of polypeptides comprise about 95% of the total hordeins (104). Genetic analysis has shown that B- and C-hordeins are encoded by two separate loci, Hor 2 and Hor 1, respectively (107). Hordeins are synthesized on membrane-bound polysomes (12, 13, 18, 19). The mRNA fraction from such polysomes were isolated employing poly U-Sepharose 4B affinity chromatography (70). A library of cDNA was constructed using the poly-(A^+) RNAs (30). The cDNA clones coding for B- and C-hordeins were identified by comparing the products synthesized in vitro with authentic hordeins (30). Partial sequence analysis of a C-hordein gene shows that an octapeptide (-Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln-) is repeated several times over in the sequence. The B-hordein sequence analysis shows the presence of two domains, i.e., a) domain 1 - repetitive, deficient in sulphur, and abundant in proline, - is homologous to the C-hordein sequence; and b) domain 2 -

non-repetitive, abundant in sulphur, and deficient in proline - is homologous to the 2S globulin of Ricinus communis and the wheat gliadins (30). The similarity of hordein polypeptides to those of zeins is not known.

Newly synthesized zein and hordein polypeptides have an extra 20-21 amino acid residue extension on the amino-terminal, the signal peptide. After synthesis of the prolamins, the signal peptide is cleaved and the prolamins are vectorially transported into the lumen of the endoplasmic reticulum membrane (18, 19).

Despite substantial progress in our understanding of the structure and expression of the prolamin genes in the last decade, more information is needed to answer the following pertinent questions: a) how is a synchronous transcription of the prolamin genes, which are scattered throughout the genome, achieved?; b) what specific signal(s) turn on the expression of prolamin genes during grain development?

3. Proteolytic Systems:

The proteases, i.e. carboxypeptidase A, thermolysin, elastase, and chymotrypsin, were among the first enzymes known and purified (110, 111). The susceptibility of proteolytic activities to different inhibitors defines their active sites and mechanism of action. This criterion has been the basis of the classification of porteases into four

major groups as listed below:

a) serine proteases have serine at their active sites and are represented by trypsin and chymotrypsin. They are inhibited by leupeptin and di-isopropylphosphofluoridate;

b) sulfhydryl proteases have (-SH) groups at their active sites and are represented by papain and cathepsin. They are inhibited by p-chloromercuribenzoate and N-ethylmaleimide;

c) metalloproteinases require metals at their active sites and are represented by collagenases and thermolysin. They are inhibited by EDTA and phosphoramidon;

d) acid proteinases are active at highly acidic pH and are represented by pepsin and chymosin. They are inhibited by pepstatin (115).

The proteases have also been broadly classified on the basis of the type of substrate they act upon, e.g., a) endoproteases, which cleave internal peptide bonds to yield smaller fragments, are assayed using substrates such as casein, azocasein, hemoglobin; and b) exopeptidases, i.e. carboxypeptidase and aminopeptidase, which cleave the carboxyl (C-) or amino (N-) terminal residue, respectively. These are generally assayed by using synthetic dipeptides blocked at either N- or C-terminal, respectively (97).

The proteolytic enzymes cleave a peptide bond between two consecutive amino acid residues. Extensive work has been done on the specificity and mechanism of action of the serine proteinases, e.g. trypsin and chymotrypsin. Such studies were made possible by the availability of a) highly purified enzyme preparations; and b) synthetic substrates with defined amino acid residues and sequences (31, 110).

In the case of small proteins used as substrate, the cleavage sites are specified by particular amino acid residues in the protein (sequence-specific), which are recognized by the active sites of a protease enzyme. Trypsin, for example, preferentially catalyzes the hydrolysis of Lys/Arg-x (x being any amino acid) peptide bonds (60, 110). On the other hand, thermolysin and pepsin catalyze the hydrolysis of y-Leu and y-Trp/Tyr/Phe (y being any amino acid) peptide bonds, respectively (31).

It has been known for a long time that in the case of large protein substrates, accessibility of the susceptible cleavage sites to active sites of the enzyme is an important factor. In general, denatured proteins are better substrates for the proteases than the native proteins (67, 86). The susceptible amino acid residues, between which the peptide bond is cleaved, are exposed in the denatured proteins. Based on this idea, Linderstrom-Lang (67) proposed the concept of 'limited hydrolysis', in which the peptide bonds are cleaved one-by-one. The conformation or tertiary

structure is, therefore, quite important in determining whether the protein substrate will be attacked by a protease.

3.1. Animals: Cellular proteins are in a dynamic state of constant turnover. The steady state concentration of an enzyme in the cells is determined by its rates of synthesis and degradation (35). As seen in Table 2, the half-life of different enzymes vary from 12 min for ornithine decarboxylase to 15 hours for glucose 6-phosphate dehydrogenase and 3-phosphoglycerate dehydrogenase. In addition to the degradation of normal proteins, abnormal proteins synthesized after specific mutations are also selectively recognized and rapidly degraded (45). In general, the degradation of proteins in animal cells is mediated by two proteolytic systems, lysosomal and non-lysosomal, which are described below.

3.1.1. Lysosomal: The lysosomes are small organelles containing a set of acidic proteases with pH optima of around 4-5 (4, 26, 111, 112). The cellular constituents are probably engulfed and degraded by these organelles (26). The participation of lysosomes in protein turnover was suggested by the following observations: a) the change in osmotic sensitivity (increased fragility) and sedimentation properties of lysosomes prepared from liver cells under conditions which promoted protein degradation, e.g., nutrient-depletion (81, 85); b) the release of TCA-

Table 2. Half-life of Various Enzymes in Mammalian and Plant Cells. Adapted from Goldberg and John (35).

Enzyme	Half-life (hour)
1. Ornithine decarboxylase	0.2
2. RNA polymerase I	1.3
3. Tyrosine aminotransferase	2.0
4. Ribulose bisphosphate carboxylase, large subunit**	3.0
5. Alpha-Amylase	4.3
6. Nitrate reductase*	4.5
7. Phosphoenolpyruvate carboxykinase	5.0
8. Glucokinase	12.0
9. RNA polymerase II	12.0
10. Glucose-6-phosphate dehydrogenase	15.0
11. 3-Phosphoglycerate dehydrogenase	15.0

* -- From corn leaves, Oaks et al. (86b).
** -- From barley leaves, Miller and Huffaker (78). All other enzymes were from mammalian cells (35).

soluble peptides and amino acids from isolated lysosomes; and c) the addition of nutrients, such as amino acids, to nutritionally-depleted liver tissues, prevented changes in osmotic sensitivities and the release of soluble peptides by isolated lysosomes. The direct evidence was provided when the proteases were isolated from lysosomes and characterized. The lysosomes of most tissues contain at least 3 endoproteases, Cathepsin B1, Cathepsin D, and Cathepsin G, exopeptidases, Cathepsin B2, Cathepsin A1, and Cathepsin C, and peptidase, Cathepsin A2 (4, 26, 71, 112).

3.1.2. Non-Lysosomal: Two observations were made quite early in the literature, which suggested the presence of an additional proteolytic system, non-lysosomal. Firstly, cellular enzymes have highly variable half-lives. In addition, their rates of degradation were not altered by additions of inhibitors of lysosomal proteases (35, 45). Secondly, an apparent dependence of intracellular protein degradation on the presence of ATP was observed (35, 45, 46). The latter observation was quite unexpected because hydrolysis of a peptide bond is an exergonic reaction. Furthermore, none of the proteolytic enzymes known so far require energy-rich cofactors. Also, in contrast to the acidic proteases of lysosomes, proteases with a pH optimum between 8 and 9 were isolated from skeletal muscles (27, 35, 45).

More direct evidence came from the studies of De Martino and Goldberg (27), who isolated an ATP-dependent alkaline protease from rat liver tissue. ATP stimulated protease activity by about 3-fold. A cell-free ATP-dependent proteolytic system was later prepared from reticulocyte cells (45). The characteristic features of this system were: a) it required the presence, but not hydrolysis, of an energy-rich compound, such as ATP; b) it degraded only the abnormal proteins; and c) it required the presence of a heat-stable polypeptide of around 9 kDa. The latter was termed an APF-1 or ATP-dependent proteolysis factor 1 (27, 45). This factor was found to be similar to another peptide, ubiquitin (121).

Ubiquitin, first discovered during the lymphocyte differentiation studies, is a polypeptide of 8.5 kDa (99). It induces the differentiation of precursor B- and T-lymphocytes into mature cells of the respective types (36). The remarkable features of this peptide are as follows: a) it is present in all the life forms tested, e.g. microbes, animals, and plants; b) it exhibits an extraordinary evolutionary conservation in terms of its structural, biochemical and immunochemical properties; and c) it is highly resistant to trypsin digestion, despite the fact that it contains 7 lysine and 4 arginine residues (99). A detailed characterization of ubiquitin showed that it exists in two forms, I and II. Form II, which is 74 amino acid residues long and inactive in stimulating the ATP-dependent proteolysis, is a tryptic

product of form I. The latter has 2 extra amino acids at the C-terminal (121). The amino acid sequence at the C-terminal of form I is -Arg-Leu-Arg-Gly-Gly in contrast to that of Arg-Leu-Arg of form II. Ubiquitin is enzymatically ligated to cytoplasmic and chromosomal proteins, e.g. histones H_2A (45). Ligation to ubiquitin, which is an intermediate step, has been regarded as a recognition signal for a selective protein degradation by the ATP-dependent system. Since ubiquitin is also preferentially associated with actively transcribing genes, a site-specific proteolysis of chromosomal proteins by ubiquitin is regarded as a possible mode of gene activation.

Recently, the presence of a ubiquitin-dependent proteolytic pathway has also been reported in higher plants (62). Using anti-human-ubiquitin serum in western immunoblots, the presence of both free and protein-conjugated ubiquitin was shown in dry kernels and in green and etiolated leaves of Avena sativa. It is, however, not known whether; a) the ubiquitin-dependent proteolytic pathway in plants is dependent on the presence of ATP; and b) it is involved in processes such as the hydrolysis of storage proteins.

Even though the ATP- and ubiquitin-dependent proteolytic pathways have been well characterized in animal cells, the nature of the protease(s) activity mediating this pathway remains elusive. Also, it is not clear why only

cellular proteins conjugated to ubiquitin are preferentially degraded. Extreme structural conservation of ubiquitin across the whole range of life forms suggests that it must have a very fundamental function in cells. It remains to be seen if selective protein hydrolysis is such a function.

3.2. Plants: Even though the use of plant proteases, such as ficin and papain in food processing and meat tenderization have been in practice for a long time, our knowledge of other plant proteases with regards to their structure, mechanism of action, and specificity is very fragmentary (20, 75). This is partly due to the fact that, in general, the substrates used to assay the plant proteases are the same as those used for animal proteases. The method of analysis has also been similar to that of animal proteases, e.g., a) increase in the ninhydrin-positive material in TCA-soluble fraction; b) increase in the absorbance at 280 nm of the TCA-soluble fraction, which is due to the presence of aromatic amino acids, and c) changes in the viscosity of the substrate after incubation with the enzyme. Active proteolysis has been examined in detail at two stages, i.e. a) mobilization of the protein reserves during seed germination; and b) chlorophyll protein degradation during leaf senescence (78). The proteolysis during seed germination is reviewed below.

3.2.1. Non-Cereals: A great deal of work has been

done in a variety of non-cereal systems, such as pea, pumpkin, mung bean, and castor bean. The major storage proteins in these systems belong to the general class of globulins (91). Use of the native proteins as substrate for the assay of protease activity, in conjunction with the analysis of breakdown products on SDS-PAGE has provided unequivocal evidence for the involvement of endoproteases in the degradation of protein reserves in these systems.

In pea, isolation of the storage proteins from cotyledons at different times after imbibition and their analysis on SDS-PAGE showed that large globulin polypeptides are first cleaved to produce fragments of smaller sizes, with no quantitative loss of the total globulins. The initial changes could be mediated by the activity of a specific endoprotease or a general peptide hydrolase, a point not examined by these workers. Subsequent mobilization of these proteins was, however, correlated with a concomitant accumulation of casein-hydrolytic activity. This proteolytic activity also released labelled amino acids when tritiated pea globulins were used as substrate (5).

In another system, pumpkin, <u>in situ</u> alteration in storage proteins during germination was correlated with the appearance of two proteolytic activities, I and II, in the cotyledons (38, 39). Activity I brings about limited proteolysis of the α- and β- subunits of native globulins,

resulting in the release of fragments of smaller size, $F_{\alpha\beta}$ (38, 41). The $F_{\alpha\beta}$ fragments were readily soluble in the saline buffers. Activity II, in conjunction with other peptidases, was thought to be involved in further breakdown of the modified globulins into small peptides and amino acids (39). Of the two proteases, i.e. activity I and activity II, only the former was present in dry cotyledons. It was not synthesized de novo, since the cycloheximide additions during imbibition, which prevented the appearance of activity II, had no effect on activity I (38). The initial hydrolysis of globulins may occur within protein bodies, since a major proportion (two-thirds) of activity I was present within the protein bodies isolated from dry cotyledons (40).

In mung bean, 7S vicilin and 11S legumin comprise the major storage proteins. In this system, a loss of globulins during germination was correlated with the development of an (-SH) endoprotease activity which hydrolyzed gelatin (23). The protease, vicilin peptidohydrolase, was purified following affinity column chromatography (6). This protease activity mediated the hydrolysis of vicilin in vitro into peptides of smaller sizes, which were then resolved on SDS-PAGE (6). In conjunction with the activity of carboxypeptidase, vicilin peptidohydrolase brings about a complete hydrolysis of vicilin into amino acids. The specificity of vicilin peptidohydrolase activity was not like that of trypsin, as it readily hydrolyzed the ester linkages

involving glutamine and asparagine instead of lysine/arginine residues at the C-terminal. Antibodies were raised against the purified enzyme and used in immuno-electron microscopy and immunofluorescence studies (7, 22, 120). Results from the immuno-electron microscopic studies showed that the enzyme was not present in protein bodies of dry seeds, and that it first appeared in these organelles two days after imbibition (22). The enzyme was synthesized de novo in the cytoplasm and later deposited in the protein bodies (7). Immunofluorescence studies showed that the newly synthesized enzyme was associated with the vesicles derived from rough-endoplasmic reticulum, suggesting that the endoplasmic reticulum may play a key role in the transfer of this enzyme into protein bodies (120).

Castor bean is another system that has been studied in detail. The major proteins in this case are insoluble. Following imbibition, they are mobilized into peptides and amino acids (1, 2). Beevers and co-workers (1, 2, 114) have shown development of the following proteolytic activities: a) endoproteases, which hydrolyze hemoglobin; b) peptidases, which hydrolyze N-benozyl-DL-arginyl-p-nitroanilide and N-benzoyl-DL-arginyl-β- naphthylanilide; c) aminopeptidases, which hydrolyze leucyl-naphthylamine and prolyl-β-naphthylamide; and lastly d) carboxypeptidases, which hydrolyze carbobenzoxy-phenylalanyl-alanine. Since the

appearance of aminopeptidases closely paralleled the hydrolysis of storage proteins, it was concluded that they were the key enzymes involved in the early hydrolysis of crystalloid storage proteins (114). The other proteases were thought to play a minor role. The basic drawback of this study was that the globulins were not used as substrate for assaying proteolytic activities and that the early hydrolytic products were not analyzed. Contrary to the above results, Gifford et al. (34) provided evidence for the involvement of endoproteases in the hydrolysis of crystalloid proteins. They analyzed the early products released in situ from the hydrolysis of crystalloid proteins. Antibody was raised against the storage proteins of castor bean. This was used in western immunoblots to detect the products released at early times. A set of polypeptide fragments of discrete sizes were released from storage proteins. Release of these fragments was mediated by an endoprotease. Since cycloheximide additions during the imbibition period did not prevent the appearance of these fragments, it was concluded that an inactive protease precursor was present in the dry seeds (34).

3.2.2. Cereals: Among cereals, barley and corn have been investigated in detail. As mentioned in preceding sections, prolamins, the storage proteins in cereals, are insoluble in aqueous buffers. This property has precluded the use of these proteins as substrate in conventional assays for protease activity. With the use of animal proteins or synthetic dipeptides as substrate, a number of proteolytic activities have been described in cereals.

In barley, the induction of a gliadin degrading protease activity by GA_3 in embryoless endosperm pieces was reported by Jacobsen and Varner (56). Since an increase in ninhydrin-positive material was used as the method to estimate the level of protease activity, it is not known whether this protease was an endoprotease(s) or an exopeptidase(s). In crude extracts prepared from endosperms, aleurone layers, and scutellum of barley seedlings, Mikola (75, 76, 77), and Schroeder and Burger (102) identified a number of protease activities. Aleurone layers from dry kernels, for example, contained 5 aminopeptidases and 3 carboxypeptidases. Whereas the aminopeptidase activity did not change during the early seedling growth, the activity of carboxypeptidase increased slightly (76). The endosperm tissue from dry kernels, on the other hand, contained only carboxypeptidases, whose levels increased appreciably during early seedling growth. The scutellum tissue from dry kernels contained high activities of both aminopeptidases and

carboxypeptidases. Levels of carboxypeptidase activities increased following the imbibition of the caryopsis. Since, carboxypeptidases increased both in endosperm and scutellum tissues during early seedling growth, these proteases were regarded as key enzymes in the mobilization of endosperm protein reserves.

In corn, Harvey and Oaks (42, 43, 44) reported an increase in the activity of a (-SH) endoprotease following the imbibition of the kernels. This protease activity also hydrolyzed the denatured zeins (43). The zein degrading activity increased between 3 and 8 days after imbibition. A number of other proteins, e.g. gliadin, hemoglobin, edestin, and BSA were also degraded by this protease. The development of this protease was correlated with the loss of insoluble protein reserves during germination. The presence and development of a (-SH) protease activity in corn endosperms was later also confirmed in other labs (32, 80).

It is clear from the brief account given above that the cereal proteases (76, 77, 80, 92, 93) have not been characterized to the same extent as protease from mung bean (6) or trypsin from mammalian cells(59). The mechanism of action and specificity of the cereal proteases are also not understood.

PART II. RATIONALE AND SPECIFIC QUESTIONS

Hordeins, the storage proteins in barley, are water insoluble. Overall mobilization of these insoluble proteins must involve a mechanism(s), which results in the release of soluble products during the initial stages of hydrolysis. In order to elucidate the underlying mechanism(s) one needs to characterize either the proteolytic activity(ies) that develop during germination, or the products released into the soluble media. Even though several proteases appear during germination, their role in the initial stages of mobilization is not known (56, 76, 76, 77, 102, 124). Alternatively, a detailed characterization of the hydrolytic products released into the soluble media may provide information concerning the nature of proteolytic activity(ies) involved in this process. The soluble products were, therefore, analyzed in this project, and the specific questions addressed are listed below:

(a) What is the mode of hydrolysis of hordeins?

(b) What is the role of GA3 in the early and overall hydrolysis of hordeins?

(c) Is the initial step of hydrolysis of the hordein polypeptides mediated by a specific protease?

MATERIALS AND METHODS

1. Plant Materials: Kernels of hybrid maize (Zea mays cv. W64A X W182E) were obtained from the University of Wisconsin, College of Agriculture, Madison, Wisconsin. Barley (Hordeum vulgare cvs Bruce, Elrose, Herta, and Perth) kernels were obtained from Dr. E. Reinbergs, Department of Crop Sciences, University of Guelph, Guelph, Ontario. One other cv. of barley, Bonanza was obtained from Dr. A. W. MacGregor, Grain Research Laboratory, Winnipeg, Manitoba.

Kernels were planted on moistened cotton layers and germinated in growth chambers. Maize seeds were germinated at 28 $\pm 2^{o}C$ and barley seeds at 20 $\pm 2^{o}C$ with a light/dark cycle of 16/8 hours. Plants were grown in mixed light (fluorescent and incandescent bulbs) with a flux density of 300 $\mu E.m^{-2}s^{-1}$. Endosperms were harvested at different times after imbibition. Husks and embryo including scutellum were excised, and the remaining endosperm pieces were quick-frozen in liquid nitrogen and stored at $-20^{o}C$ until use. Routinely, the endosperms were used within 7 days of harvesting.

2. Half-Kernel Test: The embryonal and the distal parts of barley kernels were transversely cut and discarded (116). The embryo-less endosperm pieces were first sterilized with 2%

sodium hypochlorite solution for 30 min, washed with 0.02 N HCl for 30 min, and finally rinsed several times with sterile distilled water. All subsequent steps were carried out under aseptic conditions using sterilized glass-ware and media. Thirty endosperms were incubated in 10 ml of 1 mM acetate buffer, pH 4.8, containing 0.01 M $CaCl_2$ and 20 ul of a solution of antibiotics, in 125 ml Erlenmeyer flasks. The antibiotic solution contained 0.5 mg/ml each of streptomycin, penicillin, and chloramphenicol. The endosperms were incubated for 24, 48, and 72 hours in the absence (-) and presence (+) of GA_3. GA_3 at a concentration of 5 μM was used routinely. The flasks were kept on a shaker at 25 $\pm$ 2°C. The endosperms and media were separately quick-frozen in liquid nitrogen. They were stored at -20°C until use. Bacterial contamination was checked by streaking the nutrient-agar plates with the samples of media and the contaminated samples were discarded.

3. Enzyme Assays: For endopeptidase assays, the endosperm pieces were homogenized in a mortar and pestle with 0.2 M sodium acetate buffer, pH 3.8, containing 5mM 2-mercaptoethanol (122). Two millilitres of buffer were used for each g of tissue. The crude homogenate was filtered through 2 layers of Miracloth and then centrifuged at 20000 xg for 15 min at 4°C. The supernatant solution was used as the crude enzyme extract. The standard assay contained 1 ml

of a solution of hemoglobin (50 mg/ml); 1 ml of 0.05 M sodium acetate buffer, pH 3.8, containing 2.5 mM EDTA; 0.3 ml of 0.2 M sodium acetate buffer, pH 3.8. The reaction mixture was pre-incubated for 30 min at a temperature of 38°C. The reaction was then started by adding 200 µl of extract to 2.3 ml of the substrate solution. After 30 min, the reaction was stopped by adding 2.5 ml of 10% cold TCA solution and the tubes were kept for 30 min in ice. The samples were centrifuged in a clinical centrifuge at top speed for 30 min. The supernatant solutions were decanted and the absorbance was read at 280 nm.

For aminopeptidase and carboxypeptidase, endosperms were homogenized as above but in sodium phospate buffer (0.05 M; pH 6.7). The crude homogenate was centrifuged at 20,000 xg for 15 min at 20°C. The supernatant solution could be used directly for aminopeptidase activity.

For the carboxypeptidase activity, the supernatant solution was dialyzed overnight against several changes of 0.1 M sodium acetate buffer, pH 5.2. The substrate solution was prepared by dissolving 5 mg of carbobenzoxy-L-phenylalanyl-L-alanine in 0.1 ml of DMSO and then diluting to 5 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 the above solution. After 15 min of incubation at 37°C, the reaction was stopped by adding 1 ml of 0.83% v/v ninhydrin (prepared in methyl cellosolve). The tubes, covered with marbles, were

transferred to a boiling water bath. After 15 min, they were taken out, cooled, and the solution diluted to 5 ml with 60% ethanol. The absorbance was read at 570 nm.

The substrate for aminopeptidase was prepared by dissolving 5 mg of L-leucyl-p-nitroanilide in 0.2 ml of DMSO and then adding it to 9.8 ml of 0.05 M sodium phosphate buffer, pH 6.7. The reaction was started by adding 50 µl of extract to 1 ml of the substrate solution at 37°C. The reaction was stopped after 60 min by adding 1 ml of 1 N acetic acid. The absorbance was read at 410 nm.

For α-amylase, the endosperms were homogenized to a thick paste with 0.8 ml of 0.2 M sodium chloride and then diluted to 4 ml with the same solution (25). The homogenate was centrifuged at 5,000 xg for 15 min at 4°C and the supernatant solution was used as enzyme extract. Each assay used 1 ml of substrate containing 1.5 mg of potato starch, 6 mg of potassium dihydrogen phosphate and 2 µmoles of calcium chloride. The reaction was started by adding 1 ml of enzyme extract after appropriate dilution (a dilution that gives a decrease in absorbance of about 0.4-0.8 is satisfactory). The reaction was terminated by adding 1 ml of iodine reagent. The iodine stock was prepared by dissolving 6 g of potassium iodide and 600 mg iodine in 100 ml of water, and before assay, diluting 1 ml of stock to 100 ml with 0.05 N HCl. The assay + iodine mixture was diluted to 5 ml by adding

distilled water. The activity was measured in terms of the decrease in absorbance at 620 nm.

4. Gel Filtration of Peptide Hydrolases: Ammonium sulphate was added slowly to the crude enzyme extract at 4°C, with constant stirring at low speed. The final concentration of ammonium sulphate was 60% (12.6 g/35 ml). After 60 min on ice, the suspension was centrifuged at 20,000 xg for 15 min. The protein pellet was dissolved in 3.5 ml of 0.1 M sodium acetate buffer, pH 5.2. The protein was loaded by slowly layering it on top of a Sephadex G-50 column (2.8 x 40 cm) equilibrated with 0.1 M sodium acetate buffer, pH 5.2. Proteins were eluted using the same buffer. Five ml fractions were collected and each fraction was assayed for the activity of peptide hyrolase (122).

5. Fractionation of Storage Proteins: Albumins, globulins, prolamins, and glutelins were fractionated according to the method of Shewry and co-workers (104, 105). The husks and embryonal halves of barley kernels were removed. The remaining endosperms were powdered in a 'Maulimax' coffee grinder for 15-30 min. Lipids were extracted for 24 hours by stirring the powder with 1-butanol in the cold room. A ratio of 1:5 (w/v) was maintained and the residue was washed 3 times. The defatted meal was lyophilized and the albumins were extracted for 24 hours by stirring the meal with cold water at 4°C (5 x 3 ml/g meal). Globulins were extracted

from the remaining residue with 0.5 M sodium chloride (5 x 3 ml/g residue). Finally, the prolamins were extracted with 55% propanol-1 containing 2% 2-mercaptoethanol with vigorous shaking at 60°C (5 x 3 ml/g residue). Albumins were frozen and then lyophilized. Globulins and prolamins were dialyzed against several changes of distilled water. The insoluble protein fractions were then centrifuged at top speed in a clinical centrifuge and the resultant pellets frozen and lyophilized. The different fractions, i.e., albumins, globulins, prolamins, and glutelins in the remaining residue, were analyzed for total nitrogen and polypeptide composition.

6. Fractionation of Extracts: Endosperms were extracted at 4°C with the media in the presence of 0.5 M sodium chloride using a glass homogenizer. The crude homogenate was centrifuged at 20,000 xg for 30 min in a Sorvall rotor SS 34. The supernatant solution thus recovered was termed the soluble fraction. The prolamins were extracted overnight by vigorously shaking the insoluble pellet residue with 55% propanol-1 containing 2% 2-mercaptoethanol at 60°C.

In the initial experiments, large polypeptides in the soluble fraction were precipitated overnight with 10% cold TCA at 4°C. However, when HPLC analyses were performed, polypeptides were precipitated overnight with 50% methanol, instead of 10% TCA. The suspension was then centrifuged at 10,000 xg for 30 ml in a SS 34 rotor. Total nitrogen was

determined in the following fractions: TCA-precipitable, TCA-soluble, prolamin, and the remaining residue. Polypeptides in the prolamin fraction and the TCA- or methanol-precipitable material were analyzed by SDS-PAGE.

7. Separation of Peptides and Amino Acids in the Methanol-Soluble Fraction: The methanol-soluble fraction was passed through a Sephadex G-10 column (1.3 x 90 cm). The column was equilibrated with distilled water at room temperature. The void volume containing large peptides was collected, quick-frozen in liquid nitrogen, and then lyophilized. Free amino acids and oligopeptides (less than 9 amino acid residues in length) were eluted together and thus were analyzed as one group.

Peptides in the void volume were concentrated and passed through a Sephadex G-25 column (1.3 x 90 cm). A Gilson Model 201 fraction collector was used to collect the fractions in the drop mode (90 drops, approx. 2.8 ml). Peptides and amino acids were detected by measuring the absorbance at 210 nm and 280 nm, respectively. The fractions containing larger peptides (between 50-70 residues in length) were pooled and termed 'peak I'. The fractions containing smaller peptides (between 15-35 residues in length) were also pooled and termed 'peak II'. The group containing amino acids + oligopeptides 'peak I', and 'peak II' were analyzed for total nitrogen content.

8. Total Nitrogen Determination: The samples were digested overnight with concentrated sulphuric acid. Complete digestion was achieved by adding a few drops of hydrogen peroxide and boiling the samples for 2-3 hours. The samples were then cooled and after appropriate dilution, an aliquot of digested sample was mixed with 1.5 ml of 5 N sodium hydroxide in a Conway diffusion dish. The resultant ammonia was trapped into 2 ml of 0.1 N HCl. The ammonia was then measured using Berthalot solutions according to a modified method of Kaplan (58). Solution A contained 10 g of phenol and 50 mg of sodium nitroprusside in a total volume of 1 litre of distilled water; and solution B contained 5 g of sodium hydroxide, 10 ml of 5% sodium hypochlorite, and 40.2 g of di-sodium hydrogen phosphate septahydrate in a total volume of 1 litre. Each assay contained 0.2 ml of the aliquot from the center well of the Conway dish and 1 ml each of solutions A and B. Ammonium chloride in the range of 50 to 1000 nmoles/0.5 ml was used as the standard. The absorbance was read at 625 nm.

9. α-Amino Nitrogen Estimation: A ninhydrin method modified from Moore (79) was used to measure the α-amino nitrogen levels. The ninhydrin reagent was prepared by dissolving 2 g of ninhydrin in 75 ml of methyl cellosolve, 25 ml of 4 N sodium acetate buffer, and 0.56 ml of 20% titanium chloride.

The acetate buffer was prepared by dissolving 544 g of sodium acetate trihydrate in 100 ml of acetic acid at 30-40^{o}C and then diluting to 1 litre. The ninhydrin reagent was made fresh each time. Each assay contained 0.5 ml aliquot of the sample and 2.5 ml of the ninhydrin reagent. The tubes were covered with marbles and then held in a boiling water bath for 15 min. The tubes were then taken out, cooled, and the purple colour was read at 570 nm. Alanine in the range of 0 to 0.4 µmoles/ml was used as a standard.

10. Proline Estimation: Free proline was estimated by the ninhydrin method under highly acidic conditions (21a). The reagent was prepared by dissolving ninhydrin at 60^{o}C in a mixture of of 6 M phosphoric and concentrated acetic acid with constant stirring. The ninhydrin was used at a concentration of 25 mg/ml. The acid mixture was prepared by mixing 32.7 ml of concentrated phosphoric acid (14.7 M) to 120 ml of acetic acid and diluting to 200 ml. One ml of sample was added to 1 ml of acetic acid and mixed. One ml of ninhydrin reagent was also added and the tubes covered with marbles. The tubes were then held in a boiling water bath for 60 min. After 60 min the tubes were taken out, cooled, and the colour was read at 515 nm. Proline in the range of 0 to 100 nmoles/ml was used as a standard.

11. HPLC Separation of Amino Acids: The amino acids were first derivatized with OPA (o-phthaldialdehyde) before

injection. The latter was prepared by dissolving 50 mg of OPA in 1 ml of methanol, diluting to 10 ml with 9 ml of 400 mM sodium borate buffer of pH 9.4, and finally adding 0.2 ml 2-mercaptoethanol. It was stored in the dark at room temperature and made fresh each week. Twenty microlitres of 2-mercaptoethanol was added every 24 hours.

The OPA-derivatized amino acids were passed through an Altex 5µ Ultrasphere ODS column (4.6 x 250 mm) attached with a 10µ Ultrapore pre-column (4.6 x 45 mm) to a Beckman HPLC. A twenty µl sample loop was used. The separation of amino acids was achieved in reverse phase mode, using buffer A, 50 mM acetate buffer of pH 5.85/methanol/THF (84:15:1) and buffer B, buffer/methanol (20:80) gradient according to Winspear and Oaks (123). The loop was washed several times with 33% methanol between two consecutive samples. The gradient used is as follows: at 0 min the integrator was turned on; at 3.2 min the sample was injected and the flow rate increased from 0 to 0.8 ml/min, at the same time buffer B increased from 0 to 12% over 8 min and remained constant for the next 14 min; at 25 min buffer B increased to 40% over 30 min; at 55 min buffer B increased to 80% over 15 min and remained constant for next 10 min, at 80 min buffer B increased to 100% over 5 min and remained constant for the next 15 min, at 100 min buffer decreased to 0% over 1 min and remained constant for 9 min, at 105 min the integrator

stopped, and the run was completed at 110 min. Amino acids were detected by Gilson Spectra-Glo fluorometer equipped with OPA filters.

Each amino acid was made up in 30% methanol at 1 mM concentration and stored at -20°C. The concentration of amino acids in the final mix was 50 µM, except for that of ammonium chloride, whose concentration was 500 µM. β-alanine was used as an internal standard. All the reagents were HPLC grade. Nanopure water was used to prepare the buffers. Buffers, water, and methanol were filtered through a 0.45 µ Millipore filter and thoroughly degassed.

12. Protein Estimation: Biorad reagent was used to estimate the content of protein (10). The stock reagent was diluted with distilled water in a ratio of 1:4 and filtered through Whatman #1 filter. The diluted reagent was made fresh each week and stored in a brown bottle. Five millilitres of diluted reagent was added to 0.1 ml of the sample and shaken gently. The colour was read at 595 nm and between two consecutive readings, the cuvette was rinsed with 1 N HCl. BSA in the range of 0 to 140 µg/0.1 ml was used as the standard.

13. SDS-PAGE: A discontinuous slab gel system of Laemmli (61) with 12.5% or 15% running and 4% stacking gel was used for separation of polypeptides in the presence of SDS. The running gel buffer was prepared by adding 18.3 g of Tris to

45 ml of 1 N HCl, adjusting the pH to 8.8, and diluting to 100 ml. The stacking gel buffer was prepared by adding 6.057 g of Tris to 48 ml of 1 N HCl, adjusting the pH to 6.8, and diluting it to 100 ml. The acrylamide monomer was prepared by dissolving 39 g of acrylamide and 1 g of bisacrylamide in a final volume of 100 ml distilled water.

For a running gel of 12.5% concentration, a total volume of 60 ml gel solution contained 25.7 ml of distilled water, 15 ml of the running gel buffer, and 18.75 ml of the monomer. The mixture was degassed for 10 min with slow stirring, after which 0.3 ml of 20% SDS, 190 µl of 10% ammonium persulfate and 35 µl of TEMED were added. After gently mixing, an appropriate volume (just enough to fill 4/5 of the space) was carefully poured between the two glass plates. A few drops of distilled water were then carefully layered on top of the gel. After the polyacrylamide gel was set, the water was pipetted out, and the remaining space rinsed a few times with stacking gel buffer.

For a stacking gel of 4% concentration, a total volume of 30 ml gel solution contained 19.2 ml of distilled water, 7.5 ml of stacking gel buffer, and 3 ml of monomer. The mixture was degassed for 10 min with slow stirring, after which 0.15 ml of 20% SDS, 0.12 ml of 10% ammonium persulfate, and 35 µl of TEMED were added. After gently mixing, it was poured on top of the running gel, and the comb was inserted

before the gel had set. The water was layered on top, and after the gel was set, the comb was pulled out carefully to avoid shearing the stacking gel and the slot margins. The slots were rinsed a few times with distilled water and emptied before the samples were loaded.

The protein samples were dissolved in 850 µl of 0.0625 M Tris-HCl buffer, pH 6.8, containing 10% glycerol and 0.001% bromophenol blue tracking dye. 100 µl of 20% SDS and 50 µl of 2-mercaptoethanol were added to it. The suspension was then boiled for 5-10 min. Approximately 50-100 µg of protein was loaded in each slot. The electrophoresis was performed at room temperature. A current of 1.5 mA/slot was used until the samples migrated through the stacking gel. It was then increased to 3 mA/slot. The protein bands were stained overnight with slow shaking in a solution of 0.125% Coomassie Brilliant Blue R (prepared in 50% methanol and 10% acetic acid). Destaining was done by slowly shaking the gel overnight in a solution of 7.5% acetic acid containing 5% methanol. One or two sponges were also placed in the glass tray to hasten the destaining process. The gels were stored in Ziplock bags with few mls of 7.5% of acetic acid until they were the photographed.

14. Preparation of Hordein Antigens: Hordeins were extracted from dry kernels with 55% propanol-1 containing 2% 2-mercaptoethanol as described above in section 5. The

lyophilized protein was dissolved in the sample buffer as described above in section 13. Polypeptides were electrophoresed through a 12.5% polyacrylamide gel. The major B and C bands (43-67 kDa) were then cut out. The gel was chopped into small pieces, quick-frozen in liquid nitrogen, and then ground into a fine powder. The hordein polypeptides were eluted from the powdered gel using 50 mM sodium acetate buffer, pH 4.8, containing 3-4 M urea, by vigorously shaking the suspension overnight at 37°C.

15. Immunization and Serum Preparation: A modified immunization protocol of Lauriere (66) was followed. Hordein antigens (eluted polypeptides), at a concentration of 4-5 mg/ml, were emulsified with an equal volume of Freund's incomplete adjuvant. The emulsion was injected subcutaneously into 2 New Zealand White rabbits at 4-5 sites. Subsequent injections were made with antigen emulsion prepared with Freund's complete adjuvant. Booster injections were given with 1.25 mg of antigen 21 and 31 days after the first injection. The rabbits were canulated 7 days after the final injection, and blood was collected. For pre-immune serum, 10 ml of blood was collected prior to the first injection. The blood samples were allowed to stand at 4°C for 4-5 hours. Then using an applicator stick, the clot was slowly swirled along the periphery. Finally the clot was pelleted by centrifuging the samples at 10,000 xg for 10 min.

The serum, i.e., clear supernatant solution, was carefully pipetted out, and spun once again for 10 min. The serum was stored at -60°C until use.

16. Double Immunodiffusion Assay: Hordein antigens were dissolved in 50 mM sodium acetate buffer, pH 4.8, containing 3-4 M urea. The immunodiffusion was carried out according to the method of Ouchterlony (89), in 1% (w/v) agarose gel dissolved in veronal buffer of pH 8.6. The diffusion was allowed at room temperature for 24-36 hours, after which the gel was extensively washed with several changes of 0.9% saline solution for 36-48 hours. The gel was then dried completely. Immunoprecipitates were stained for 5 min with 1% amido black solution, after which they were destained in 7.5% acetic acid.

17. Enrichment of Crude Serum for Hordein-IgG's: Precipitin lines were noted when the crude serum was challenged against both types of proteins, i.e. hordeins and non-hordeins (soluble), extracted from ungerminated kernels. This result suggested the presence of non-hordein related IgG's in the crude serum. Removal of contaminating IgG's was achieved by pre-treating the crude serum with soluble proteins. One millilitre of a solution containing 125, 250, 500, 1000, 2000, and 4000 µg of soluble proteins was added to 1 ml of the crude serum and incubated at 4°C. After 48 hours, the samples were cenrifuged, resulting in removal of the

complexes between soluble proteins and non-specific IgG's. The resultant serum from each treatment was challenged again with both types of proteins. As seen in Figure 27, no precipitin line was noted against soluble proteins when the antisera pre-treated with 1000 µg or more of the soluble proteins were used. A precipitin line was, however, noted when the same antisera were challenged with hordeins. This result suggested that the hordein-unrelated IgG's had been removed from the crude serum.

18. Western Immunoblotting: Polypeptides were electrophoresed through a 15% polyacrylamide gel as described above in section 13. They were then electroblotted onto a nitrocellulose membrane using a Biorad Trans-blot cell. The stock solution of electroblotting buffer was prepared by dissolving 15.15 g Tris and 72.0g glycine in 250 ml of distilled water, adjusting the pH to 8.3 with acetic acid, and then diluting to 1 litre. Before electroblotting, 600 ml of stock buffer was added to 600 ml of methanol and then diluted to 3 litres. Four sheets of 3MM filters, 2 sponges, and nitrocellulose membrane were submerged in the buffer and cooled. The blotting was done for 4 hours at 4°C using 60 v and 0.25 amp (113). The buffer was stirred during electroblotting. All subsequent operations were performed at room temperature with slow shaking.

The nitrocellulose membrane, after electroblotting, was incubated overnight (1 hour is sufficient) in a blocking solution, which contained 3% gelatin (BSA and Carnation milk were also tried, but results were not as good as with gelatin) prepared in 50 mM Tris-HCl containing 150 mM sodium chloride, pH 7.5 (Tris-saline buffer). Next, the nitrocellulose membrane was incubated for 3 hours in a solution of primary antibody (hordein IgG enriched serum) diluted 1/1000 with the blocking solution. After the membrane was washed twice for 10 min each with Tris-saline buffer, pH 7.5, it was incubated for 1 hour in a solution of Goat Anti-Rabbit IgG (H+L) Horseradish Peroxidase conjugate (GAR-HRP, diluted 1/1000 with the blocking solution). It was again washed twice for 10 min each with Tris-saline buffer. Immunoreactive bands were visible after treating the nitrocellulose membrane with the colour reagent. The reagent was prepared by dissolving 60 mg of 4-chloro-1-naphthol in 20 ml of methanol, and then adding it to 100 ml of Tris-saline buffer containing 30 µl of hydrogen peroxide. The membrane was incubated in this reagent and the colour development was stopped by replacing the reagent with cold distilled water.

19. Immunoaffinity Column Chromatography: An immunoaffinity column was prepared according to the method of Livingston (68). Proteins in the crude serum were precipitated with ammonium sulphate at 4°C with slow shaking (final saturation

33%, 2 g/10 ml serum). After 60 min, the suspension was centrifuged at 4°C, and the pellet was dissolved in 0.1 M sodium bicarbonate buffer, pH 8.3, containing 0.5 M sodium chloride. The protein solution was then dialyzed at 4°C against 1 litre of the same buffer. The IgG's were enriched and partially purified by passing the protein sample through a DEAE-cellulose (DE-23, Whatman) column. The column was prepared in a 10 ml syringe, and equilibrated with 10 mM potassium phosphate buffer, pH 6.8. About 150 ml of the buffer was passed over the column at 52 ml/70 min. After loading protein samples, the buffer was passed through the column, and 80 fractions each of approx. 2.1 ml were collected. Absorbance was read at 280 nm, and the IgG containing fractions were pooled. Proteins were again precipitated with ammonium sulphate (50% final saturation, 31.3 g/100 ml). The pellet containing the IgG's was dissolved and dialyzed overnight at 4°C against several changes of 10 mM potassium phosphate buffer, pH 6.8. The IgG's were then dialyzed against coupling buffer, 0.1 M sodium bicarbonate buffer, pH 8.3, containing 0.5 M sodium chloride.

The IgG's (hordein IgG enriched serum) were then coupled to cyanogen bromide-activated Sepharose 4B beads. Two g of dry cyanogen bromide powder (after swelling, each g gives approx. 3.5 ml gel) was first added to 10 ml of 1 mM HCl. The gel was washed for 15 min with about 150 ml of 1 mM

HCl on a sintered glass filter. After washing, the IgG solution dissolved in the coupling buffer was added to cyanogen bromide gel suspension (approx. 5-10 mg of IgG's/ml of the gel were used). Coupling was allowed for 4-5 hours at 4°C with gentle shaking on a rotary shaker. The IgG-coupled cyanogen bromide gel was then packed in a 10 ml syringe, and then washed with 3 cycles of alternating solutions (100 ml) of coupling buffer and 0.1 M sodium acetate buffer of pH 4 containing 0.5 M sodium chloride. Finally it was washed with 10 mM sodium bicarbonate buffer of pH 8.0.

The protein sample from incubated half-kernels was loaded by cycling the solution through the gel for 5-6 hours using a peristaltic pump. About 150 ml of 10 mM sodium bicarbonate buffer, pH 8, was then passed through the column to wash off the unligated antigens. Finally, the bound antigens were eluted with 0.1 M glycine-HCl buffer, pH 2.8, containing 8 M urea. Thereafter, about 150 ml of 10 mM sodium bicarbonate buffer, pH 8, was passed through the column to wash off urea and other ions. Fractions containing the eluted proteins were identified by measuring the absorbance at 280 nm, and pooled. The pooled fractions were dialyzed extensively against several changes of distilled water to rid the samples of urea and other salts. The samples were then lyophilized.

20. <u>High Performance-Size Exclusion Chromatography</u>: The

eluted proteins were dissolved in 10 mM sodium phosphate buffer, pH 6.8, and then passed through a Spherogel™-TSK G2000 SW column (7.5 mm x 30 cm) attached to a Beckman HPLC (33). A one hundred µl sample loop was used, and between two consecutive samples, the loop was washed with distilled water. Proteins were eluted with 10 mM sodium phosphate buffer, pH 6.8, containing 150 mM sodium chloride, and detected at 215 nm using a Beckman Model 160 UV detector. A flow rate of 0.5 ml/min was used. Sixty fractions were collected. The fractions were also read manually at 214 nm and pooled in five groups as shown in Figure 33. The pooled fractions were dialyzed against several changes of distilled water at 4°C to rid the samples of salts, and then lyophilized.

21. Determination of the N-Terminal Residues: The samples were dansylated according to the method of Oray (87). The dansyl reagent was prepared by dissolving 2.5 mg dansyl chloride in 1 ml of acetonitrile. It was made fresh each week and stored in the dark at -20°C. 100 µl of 50 mM potassium borate buffer, pH 9.5, was added to the dried samples and mixed. Fifty microlitres of the dansyl chloride reagent was added and mixed again. The tubes were immediately sealed with parafilm, covered with aluminum foil, incubated for 60 min. at 42°C, and transferred to small glass tubes. The samples were then lyophilized.

After dansylation, 100 µl of 6 N HCl was added to the lyophilized samples. The tubes were flame-sealed under vacuum, and kept in the oven for 8 hours at 110°C. After cooling the seals were broken and the samples dried. To wash off the excess acid, samples were taken up in 100 µl of 33% acetonitrile and dried again. This cycle was repeated 4-5 times. The dansylated residues were passed through an Altex 5µ Ultrasphere ODS column (4.6 x 250 mm) attached with a 10µ Ultrapore pre-column (4.6 x 45 mm) to a Beckman HPLC. A twenty µl sample loop was used. The separation was achieved by passing a gradient of buffer A (30 mM sodium phosphate, pH 6.5, containing 2% THF) and buffer B (acetonitrile). Between consecutive samples, the loop was washed with 33% acetonitrile. The gradient used is as follows: at 0 min, the integrator is started and sample loaded; at 1 min, the concentration of buffer B is 12%, and the flow rate increased to 1 ml/min over a 1 min period; at 2 min the concentration of B increased from 12% to 20% over the next 38 min period; at 40 min buffer B increased from 20% to 35% over the next 40 min; at 80 min, buffer B increased from 35% to 60% over the next 10 min period; at 90 min, buffer B decreased from 60% to 12% over the next 2 min period to restore the column to the starting conditions; at 95 min, the integrator stopped and the data was printed; and finally, at 100 min, the flow rate stopped. Dansyl-derivatized amino acids were detected using a Gilson fluorometer equipped with [illegible]amine filters.

For standards, a 1 mM solution of each of the common dansylated amino acids (Sigma) was made up in 30% methanol. The concentration of each amino acid in the mix was 50 μM. All the reagents were HPLC grade. Nanopure water was used for preparing the buffers. Water, acetonitrile, and buffers were filtered through a 0.45μ Millipore filter, and then thoroughly degassed.

RESULTS

1. Initial Standardization:

Cereal endosperm reserves are degraded to sugars and amino acids during the early seedling growth. α-amylase, β-amylase, and debranching enzyme activities are responsible for the breakdown of starch. Activities of various proteases (endoprotease, carboxypeptidase, and aminopeptidase) are considered to be involved in the overall hydrolysis of the storage proteins.

1.1. Peptide hydrolase activities in barley and corn endosperms: The initial measurement of endopeptidase, carboxypeptidase, and aminopeptidase activities was done using model substrates, i.e. hemoglobin, carbobenzoxy-phenylalanyl-alanine, and L-leucyl-p-nitroanilide, respectively. The activities of endopeptidase, carboxypeptidase, and aminopeptidase were measured with extracts prepared from the endosperms of corn and barley (cv. 'Bonanza'), six days after imbibition. Since the activity of aminopeptidase was barely detectable in these extracts, the extracts prepared from dry kernels were used for measuring the enzyme activity. Dialyzed and undialyzed hemoglobin solutions were used as the substrate for measuring the activity of endopeptidase. The dialysis was done to rid the substrate of small molecular weight UV-absorbing material.

The activity of endopeptidase in both corn and barley is linear for the initial 30-45 min. (Fig. 1A and 1B). Overnight dialysis of the substrate had no effect on its susceptibility to hydrolysis.

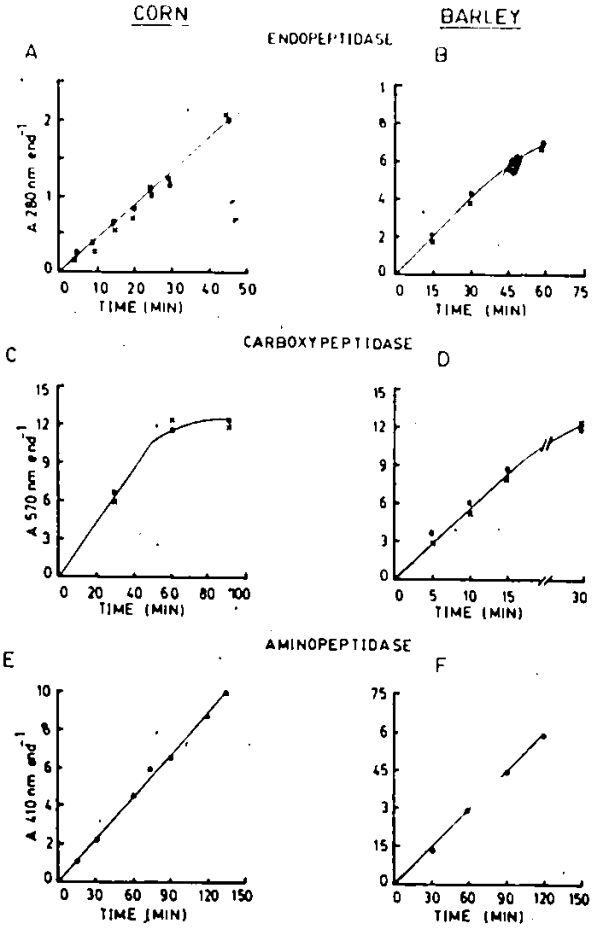
For measuring the activity of carboxypeptidase, both dialyzed and undialyzed crude extracts were used. Dialysis was performed to rid the extracts of amino acids and small peptides, which results in low background colour. Whereas in corn, the activity of carboxypeptidase is linear up to 50-60 min, it starts to plateau within 15 min when barley extracts were used (Fig. 1C and 1D). Dialysis of the extracts had no effect on their activities.

The activity of aminopeptidase is constant up to 120 min, in both corn and barley (1E and 1F). The activities of endopeptidase and carboxypeptidase in extracts of endosperms from corn and barley, six days after imbibition, are summarized in Table 3. The extracts from barley compared to those from corn, have a much higher proportion of carboxypeptidase, since the ratio between the activities of carboxypeptidase and endopeptidase is respectively 41.5 and 5.5 in the two systems. Corn was used as a control system in the initial activity measurement experiments only. Subsequent work was restricted to barley.

1.2. Gel filtration of crude extract through Sephadex G-50 column: The activities of endopeptidase and carboxypeptidase eluted in two separate peaks, when the

Figure 1. Initial standardization of the peptide hydrolase activities in extracts of corn (W64A x W182E) and barley (cv. Bonanza) endosperms. The endosperms were harvested six days after imbibition. The extraction and measurement of the enzyme activity were performed as described in the Materials and Methods. Saturating levels of substrates were used to assay the 3 enzyme activities. The rate of reaction was also linear with the increase in enzyme concentration. o_____o and x_____x in A and B refer to undialyzed and dialyzed hemoglobin, respectively. In C and D, these symbols refer to undialyzed and dialyzed enzyme extracts, respectively. end. = endosperm.

The experiment was performed once with corn extracts and twice with barley extracts. Enzyme assays were performed in triplicate. Each point represents the mean of three readings. The mean variation was less than 5%.


CORN
BARLEY
ENDOPEPTIDASE
A
B
A 280 nm end^{-1}
TIME (MIN)
CARBOXYPEPTIDASE
C
D
A 570 nm end^{-1}
TIME (MIN)
AMINOPEPTIDASE
E
F
A 410 nm end^{-1}
TIME (MIN)

extracts from corn endosperms, harvested six days after imbibition, were passed through a column of Sephadex G-50 (Fig. 2B, 122). A barley endosperm extract was also passed through a similar column of Sephadex G-50. Co-elution of both the activities occurred showing that the elution behaviour of peptide hydrolases is different in barley and corn (Figs. 2A and 2B).

1.3. Time-course of the appearance of peptide hydrolase and α-amylase activity: The appearance of peptide hydrolase and α-amylase activity in barley (cv. Bonanza) endosperms, following imbibition, is shown in Figure 3. Levels of both α-amylase and carboxypeptidase activities are low in the extracts from dry kernels. After a lag of 1-2 days, their levels increase sharply and reach a peak between 4 and 6 days. Endopeptidase activity, on the other hand, is barely detectable in the dry kernels. It starts to increase after a lag of 3 days and peaks between 7 and 8 days.

The appearance of carboxypeptidase and endopeptidase activity following imbibition in another cv. of barley, Perth, is shown in Figure 4. Reasons for selecting this cultivar will be evident in the following section. The level of carboxypeptidase activity is low in the extracts from dry kernels. After a lag of 1-2 days, its activity starts to increase sharply and peaks between 4 and 5 days. The activity of endopeptidase is barely detectable in the extracts from dry kernels. It starts to increase sharply

Table 3: Comparison of Peptide Hydrolase Activities in Extracts of Corn and Barley Endosperms Germinated for Six Days.

System	Enzyme Units*/endosperm			Enzyme Units/g fresh weight		
	EP	CP	Ratio	EP	CP	Ratio
Corn	0.6 ± 0.1	3.3 ± 0.4	5.5	4.2 ± 0.4	23.1 ± 2	5.5
Barley	0.2 ± 0.1	8.3 ± 0.8	41.5	6.0 ± 0.9	249.0 ± 24	41.5

The extracts were prepared from endosperms of seedlings grown for six days after imbibition. Extractions and the enzyme assays were performed as described in the Materials and Methods. Saturating levels of the substrates were used for assaying enzyme activities. The experiment was performed once with corn extracts and two times with barley extracts. Mean values ± 1/2 range between minimum and maximum is given.

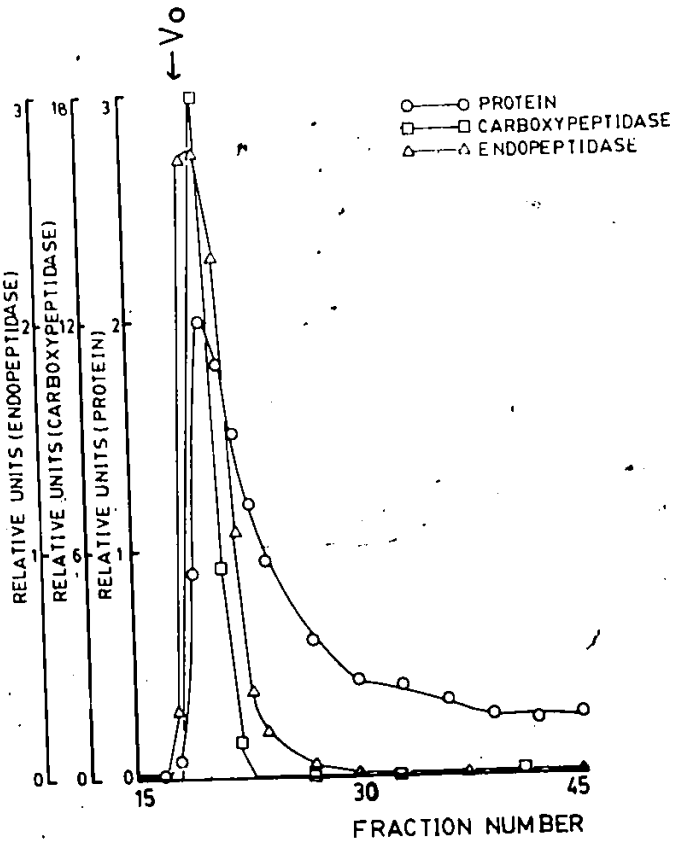
EP = endopeptidase, CP = carboxypeptidase.

* 1 unit of EP = Δ A280 nm/15 min.

1 unit of CP = Δ A570 nm/15 min.

Figure 2a. Elution profile of barley (cv. Bonanza) crude extract through Sephadex G-50 column (2.8 x 40 cm). The extract was prepared from 250 endosperms of seedlings grown for 6 days. The extraction, protein concentration, and gel filtration were performed as described in the Materials and Methods. Flow rate was 0.7 ml/min. Each fraction was approximately 4.2 ml. Void volume (Vo) was approximately 66 ml. One relative unit for endopeptidase, carboxypeptidase and protein is; Δ A 280 nm/fraction/hour, $\tilde{\Delta}$ A 570 nm/fraction/hour, and A 280 nm/fraction, respectively. The experiment was performed once. Enzyme assays were performed in duplicate. Each point represents the mean of two readings. The mean variation was less than 5%.

Figure 2b. Elution profile of corn (W64A X W182E) crude extract through Sephadex G-50 column (3 X 40 cm). (Courtesy Winspear, 122). The extract was prepared from 30 endosperms. Other details are same as in Figure 2a.


Vo
PROTEIN
CARBOXYPEPTIDASE
ENDOPEPTIDASE
RELATIVE UNITS (ENDOPEPTIDASE)
RELATIVE UNITS (CARBOXYPEPTIDASE)
RELATIVE UNITS (PROTEIN)
FRACTION NUMBER
15
30
45

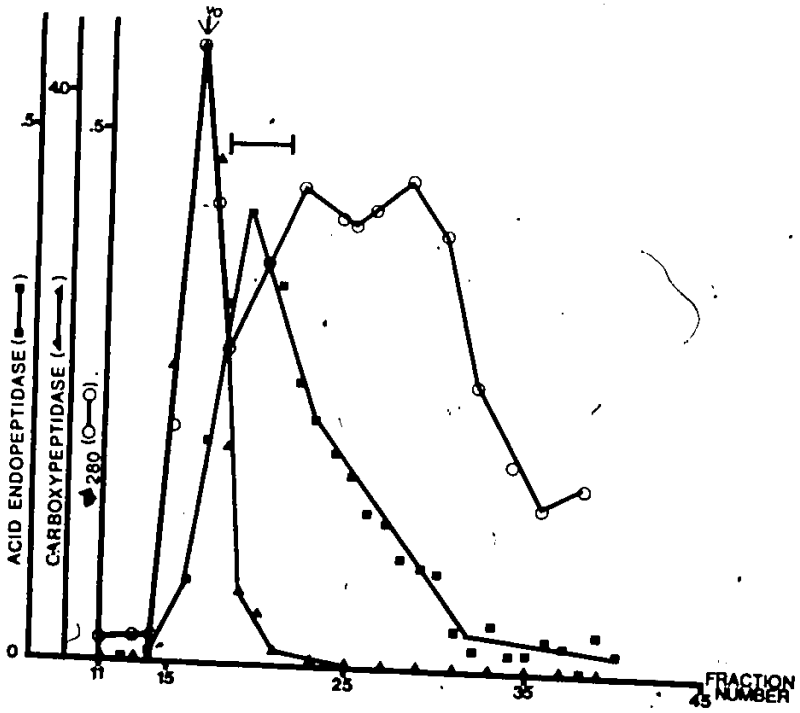

ACID ENDOPEPTIDASE
CARBOXYPEPTIDASE
280
40
5
0
11
15
25
35
45
FRACTION NUMBER

Figure 3. Time-course of the appearance of alpha-amylase, endopeptidase, and carboxypeptidase activities in barley (cv. Bonanza) endosperms. The endosperms were harvested at different times after imbibition. The extractions and enzyme assays were performed as described in the Materials and Methods. One enzyme unit of alpha-amylase, endopeptidase, and carboxypeptidase is: Δ A 620 nm/endosperm/hour, Δ A280 nm/endosperm/hour, Δ A 570 nm/endosperm/hour, respectively. The whole experiment was performed once. But for early period, i.e. up to 72 hours after imbibition, it was repeated one more time and a similar trend was observed. Enzyme assays were performed in duplicate. Each point represents the mean of two readings. Bars represent the range between minimum and maximum values.

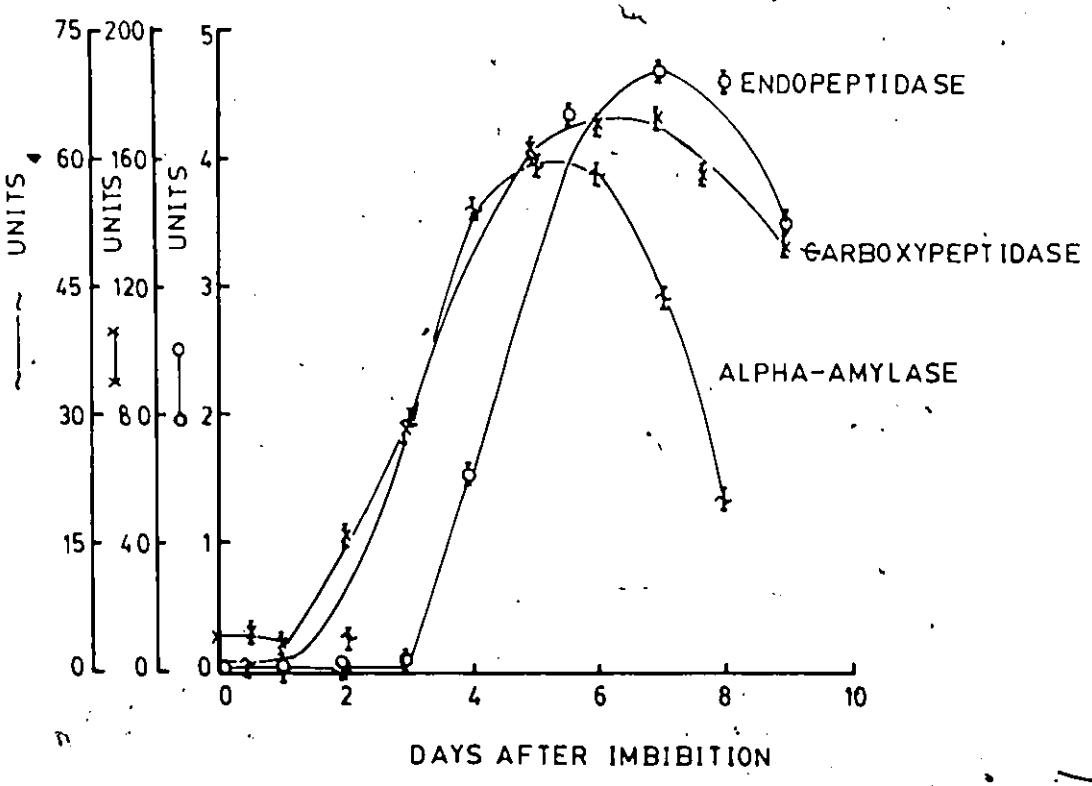
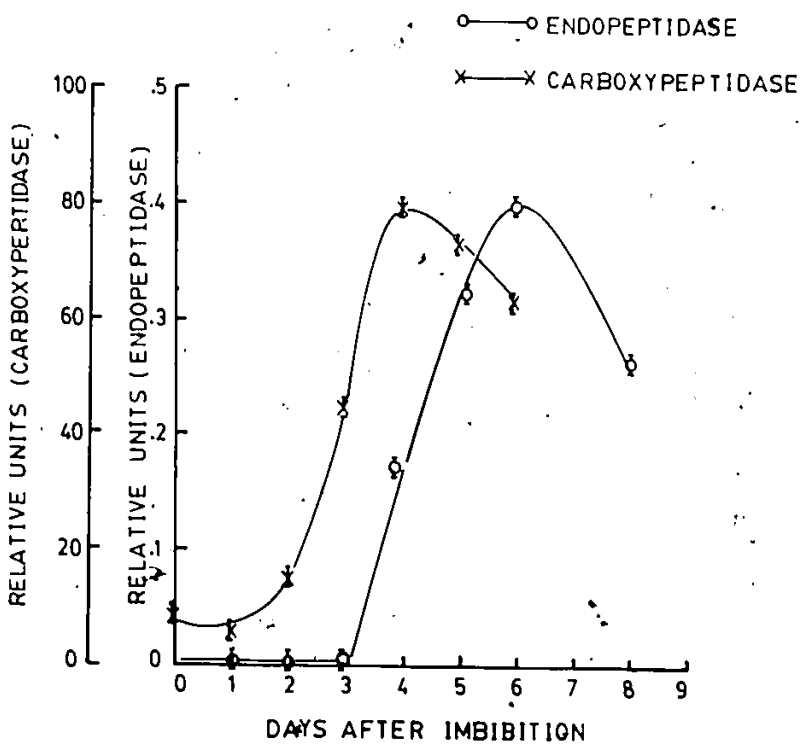

75
60
45
30
15
0
UNITS
200
160
120
80
40
0
UNITS
5
4
3
2
1
0
UNITS
0
2
4
6
8
10
DAYS AFTER IMBIBITION
ENDOPEPTIDASE
CARBOXYPEPTIDASE
ALPHA-AMYLASE

Figure 4. Time-course of the appearance of endopeptidase and carboxypeptidase activities in barley (cv. Perth) endosperms. The extractions and enzyme assays were performed as described in the Materials and Methods. One enzyme unit of endopeptidase and carboxypeptidase is: Δ A 280 nm/endosperm/hour and Δ A 570 nm/endosperm/hour, respectively.

The whole experiment was performed once. But for early period, i.e. up to 72 hours after imbibition, it was repeated one more time and a similar trend was observed. Enzyme assays were performed in duplicate. Each point represents the mean of two readings. Bars represent the range between minimum and maximum values.


ENDOPEPTIDASE
CARBOXYPEPTIDASE
RELATIVE UNITS (CARBOXYPERTIDASE)
RELATIVE UNITS (ENDOPEPTIDASE)
100
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0
0 1 2 3 4 5 6 7 8 9
DAYS AFTER IMBIBITION

after a lag of 3 days and peaks between 6 and 7 days.

1.4. Screening of barley cultivars: The induction of hydrolases, such as α-amylase and protease by GA_3 in barley (cv. 'Himalaya') is well documented (24, 25, 116). The GA_3-induced proteases have been implicated in the mobilization of stored carbohydrates and proteins in barley endosperms (8, 56, 102). A dependence of the initial steps of protein hydroysis on GA_3 has, however, never been examined. A GA_3-responsive cv. of barley was therefore sought to examine this point in detail. Five cvs of barley, i.e., Bonanza, Bruce, Elrose, Herta, and Perth, were screened to test whether the induction of α-amylase was dependent on the presence of GA_3. Of these, cv. 'Perth' showed a good response in the presence of GA_3, with low background activity in the control samples (Table 4). This cultivar was chosen for detailed work.

1.5. Time-course of the appearance of α-amylase and protease activities in embryo-less endosperm pieces: The hydrolysis of storage proteins can be initiated under two conditions: a) following imbibition and normal germination; and b) incubation of the embryoless endosperms in an appropriate buffer containing GA_3. The technique of half-kernel incubation has been used extensively in the induction studies of α-amylase by GA_3. The subsequent series of experiments designed to investigate protein hydrolase were also performed using the same technique.

Table 4. Induction of α-amylase Activity in Five Cultivars of Barley Half-Kernels by Gibberellic Acid.

	α-amylase Activity					
	Medium		Half-kernels		Total	
Cultivars	$-GA_3$	$+GA_3$	$-GA_3$	$+GA_3$	$-GA_3$	$+GA_3$
Bonanza	0.9 ± 0.2	7.9 ± 0.3	1.2 ± 0.5	2.2 ± 0.1	2.1	9.1
Bruce	0.1 ± 0.1	0.4 ± 0.1	-	-	-	0.4
Elrose	-	1.5 ± 0.2	0.9 ± 0.1	1.4 ± 0.2	0.9	2.9
Herta	-	2.0 ± 0.1	-	2.7 ± 0.1	-	4.7
Perth	0.5 ± 0.3	14.3 ± 0.9	0.3 ± 0.1	11.6 ± 0.8	0.8	26.0

Half-kernels were incubated for 48 hours in the presence (+) and absence (-) of GA_3 (5 μM) as described in the Materials and Methods. α-amylase activity was determined using the starch hydrolysis test. The activity is represented as change in absorbance (A620 nm)/min. 10 endosperms or media). The experiment with Perth has been repeated many times, and a similar trend noted. Values are represented as means ± range between minimum and maximum of three determinations of one experiment. Dashes (-) denote barely detectable activity.

Table 5. Effect of Gibberellic Acid on α-Amylase and Protease Activity in Barley (cv. Perth) Half-Kernels.

Treatment (days)	α-amylase μg starch/min. endosperm		Protease μg tryptophan/min. endosperm	
	$-GA_3$	$+GA_3$	$-GA_3$	$+GA_3$
0	2.7 ± 0.1		-	
2	3.4 ± 0.1	140 ± 6	0.1 ± 0.1	0.3 ± 0.1
4	2.5 ± 0.1	240 ± 14	0.1 ± 0.0	0.6 ± 0.1
6	3.7 ± 0.3	371 ± 18	0.1 ± 0.0	0.6 ± 0.1

Half-kernels were incubated for 2, 4, and 6 days in the presence (+) and absence (-) of GA_3 (5 μM). α-amylase activity was determined using the starch hydrolysis test and protease activity was measured using hemoglobin as described in the Materials and Methods. The experiment has been repeated several times and a similar trend observed. Values are represented as means ± standard deviation of three experiments.

(-) dash denotes barely detectable activity

Embryo-less endosperm pieces were incubated in the presence and absence of GA_3 for different times, and the activities of α-amylase and protease were measured. In the absence of GA_3, levels of both the activities remained low up to 2, 4, and 6 days after incubation (Table 5). In its presence, however, the α-amylase activity increased about 45-fold within 2 days of incubation. A 200-fold increase in its activity was noted with a prolonged incubation period. The protease activity shows a 2-fold increase within 2 days of incubation. Maximum activity was noted after 4 days of incubation. A 5-fold increase in the activity of carboxypeptidase was noted after a 6 day incubation period.

1.6. Optimization of embryo-less endosperm incubation test for protein hydrolysis: Changes in the concentration of α-amino nitrogen in the media was chosen as a criterion to estimate the extent of protein hydrolysis. The ninhydrin method was used to measure its amounts. A standard curve of alanine between 0 to 0.200 μmoles/0.5 ml concentration showed a linear relationship with the absorbance (Fig. 5).

1.6.1. pH optima: In order to determine the optimum pH of the incubation medium, endosperm incubations were set up at different pH's, 4.1, 4.8, 5.2, 6.0, and 6.5, in the absence and presence of GA_3. The levels of α-amino nitrogen were measured both in the media and soluble extract of endosperms. In the absence of GA_3, similar levels of α-amino

nitrogen were observed at different pH's (Fig. 6). Approximately the same levels of α-amino nitrogen were present in the incubation media and endosperm extracts. In the presence of GA_3 and at pH 4.1, however, a 2.5-fold increase in the levels of α-amino nitrogen was noted (Fig. 6). At pH 4.8 and 5.2, the levels increased by 4.5-fold. Up to 75% of the α-amino nitrogen was released into the media in GA_3-treated samples. At higher pH's, i.e. 5.9 and 6.5, however, GA_3 additions did not result in increased rates of protein hydrolysis, since a similar level of α-amino nitrogen was noted in the control and GA_3-treated samples. An incubation medium of pH 4.8 was used in subsequent experiments.

1.6.2. Optimum GA_3 concentration: Endosperms were incubated in the presence of 1, 5, 10, 50, and 100 μM concentration of GA_3, to determine the optimum dose of this growth regulator. A control set was incubated in its absence. A 3.5-fold increase in the levels of α-amino nitrogen was noted when half-kernels were incubated in the presence of 1 and 5 uM concentration of GA_3 (Fig. 7). Reduced levels were noted at higher concentrations of GA_3. A five μM concentration of GA_3 was used in the subsequent experiments.

1.6.3. Time-course: Endosperms were incubated in the presence and absence of GA_3 for different times. The release of α-amino nitrogen into the incubation media is

Figure 5. Linear relationship between different concentrations of alanine and absorbance. The alpha-amino nitrogen was estimated using ninhydrin reagent. The preparation of ninhydrin reagent and the assay was performed as described in the Materials and Methods.

The standard curve was performed with each experiment. The variation between different experiments was less than 10%. The test samples were performed in triplicate. Each point represents the mean of three readings. Bars represent the range between minimum and maximum values within one experiment.

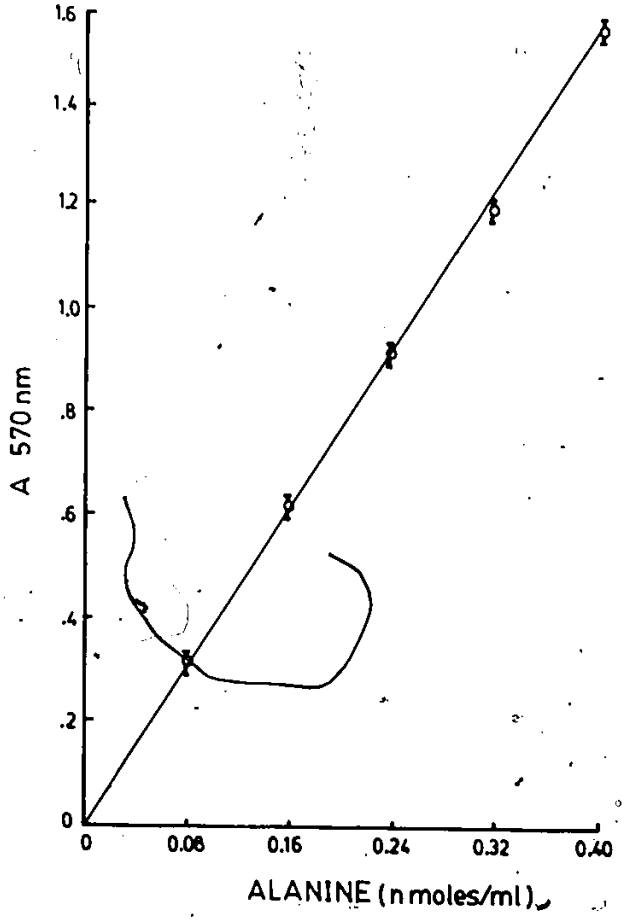

1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
A 570 nm
0
0.08
0.16
0.24
0.32
0.40
ALANINE (n moles/ml)

Figure 6. Effect of different pH's on the release of alpha-amino nitrogen in barley (cv. Perth) endosperms. Embryo-less endosperm pieces were incubated for 48 hours in the buffer media of different pH's (shown in the figure) as described in the Materials and Methods. The levels of alpha-amino nitrogen were measured using ninhydrin reagent. The stippled and clear areas of histogram represent the levels of alpha-amino nitrogen estimated in the incubation media and the extracts of endosperm pieces, respectively. - = absence of GA_3; + = presence of GA_3 (5 μM). The experiment was performed once and all measurements were performed in triplicate. Bars represent the range between minimum and maximum values.

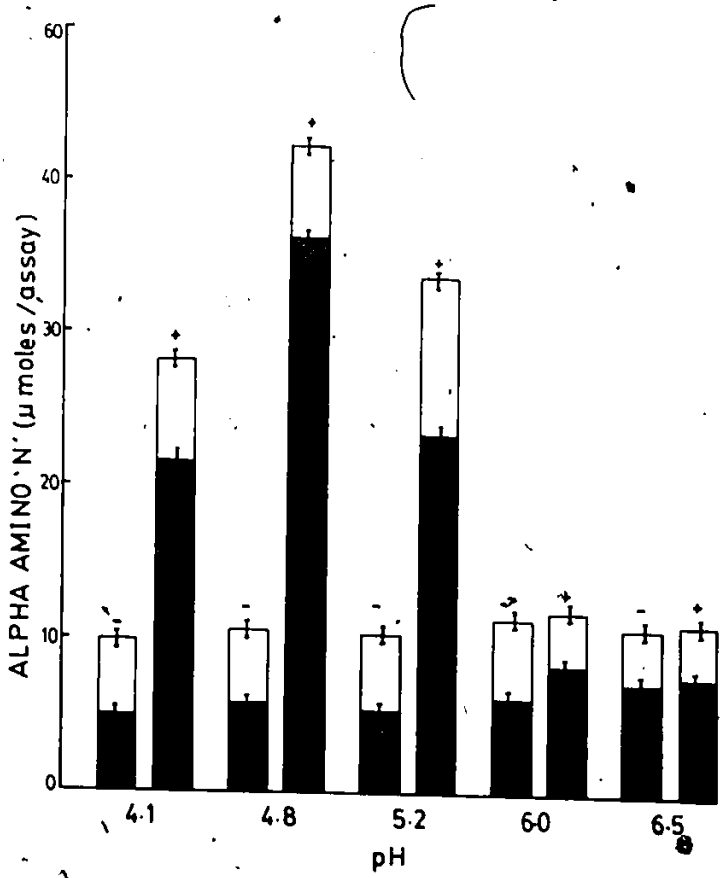

ALPHA AMINO 'N' (μ moles /assay)
60
40
30
20
10
0
4·1
4·8
5·2
6·0
6·5
pH

Figure 7. Effect of different concentrations of gibberellic acid on the release of alpha-amino nitrogen in barley (cv. Perth) endosperms. Embryo-less endosperms were incubated for 72 hours in the buffer media (pH 4.8) containing different concentrations of gibberellic acid. The estimation of alpha-amino nitrogen with ninhydrin reagent was performed as described in the Materials and Methods. The levels of alpha-amino nitrogen were measured in the media (stippled area) and the extracts of endosperms (clear area). The experiment was performed once, and all measurements were performed in triplicate. Bars represent the range between minimum and maximum values.

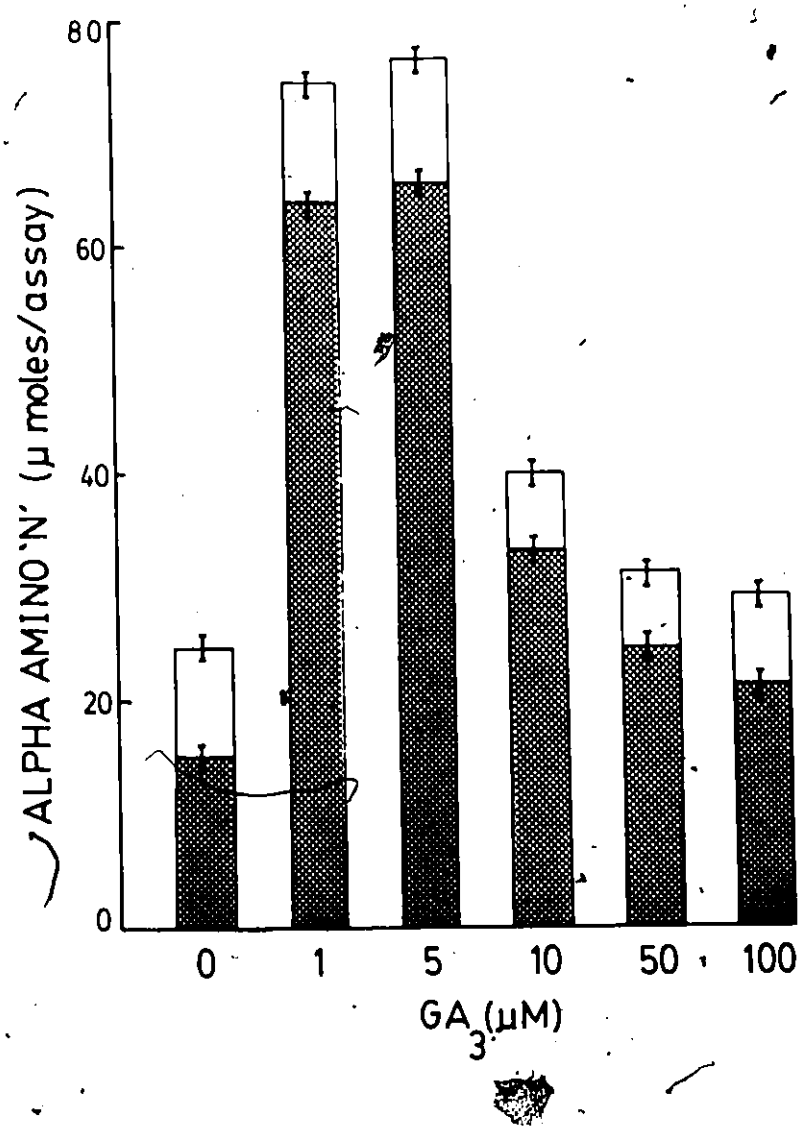
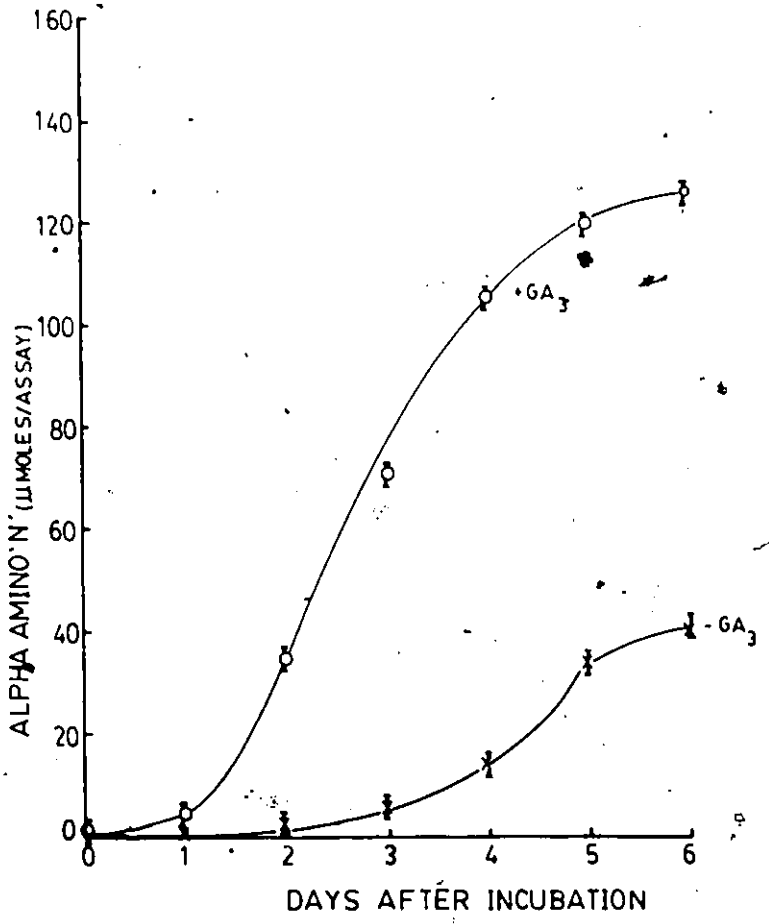

80
60
40
20
0
ALPHA AMINO 'N' (μ moles/assay)
0
1
5
10
50
100
GA3 (μM)

Figure 8. Time-course of the release of alpha-amino nitrogen in barley (cv. Perth) endosperms. Embryo-less endosperms were incubated in the buffer media (pH 4.8) for different times in the presence ($+GA_3$) and absence ($-GA_3$) of gibberellic acid. The levels of alpha-amino acid nitrogen were estimated using ninhydrin reagent in the soluble fraction (media + soluble extracts of endosperms), as described in the Materials and Methods. The experiment was performed twice and all measurements were performed in triplicate. The mean variation between two experiments was $\pm$ 5%. Each point represents the mean of three readings. Bars represent the range between minimum and maximum values.


160
140
120
100
80
60
40
20
0
ALPHA AMINO N (μMOLES/ASSAY)
+GA3
-GA3
0
1
2
3
4
5
6
DAYS AFTER INCUBATION

shown in Figure 8. In the absence of GA_3, the levels of α-amino nitrogen remained low up to 2 days, after which a slow increase was noted. The levels increased about 2-fold by 4-5 days of incubation period. In the presence of GA_3, the levels of α-amino nitrogen remained low up to 1 day, after which a sharp increase was noted up to 4 days. Compared to the controls, about 4-fold higher levels were noted by 5 days of incubation in the presence of GA_3. Since levels of α-amino nitrogen show a linear increase up to 4 days, the hydrolytic products in subsequent experiments were analyzed in samples incubated up to 72 hours.

2. GA_3 and Overall Protein Hydrolysis:

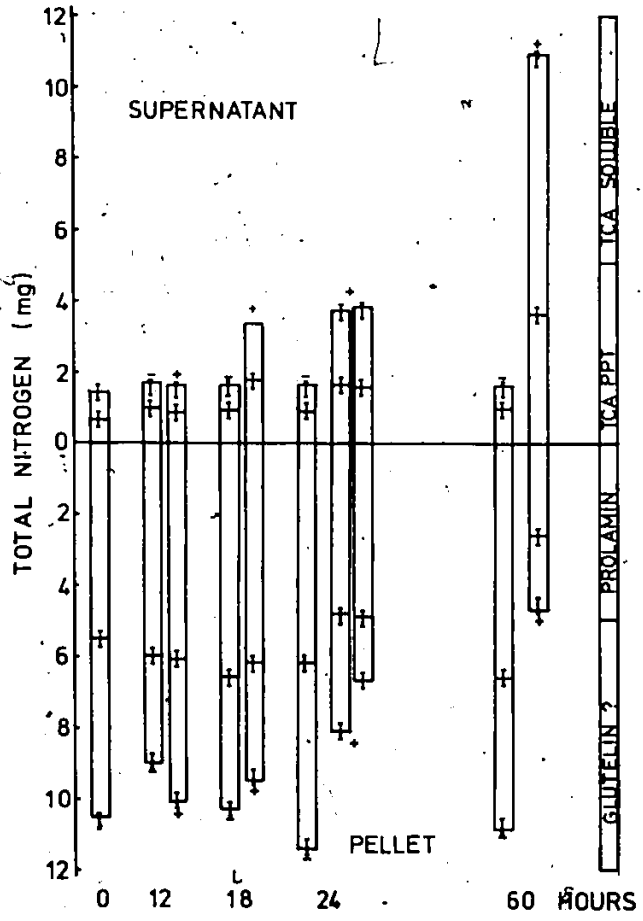
Gibberellic acid induces a number of proteolytic activities in the embryoless endosperms of barley (56, 75, 102). Such activities have been measured using model proteins, e.g. hemoglobin, casein, and synthetic dipeptides, as substrates. The implication that the hydrolysis of storage proteins is dependent on these proteases has, however, not been experimentally substantiated. In the following set of experiments, I wanted to examine: a) whether the overall protein hydrolysis was dependent on the presence of GA_3; and b) the nature of hydrolytic products.

2.1. Redistribution of total nitrogen into soluble and insoluble fractions: The total nitrogen stored in mature barley kernels is distributed in the soluble (buffer

extractable) and insoluble (propanol extractable prolamins and the alkali soluble glutelins) fractions. The hydrolysis of insoluble protein reserves should result in an increase in total nitrogen in the soluble fraction. Redistribution of total nitrogen from the insoluble to the soluble fraction is shown in Figure 9. When embryoless endosperms were used as experimental material, GA_3 was required in the media for appreciable protein hydrolysis. Approximately 12% of the total nitrogen in dry kernels was recovered in the soluble fraction. TCA-soluble and TCA-precipitable material were present in about equal proportion in this fraction. There was no significant change in the distribution of total nitrogen in the absence of GA_3 up to 60 hours. In the GA_3-treated samples, however, 35% of the total nitrogen was recovered in the soluble fraction after a 24 hour incubation period. The increase was observed in both TCA-soluble and TCA-precipitable material. A concomitant decrease in total nitrogen of the insoluble fraction (prolamins + glutelins) was also apparent. The proportion of nitrogen in the soluble fraction increased to about 70% during the next 36 hours. In the samples treated with GA_3 for 60 hours, about 60-70% of the total increase was observed in the TCA-soluble fraction, suggesting that peptides and amino acids were the predominant components in the soluble fraction. In the presence of GA_3, an increase in total nitrogen was, therefore, noted in both TCA-soluble and TCA-precipitable material. This result

Figure 9. Distribution of the total nitrogen in different protein fractions prepared from barley (cv. Perth) endosperms. Embryo-less endosperms were incubated in the presence (+) and absence (-) of gibberellic acid (5 μM) for times shown in the figure. Different fractions, i.e., albumins, globulins, and prolamins, were extracted as described in the Materials and Methods. Total nitrogen was measured in the three fractions and the remaining residue containing glutelins, as described in the Materials and Methods. SUPERNATANT = soluble fraction; PELLET = insoluble proteins (prolamins + glutelins).

The whole experiment was repeated two times and a similar trend was observed. All measurements were performed in triplicate. The means are indicated and bars represent the range between minimum and maximum values.


SUPERNATANT
PELLET
TOTAL NITROGEN (mg)
12
10
8
6
4
2
0
2
4
6
8
10
12
0
12
18
24
60
HOURS
TCA SOLUBLE
TCA PPT
PROLAMIN
GLUTELIN ?

suggests that the overall hydrolysis of proteins is dependent on the presence of GA_3.

2.2. Qualitative changes in the spectrum of polypeptides: In response to GA_3, a loss in total nitrogen from the insoluble fraction and an increase in total nitrogen in the soluble fraction were noted. It is possible that the release of one or more of the large polypeptide fragments from the hydrolysis of hordein account for the observed changes in total nitrogen in the soluble fraction. This possibility was tested by comparing the electrophoretic profile of polypeptides on polyacrylamide gels in the presence of SDS.

2.2.1. TCA-precipitable material in the soluble fraction: SDS-PAGE profile of large polypeptides precipitated from the soluble fraction is shown in Figure 10. In the samples incubated in the absence of GA_3, compared to controls (0 time, soluble proteins from unincubated kernels), no major change in the profile of polypeptides is seen up to 36 hours. In the presence of GA_3, the banding pattern remains largely unchanged at 12 hours, after which an increase in intensity of the band around 42 kDa (Fig. 10) was noted. On the basis of the electrophoretic mobility, this band was tentatively identified as α-amylase polypeptide.

2.2.2. Hordein polypeptides in the insoluble fraction: SDS-PAGE profile of hordeins from the residual pellet is shown in Figure 11. In the absence of GA_3,

Figure 10. SDS-PAGE analysis of polypeptides in the soluble fraction (media + soluble extracts) of barley (cv. Perth) endosperms. Thirty embryo-less endosperms were incubated for different times in the presence (+) and absence (-) of gibberellic acid (5 μM). Polypeptides in the soluble fractions were precipitated using 10% TCA. Electrophoresis was performed using 12.5% gel in presence of SDS, as described in Materials and Methods. Approximately 75 μl of samples (50-70 μg of proteins) were loaded in different wells. H = hours. Protein mole mass (MW) standards were BSA (68 kDa), ovalbumin (45 kDa), trypsinogen (23 kDa), and cytcochrome C (12 kDa). Arrow indicates the position of alpha-amylase polypeptide. The experiment has been repeated several times and essentially similar results were obtained.

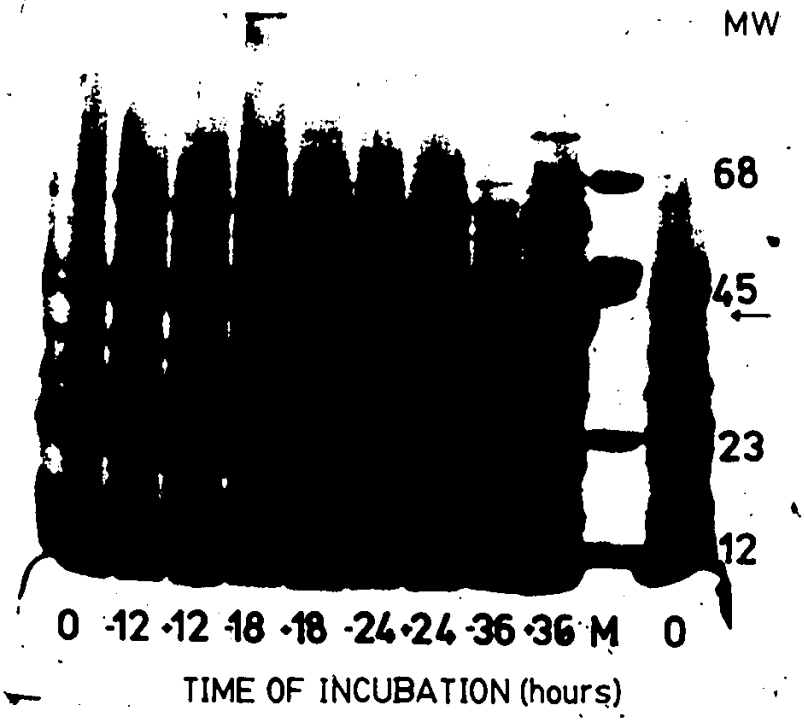
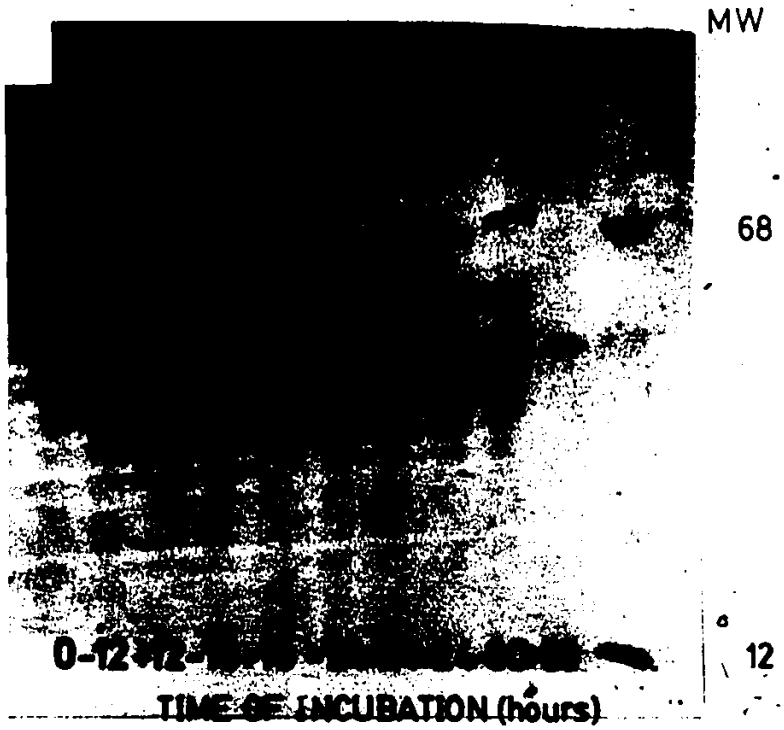

MW
68
45
23
12
0 -12 -12 -18 -18 -24 -24 -36 -36 M 0
TIME OF INCUBATION (hours)

Figure 11. SDS-PAGE analysis of hordein polypeptides in the prolamin fraction of barley (cv. Perth) endosperms. Thirty embryo-less endosperms were incubated for different times in the presence (+) and absence (-) of gibberellic acid (5 μM). The prolamins were extracted as described in the Materials and Methods. The hordein polypeptides were electrophoresed through 12.5% gel in presence of SDS and stained with Coomassie Brilliant Blue R. Equal volumes were loaded in each well. Protein content varied between 10-50 μg H = hours; 0 = prolamins from dry kernels; -12, -18, -24, and -60 = prolamins extracted from endosperms incubated for 12, 18, 24, and 60 hours in the absence of gibberellic acid; +12, +18, +24, and +60 = prolamins extracted from endosperms incubated for 12, 18, 24, and 60 hours in the presence of gibberellic acid. Protein mole mass (MW) standards were BSA (67 kDa) and cytochrome C (12 kDa).
The experiment was repeated two times, and similar results were obtained.


MW
68
12
TIME OF INCUBATION (hours)

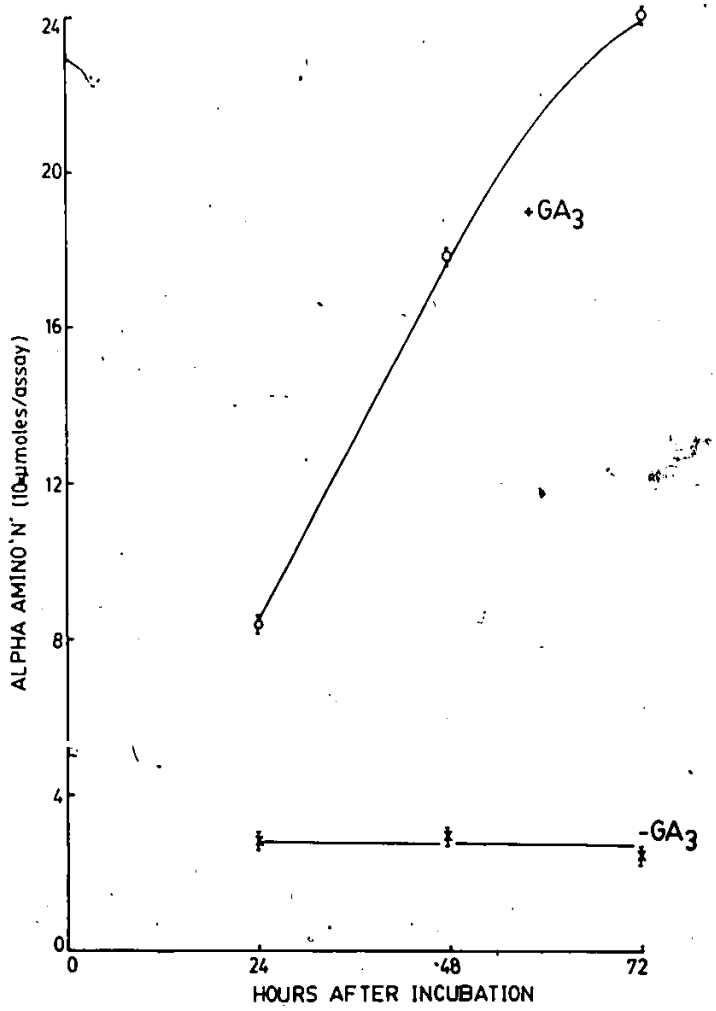
compared to controls (0, unincubated kernels), no major change is detected during the initial 60 hours of incubation. In the GA_3- treated samples, however, a gradual loss of staining intensity of the major bands with long incubation periods was noted. This result suggests that an observable loss of the hordein polypeptides requires the presence of GA_3, and that this loss is of a general nature, i.e. no one single polypeptide is preferentially degraded.

2.3. Analysis of the methanol-soluble fraction: Following the addition of GA_3, an increase in total nitrogen was noted in the methanol-soluble fraction. This increase may be due to either soluble peptides of uniform or heterogeneous sizes, and/or amino acids. Analysis of methanol-soluble fraction is summarized below.

2.3.1. Amino acid estimation: The appearance of α-amino nitrogen in the methanol-soluble fraction of the media is shown in Figure 12. In the absence of GA_3, the level of α-amino nitrogen remained low for the entire incubation period. In the presence of GA_3 however, about 2.8-fold and 8-fold higher levels were noted after a 24 hour and 72 hour incubation period, respectively. This result suggests that the major release of amino acids and peptides occur between 24 and 72 hours of incubation period.

2.3.2. Proline estimation: Proline is one of the predominant amino acids in the hordein proteins. The time-

Figure 12. Time-course of the release of alpha-amino nitrogen in the methanol-soluble fraction (media + soluble extracts) of barley (cv. Perth) endosperms. Thirty embryo-less endosperms were incubated for different times in the presence (+) and absence (-) of gibberellic acid (5 μM). The alpha-amino nitrogen was estimated using the ninhydrin reagent as described in the Materials and Methods. The experiment was performed once and all measurements were performed in triplicate. Each point represents the mean and bars represent the range between minimum and maximum values.


24
20
16
12
8
4
0
ALPHA AMINO 'N' (10µmoles/assay)
+GA3
-GA3
0
24
48
72
HOURS AFTER INCUBATION

course of its appearance in the methanol-soluble fraction of the media was followed. A standard curve of proline between 0-75 μmoles/ml shows a linear relationship between the concentration and absorbance (Fig. 13). In the absence of GA_3, the levels of proline remained low up to a 72 hour of incubation period (Fig. 14). In the GA_3-treated samples, however, the levels of proline increased slowly up to a 24 hour period, after which a sharp increase in its level was noted up to 72 hours. This result shows that the major release of proline occurs between 24 and 72 hours after the initiation of the experiment.

2.3.3. Presence of peptides: Release of peptides may also account for an increase in total nitrogen in the methanol-soluble fraction. If peptides were the initial products, a higher proportion of amino acids should be recovered in the peptide-bound form at early times. The concentration of different amino acids was estimated after their separation. Amino acid separation was achieved using an HPLC. A mixture of 20 standard amino acids and an internal standard, α-alanine were passed through an HPLC to determine their elution time. The elution profile is shown in Figure 15. Proline is not detected because of an absence of a primary amino group. Amino acid concentrations were estimated in the methanol-soluble fraction before (BH) and after (AH) hydrolysis in the presence of 6 N HCl. The data are summarized in Table 6. The ratio of amino acid

Figure 13. Linear relationship between different concentrations of proline and absorbance. Proline was measured under highly acidic conditions using ninhydrin reagent according to the method of Chinard (21a) as described in the Materials and Methods. This method is specific for proline and presence of other amino acids does not interfere with proline measurement. The standard curve was performed with each experiment, and all measurements were performed in duplicate. Variation in different experiments was less than 5%.

Each point represents the mean, and bars represent the range between minimum and maximum values.

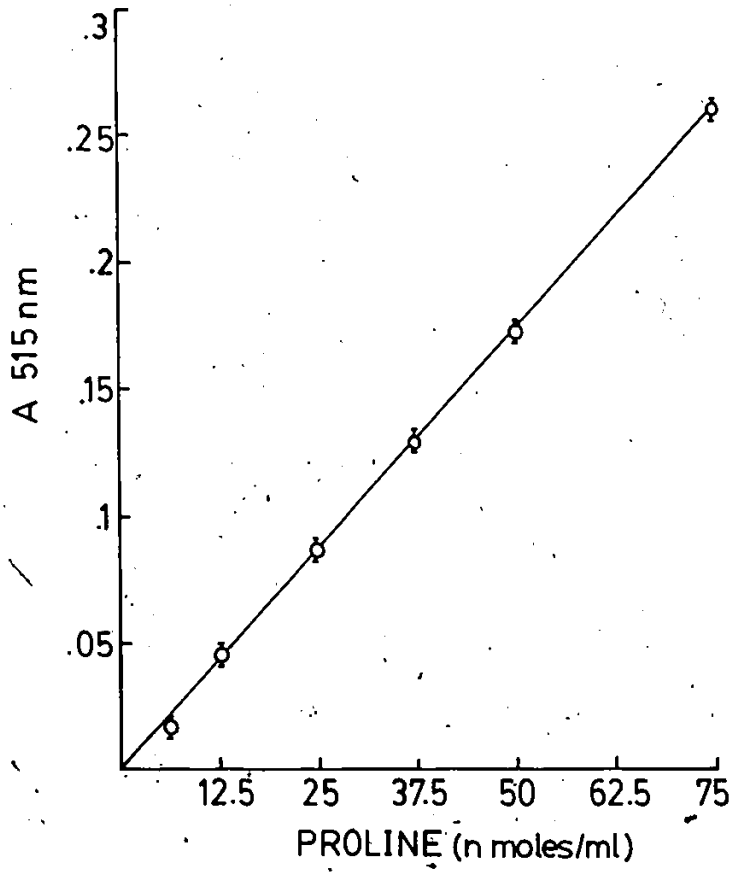

.3
.25
.2
.15
.1
.05
A 515 nm
12.5
25
37.5
50
62.5
75
PROLINE (n moles/ml)

Figure 14. Time-course of the release of proline in the methanol-soluble fraction (media + soluble extracts) of barley (cv. Perth) endosperms. Thirty embryo-less endosperms were incubated for different times in the presence ($+GA_3$) and absence ($-GA_3$) of gibberellic acid (5 μM). Proline was measured by ninhydrin reagent according to the method of Chinard (21a) as described in the Materials and Methods. The whole experiment was performed once, and all measurements were performed in triplicate. Each point represents the mean, and bars represent the range between minimum and maximum values.

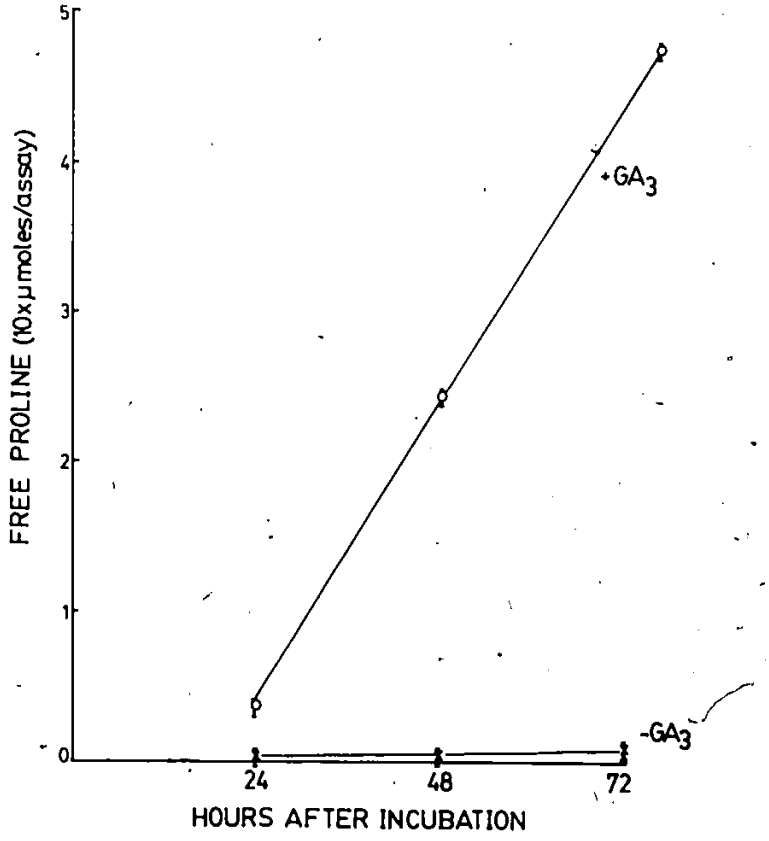

FREE PROLINE (10x µ moles/assay)
5
4
3
2
1
0
+GA3
-GA3
24
48
72
HOURS AFTER INCUBATION

Figure 15. Elution profile of OPA-derivatized amino acids. A mixture of amino acids was passed through an Altex 5μ Ultrasphere ODS column (4.6 x 250 mm) using a Beckman HPLC. Before samples were injected, the amino acids were derivatized with OPA. The gradient and detailed methodology are described in the Materials and Methods. Standards were run after 4-5 test samples.

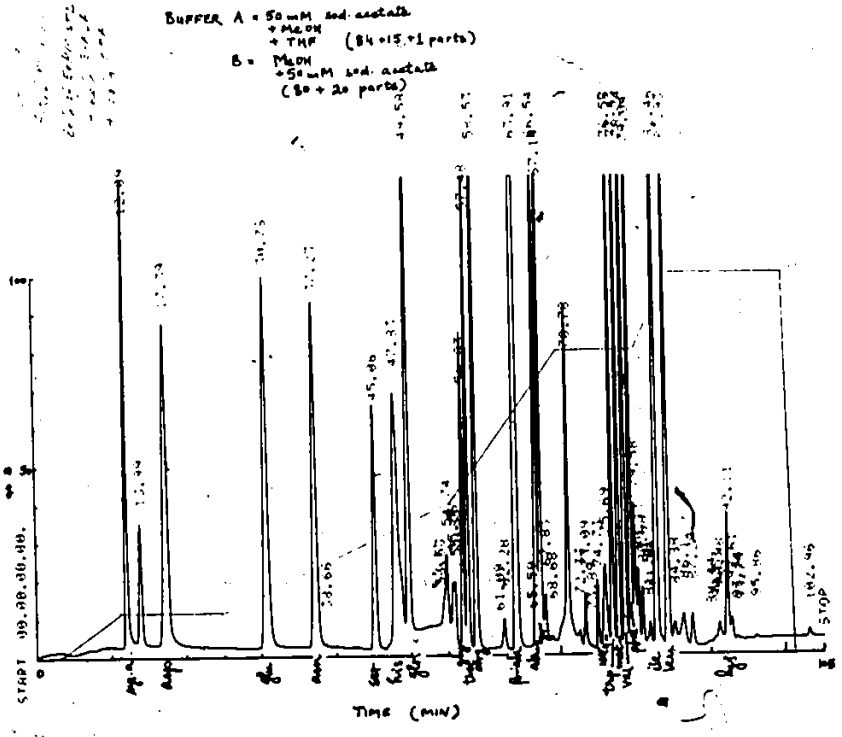

BUFFER A = 50 mM sod. acetate
+ MeOH
+ THF (84 + 15 + 1 parts)
B = MeOH
+ 50 mM sod. acetate
(80 + 20 parts)
START
STOP
TIME (MIN)

Table 6. Concentration of the Different Amino Acids in the Soluble Fractions of Barley (cv. Perth) Half-kernels

Amino Acid	Concentration of Amino Acids (μM/Assay).					
	-24 H	+ 24 H	-48 H	+ 48 H	-72 H	+72 H
Cys	0.00	0.00	0.00	0.00	0.00	0.00
Asp	122.60	111.28	126.16	209.55	97.56	279.23
Glu	138.38	139.40	114.95	357.43	93.25	512.96
Asn	109.35	89.88	102.41	132.89	74.39	163.88
Ser	8.77	116.68	18.50	521.83	17.03	853.79
His	7.27	13.69	8.76	52.77	9.81	89.26
Gln	42.85	924.11	49.10	3324.41	52.42	4014.69
Gly	3.64	22.50	4.66	127.98	0.00	229.41
Thr	6.00	40.77	7.25	202.50	3.76	315.39
Arg	15.34	133.77	19.62	545.06	18.01	763.82
BAla	800.00	800.00	800.00	800.00	800.00	800.00
Ala	73.02	263.19	55.07	879.53	38.05	1196.16
Tyr	17.04	165.36	19.37	741.78	20.04	1147.79
GAba	0.00	0.00	0.00	0.00	0.00	0.00
NH4	0.00	79.74	45.68	155.73	45.57	220.17
Trp	37.88	81.68	34.77	229.30	29.65	281.08
Met	2.93	52.80	6.54	205.14	8.03	278.45
Val	31.19	243.43	39.17	804.35	38.85	1153.32
Phe	20.28	182.27	27.57	801.30	27.07	1158.26
Ile	13.13	139.25	14.23	502.71	17.08	710.77
Leu	42.51	531.43	53.45	1648.76	59.08	2218.98
Lys	21.42	63.31	17.40	247.13	16.26	355.94

Thirty half-kernels were incubated for 24.48 and 72 hours in 10 ml media in presence (+) or absence (-) of GA_3 (5 μM). Soluble Fractions were prepared as described in the Materials and Methods. The amino acid concentration was determined after separation through an HPLC according to the method of Winspear and Oaks (123).

concentration in both the conditions, i.e. AH/BH, was determined. This ratio reflected the relative proportion of amino acids in the peptide-bound fraction. It was greater than one for most of the amino acids, when samples were incubated for 24 hours in the presence of GA_3 (Table 7). A time-course experiment showed that arginine, isoleucine, leucine, and valine are the four amino acids whose ratio (AH/BH) approaches unity within next 24 hour incubation period. This experiment suggests that amino acids are not the initial products of hydrolysis of the hordeins.

The presence of peptides was also confirmed spectrophotometrically. Peptide bonds have high absorbance between 205 and 230 nm, the UV range in which amino acids have very low absorbance (Fig. 16). UV absorption spectra of the methanol-soluble fraction are shown in Figure 17. In the absence of GA_3, the concentration of peptides remained low up to a 72 hour incubation period. In the GA_3-treated samples, however, the concentration of peptides increased by 2.5-fold and 5.5-fold after a 24 and 72 hour incubation period, respectively.

2.3.4. Separation into acidic (I), neutral + basic (II), and amide (III) fractions: The most abundant amino acid residues in hordein proteins and the methanol-soluble fractions are glutamate + glutamine, aspartate + asparagine, and proline. Whereas the glutamic acid and aspartic acid (and the peptides with these residues at the C-terminal) are

Table 7. Changes in the Relative Proportion of Amino Acids in the Peptide-Bound Form in the Soluble Fraction from Barley Half-kernels.

Amino Acid	Ratio (After Hydrolysis/Before Hydrolysis) 24 H	48 H	72 H
Ser	13.9	4.7	4.5
His	54.2	14.3	14.3
Gly	92.1	18.2	12.7
Thr	21.3	6.6	6.4
Arg	24.4	1,4	1.2
Ala	4.7	1.9	1.9
Tyr	3.4	1.3	1.1
Val	3.5	1.3	1.0
Phe	4.8	1.9	1.5
Ile	3.7	1.7	1.6
Leu	2.7	1.1	1.0
Lys	9.1	2.1	2.0
Pro	14.1	4.2	2.9

Thirty half-kernels were incubated for 24, 48, 72 hours (H) in 10 ml media in the presence (+) or absence (-) of gibberellic acid (5 μM) (see Materials and Methods). The supernatant from crude homogenate was precipitated with 50% methanol. An aliquot from methanol-soluble fraction was subjected to hydrolysis with 6 N HCl for 8 hours at 110°C. Separation of amino acids after OPA-derivatization was achieved using HPLC, according to the method of Winspear and Oaks (123). AH = after hydrolysis; BH = before hydrolysis. Peptide-proportion of amino acid (aa) was estimated as follows:

$$\frac{(\text{conc. of aa AH } (+GA_3) - \text{conc. of aa AH } (-GA_3)}{(\text{conc. of aa BH } (+GA_3) - \text{conc. of aa BH } (-GA_3)}$$

Figure 16. UV absorption spectra of oligopeptides. Polyproline (50-80 proline residues) was used at a concentration of 0.1 mM. Other peptides and glycine were used at a concentration of 1 mM.
The measurements were performed in duplicate. Each point represents the means, and bars represent the range between minimum and maximum values.

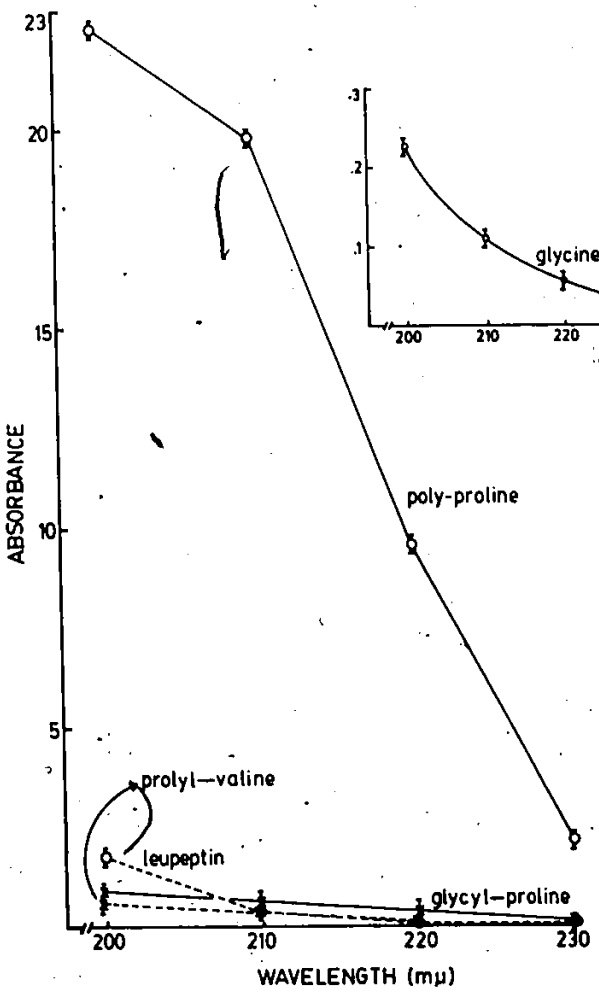
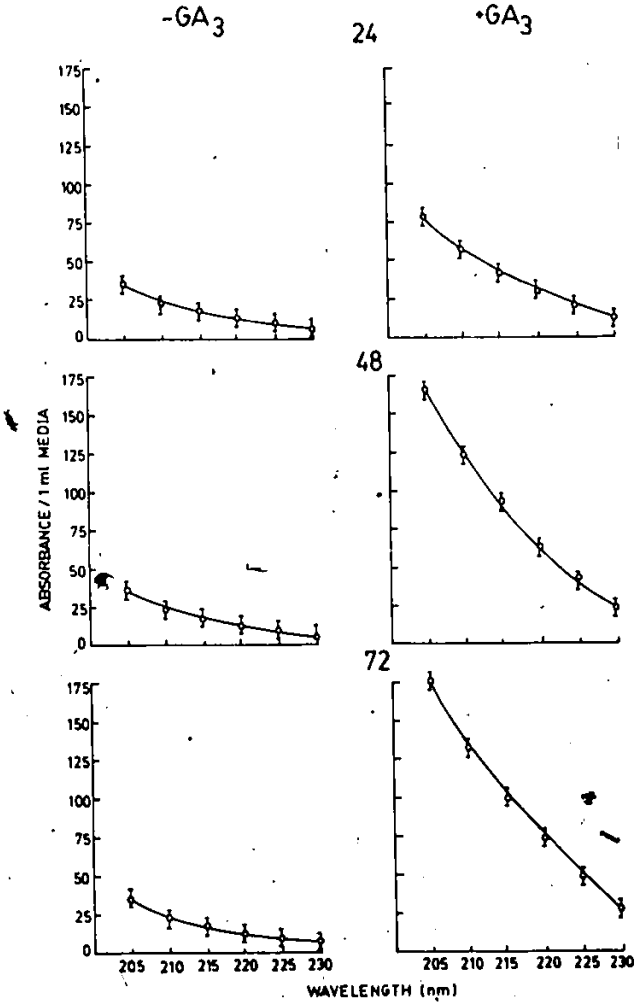

23
20
15
10
5
ABSORBANCE
poly-proline
glycine
.3
.2
.1
200
210
220
230
prolyl—valine
leupeptin
glycyl—proline
200
210
220
230
WAVELENGTH (mμ)

Figure 17. UV absorption spectra of peptides in the methanol-soluble fraction (media + soluble extracts) of barley (cv. Perth) endosperms. 30 embryo-less endosperms were incubated for 24, 48, and 72 hours in 10 ml media in the presence ($+GA_3$) and absence ($-GA_3$) of gibberellic acid (5 μM).

The whole experiment was performed once and all measurements were performed in duplicate. Means are indicated by each point and bars represent the range between minimum and maximum values.


-GA3
+GA3
24
48
72
ABSORBANCE / 1 ml MEDIA
0
25
50
75
100
125
150
175
205 210 215 220 225 230
WAVELENGTH (nm)

expected to elute in the acidic fraction, the glutamine and asparagine (and the peptides with these residues at the C-terminal) should elute in the acidic fraction after hydrolysis of the amide groups. Proline and other amino acids (and peptides with these residues at the C-terminal) however, are expected to elute in the neutral + basic fraction. Since, amino acids are not the initial products of hydrolysis, it was of interest to see: a) the fractions in which peptides elute; and b) the fractions in which peptides comprising the three predominant residues elute. Peptides and amino acids in the methanol-soluble fractions were separated into the acidic, amide and neutral + basic fractions by ion-exchange chromatography using Dowex columns (86a). The concentration of amino acids in each fraction was estimated after (AH) and before (BH) hydrolysis with 6 N HCl. In the acidic fraction, the fact that amino acids other than glutamate and aspartate appear, indicates that peptides are the major components in this fraction (Table 8). In the neutral + basic fraction the ratio for most of the amino acids was around one at 24, 48, and 72 hours of incubation period. This observation suggested the presence of either small peptides and/or predominantly free amino acids. In general, the levels of amino acids were low in this fraction. In the amide fraction, the presence of peptides was indicated by the fact that alanine, tyrosine, valine, phenylalanine, isoleucine, and leucine, were present in the hydrolyzed

Table 8. Changes in Peptide-Proportion of Amino Acids in Acidic (I), Neutral + Basic (II), and Amide (III) Fractions Separated from Methanol-Soluble Fraction.

	Ratio (After Hydrolysis/Before Hydrolysis)								
	Acidic (I)			Neutral + Basic (II)			Amide (III)		
Amino Acid	24 H	48 H	72 H	24 H	48 H	72 H	24 H	48 H	72 H
Asx	4.10	8.52	9.73	n.d.	n.d.	n.d.	0.88	0.81	0.82
Glx	9.71	13.10	7.67	n.d.	n.d.	n.d.	1.11	1.04	0.98
Ser	102.67	n.d.	n.d.	0.97	1.21	1.98	n.d.	n.d.	n.d.
His	n.d.	n.d.	n.d.	n.d.	3.04	3.72	0.40	1.13	1.09
Gly	37.89	163.00	81.70	1.01	1.08	1.83	n.d.	n.d.	n.d.
Thr	n.d.	159.00	125.00	0.95	1.62	1.95	n.d.	n.d.	n.d.
Arg	n.d.	n.d.	43.00	2.76	2.41	3.25	n.d.	n.d.	n.d.
Ala	12.00	31.50	33.25	1.03	1.05	1.51	3.75	2.85	5.50
Tyr	2.04	2.73	0.49	0.47	0.54	0.77	1.66	1.73	1.67
Val	17.44	30.92	44.83	0.78	1.32	1.21	5.76	16.57	n.d.
Phe	4.18	7.76	9.91	0.84	1.05	1.24	1.54	2.36	3.15
Ile	9.50	20.80	59.00	0.88	1.39	1.60	3.13	7.87	n.d.
Leu	5.45	12.35	32.62	0.70	0.97	1.16	1.89	5.33	20.24

Thirty half-kernels were incubated for 24, 48, and 72 hours (H) in 10 ml media in the presence or absence of gibberellic acid (see Materials and Methods). The supernatant from crude homogenate was precipitated with 50% methanol. An aliquot

from methanol-soluble fraction was separated into acidic (I), neutral + basic (II), and amide (III) fractions using ion-exchange chromatography (86a). The fractions were hydrolyzed for 8 hours with 6 N HCl at 110°C. Amino acids were analyzed in these fractions before and after hydrolysis. Amino acids were derivatized with OPA and separated on a reverse phase column using HPLC (123). Peptide proportion of the amino acids was estimated by computing the ratio of amino acid concentration (after hydrolysis/before hydrolysis). n.d. = non-detectable. Asx = aspartate + asparagine, Glx = glutamate + glutamine.

samples obtained after 24, 48, and 72 hours of incubation period. For estimating the proline concentration, a colorimetric test was used. Figure 18 summarizes its concentration in the three fractions. Proline, as expected, was recovered mainly in the neutral + basic fraction. It was released mainly as a free amino acid during an initial 48 hour incubation period, after which peptides containing proline were also released. Such peptides were also recovered in the acidic and amide fractions. The proportion of proline containing peptides was more in the amide fraction.

2.3.5. Separation of peptides using Sephadex size exclusion columns: Peptides in the methanol-soluble fraction may be of either uniform or heterogeneous sizes. To assess the complexity of peptides appearing in the media, a gel filtration technique was adopted. Peptides were first separated from the amino acids. It was achieved by passing the samples through a column of Sephadex G-10.

The elution profile of marker peptides through this column is shown in Figure 19. Cytochrome C (11.7 kDa) eluted with the void volume. Whereas the elution of poly-proline (50-80 proline residues), insulin chain A (21 amino acid residues) and chain B (30 amino acid residues) was only slightly retarded, peptides with less than 9 amino acid residues, e.g. leupeptin (3) and bradykinin (9), and amino acids co-eluted later (160 ml of the elution volume).

Figure 18. Estimation of proline in the acidic, neutral + basic, and amide fractions. The fractions were separated after ion-exchange chromatography of the methanol-soluble fraction (media + soluble extracts) of barley (cv. Perth) endosperms. Thirty embryo-less endosperms were incubated for 24, 48, and 72 hours in 10 ml media in the presence and absence of gibberellic acid (5 μM). Values in the absence of gibberellic acid were very low and therefore not shown. Dotted lines indicate estimations after hydrolysis in the presence of 6 N HCl, and solid lines indicate estimations before hydrolysis.

The whole experiment was performed once, and all measurements were performed in triplicate. Each point represents the means, and bars represent the range between minimum and maximum values.

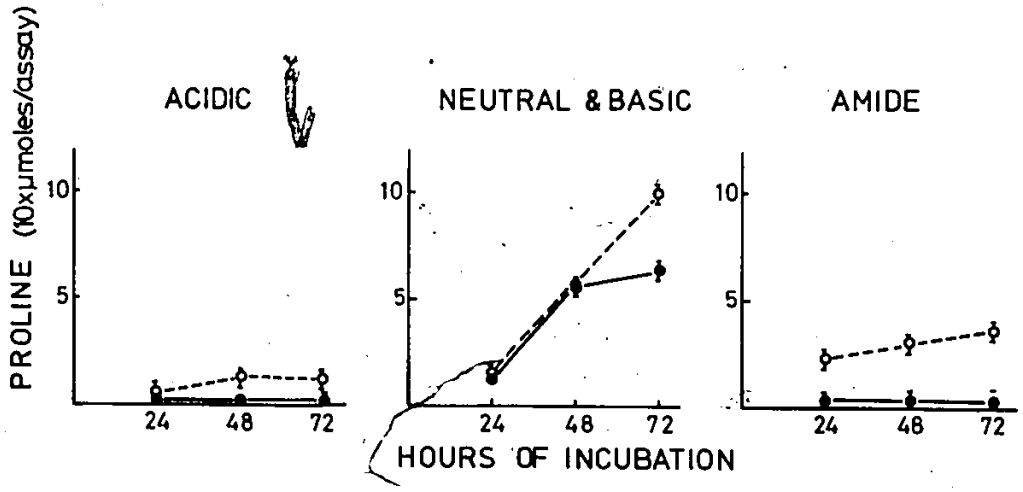
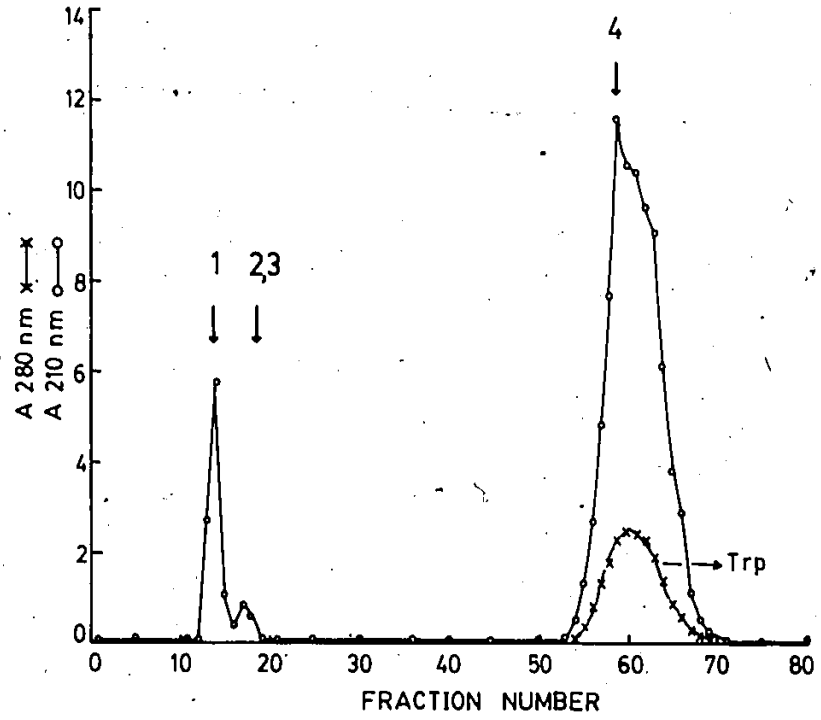

PROLINE (10xμmoles/assay)
ACIDIC
NEUTRAL & BASIC
AMIDE
10
5
24
48
72
HOURS OF INCUBATION

Figure 19. Elution profile of standard peptides and tryptophan through a column (1.3 x 90 cm) of Sephadex G-10. 1 = cytochrome C, 2 = polyproline, 3 = insulin chain A and B, and 4 = bradykinin + prolyl-glycine + leupeptin. x_____x and o_____o, show absorbance at 280 nm (tryptophan) and 210 nm, respectively. Peptide bonds were measured at 210 nm and aromatic amino acids at 280 nm.
The calibration was performed once.


14
12
10
8
6
4
2
0
A 280 nm
A 210 nm
1
2,3
4
Trp
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

Peptides of about 9 amino acid residues or less + amino acids were thus separated from larger peptides.

Sephadex G-25 column was then employed to separate peptides of different sizes. The elution profile of marker peptides through this column is shown in Figure 20. Cytochrome C eluted with the void volume. Elution of poly-proline and insulin chain A and B is retarded by 15 and 30 ml of the elution volume. The elution profile of the samples showed two broad peaks (Fig. 21 and 22). Whereas peptides eluting in the first peak range in size from 50-70 amino acid residues in length, those eluting in the second peak range are between 15 and 35 amino acid residues. Peptides of each class size were pooled and termed 'peak I' and 'peak II', respectively. In the absence of GA_3, no change in the elution profile and/or relative proportion of peptides in 'peak I' and 'peak II' was noted up to 72 hours of incubation (Fig. 21). An overall decrease in the concentration of peptides in both the peaks was, however, apparent. In the presence of GA_3, the proportion of peptides in 'peak I' decreased with longer periods of incubation (Fig. 22). A corresponding increase in the proportion of peptides in 'peak II' was also noted. Peptides in 'peak I' accounted for about 43% of total nitrogen in the methanol-soluble fraction at 24 hours, and about 11% by 72 hours (Table 9). During the same period, the proportion of total nitrogen in 'peak II' peptides increased from 42% to 50%.

Figure 20. Elution profile of standard peptides and tryptophan through a column (1.3 x 90 cm) of Sephadex G-25. 1 = cytochrome C, 2 = polyproline, 3 = insulin chain A and B, and 4 = bradykinin + prolyl-glycine + leupeptin. x_____x and o_____o, show absorbance at 280 nm (tryptophan) and 210 nm, respectively. Peptide bonds were measured at 210 nm and aromatic amino acids at 280 nm.
The calibration was performed once.

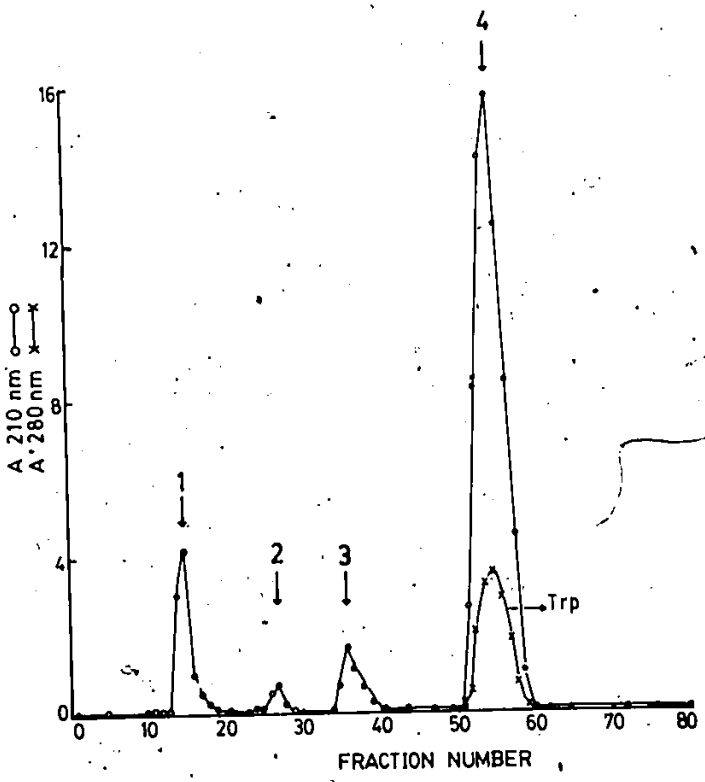

4
16
12
A 210 nm
A 280 nm
8
1
4
2
3
Trp
0
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

Figure 21. Elution profile of peptides in the methanol-soluble fraction (media + soluble extracts) of barley (cv. Perth) endosperms through Sephadex G-10 and G-25 columns (1.8 x 90 cm). Thirty embryo-less endosperms were incubated for 24, 48, and 72 hours in 10 ml media in the absence (-) of gibberellic acid. The peptide bonds were measured at 210 nm. A = absorbance.

The whole experiment was performed two times and a similar trend was observed.

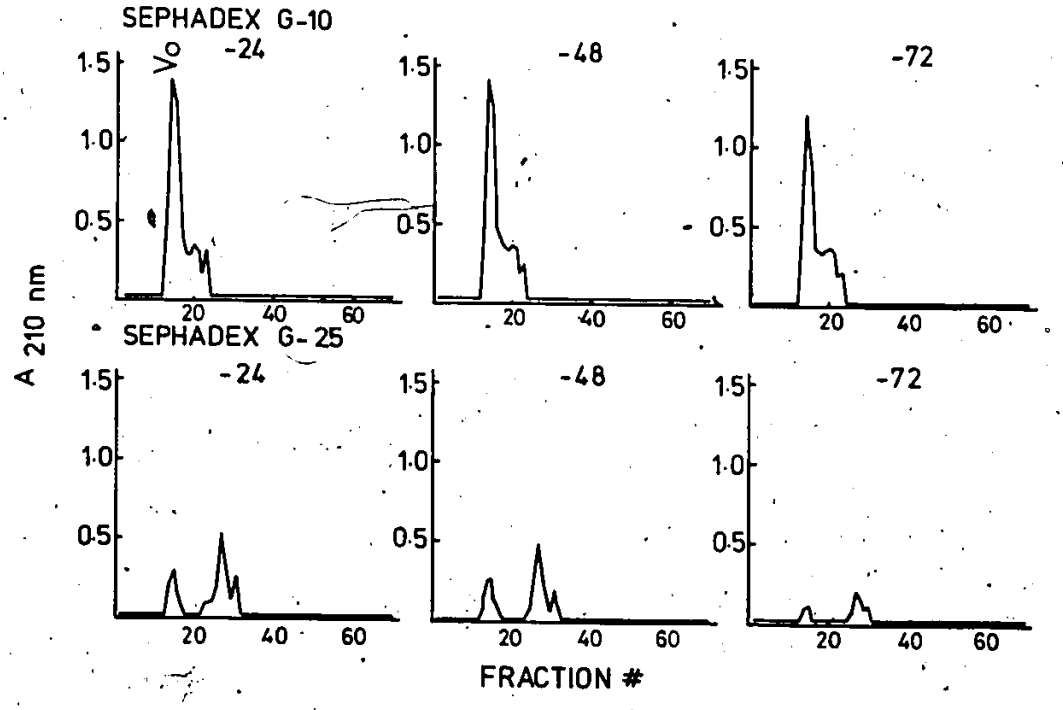

SEPHADEX G-10
-24
-48
-72
Vo
SEPHADEX G-25
-24
-48
-72
A 210 nm
1.5
1.0
0.5
20
40
60
FRACTION #

Figure 22. Elution profile of peptides in the methanol-soluble fraction (media + soluble extracts) of barley (cv. Perth) endosperms through Sephadex G-10 and G-25 columns (1.8 x 90 cm). Thirty embryo-less endosperms were incubated for 24, 48, and 72 hours in 10 ml media in the presence (+) of gibberellic acid (5 μM). I_____I with numbers I and II indicate the fractions pooled in 'peak I' and 'peak II', respectively. The peptide bonds were measured at 210 nm. A = absorbance.

The whole experiment was performed two times and a similar trend was observed.

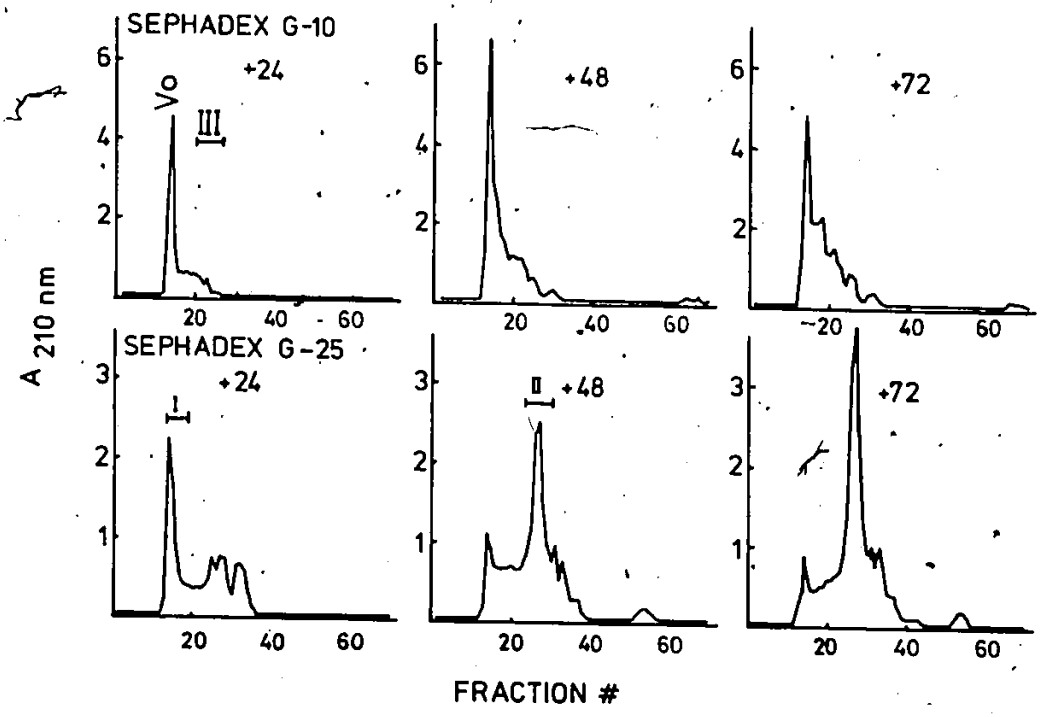

SEPHADEX G-10
+24
+48
+72
Vo
III
SEPHADEX G-25
+24
+48
+72
I
II
A 210 nm
FRACTION #

Table 9. Balance Sheet of Total Nitrogen in Pellet (Prolamin and Glutelin) and Soluble Fraction (Methanol-Soluble Fraction, 'peak I', 'peak II', and the Fraction Containing Oligopeptides plus Amino Acids) Prepared from Barley (cv. Perth) Endosperms.

	Total Nitrogen (mg/30 endosperms)					
Sample	Pellet	SF	MSF	'peak I'	'peak II'	OAA
Initial	10.5	1.4	-	-	-	-
-24 H	11.4	1.6	0.7	0.2	0.4	0.03
+24 H	8.1	3.7	2.1	0.9	0.8	0.04
-72 H	10.5	1.6	0.1	0.1	0.4	0.05
+72 H	2.3	10.9	7.2	0.8	3.5	1.90

Extracts were prepared from 30 half-kernels incubated for 24 and 72 hours in 10 ml of media in the presence (+) and absence (-) of GA_3. Large polypeptides were precipitated using 50% methanol. The methanol-soluble fraction was successively passed through Sephadex G-10 and G-25, and 'peak I', 'peak II', and oligopeptides plus amino acids (OAA) were collected as described in the Materials and Methods. Total nitrogen content was measured in these fractions according to the method of Kaplan (58). The experiment was repeated once more and a similar trend was noted. Variation between the two experiments was less than 10%. SF = soluble fraction; MSF = methanol-soluble fraction; OAA = oligopeptides plus amino acids. Dashes (-) = sample not done.

The proportion of total nitrogen in the fraction containing oligopeptides and amino acids increased from 2% to 26%. This result clearly establishes that: a) peptides of heterogeneous sizes are present in the methanol-soluble fraction of the media; b) the elution profile and the relative proportion of peptides in 'peak I' and 'peak II' do not exhibit significant change, when endosperms are incubated for 24, 48, and 72 hours in the absence of GA_3; c) in the presence of GA_3 peptides of large sizes appeared in the media at early times, i.e. 24 hours; and d) with longer periods of incubation, i.e. 72 hours, in the presence of GA_3, the proportion of small size peptides and amino acids increased.

2.3.6. N-terminal residues of peptides in 'peak I' and 'peak II': The N-terminal residues were determined to: a) further assess the complexity of peptides in the two peaks, and b) see if the peptides have few or many types of amino acids as N-termini. The determination was done after dansyl derivatization and separation of the derivatives was achieved on the reverse phase mode using an HPLC (87). The separation of a standard mixture of 18 dansylated amino acids is shown in Figure 23. The N-terminal residues of peptides in 'peak I' and 'peak II' are summarized in Table 10. Valine, glutamate plus glutamine, alanine, and glycine/threonine are the major N-terminal residues (comprising about 68% of the estimated N-terminal residues) of peptides in 'peak I' after a 24 hour incubation period.

Figure 23. Elution profile of a mixture of standard dansylated amino acids. A mixture of amino acids was passed thourgh an Altex 5μ Ultrasphere ODS column (4.6 x 250 mm) using a Beckman HPLC. The standards were run after 4-5 test samples.

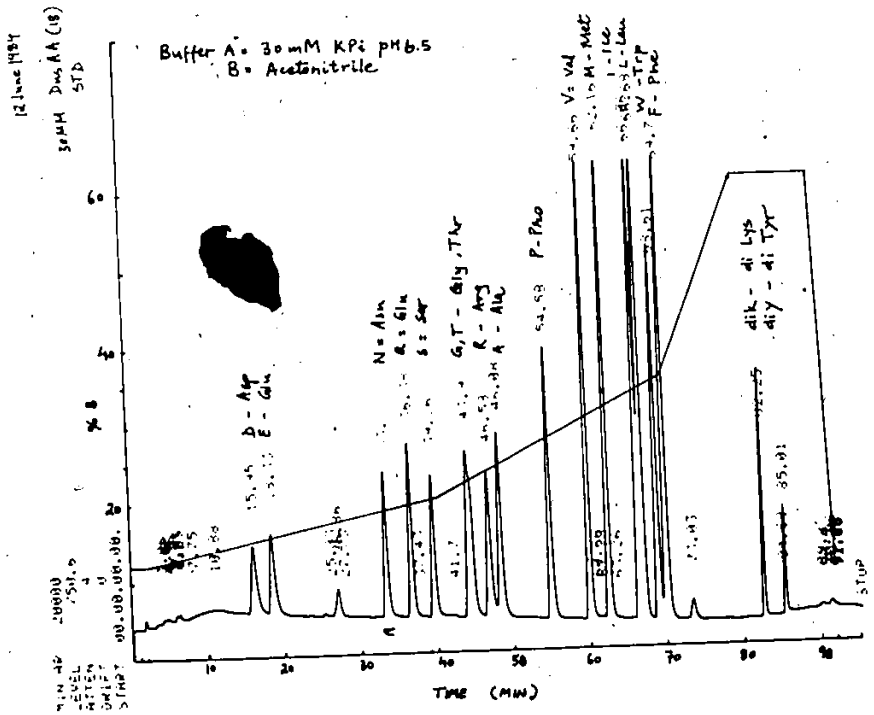

Buffer A = 30mM KPi pH6.5
B = Acetonitrile
12 June 1984
50pM DansAA (18)
STD
% B
TIME (MIN)
D - Asp
E - Glu
N = Asn
Q = Gln
S = Ser
G,T - Gly, Thr
R - Arg
A - Ala
P-Pro
V = Val
M - Met
I - Ile
L - Leu
W - Trp
F - Phe
dik - di Lys
diy - di Tyr
STOP

Table 10. Estimation of N-Terminal Residues of Peptides in 'peak I' and 'peak II' Fractions.

Amino Acid	p moles per endosperm			
	-24 (I)	+24 (II)	-72 (I)	+72 (II)
Asx	-	922 ± 23	722 ± 19	-
Glx	533 ± 17	4082 ± 118	-	60866 ± 2078
Ser	-	1135 ± 85	-	8053 ± 450
Gly/Thr	-	1598 ± 85	2578 ± 210	15360 ± 750
Arg	-	1288 ± 70	-	4660 ± 190
Ala	-	3293 ± 110	-	8823 ± 300
Pro-	126 ± 15	-	-	10293 ± 453
Val	120 ± 10	5835 ± 195	1995 ± 58	7786 ± 352
Met	-	692 ± 35	-	-
Ile	-	775 ± 35	546 ± 24	-
Leu	-	1420 ± 58	-	-
Phe	93 ± 8	478 ± 21	-	-

Thirty half-kernels were incubated for 24 and 72 hours in a buffered media in the presence (+24 and +72) and absence (-24 and -72) of GA_3 (5 μM). Polypeptides in the soluble fractions were precipitated with 50% methanol. Peptides in the methanol-soluble fraction were separated using Sephadex G-10 and G-25 columns as described in the Materials and Methods. 'peak I' (I) and 'peak II' (II) peptides were dansylated and

hydrolyzed using 6 N HCl for 8 hours. N-terminal residues were separated using an HPLC. The experiment was performed two times and the values are represented by the means ± range between minimum and maximum. Dashes (-) represent the values below 25 p moles per endosperm.

Varying levels of other amino acids, e.g., aspartate plus asparagine, serine, arginine, methionine, isoleucine, leucine, and phenylalanine, as N-termini were also detected. A similar, but somewhat less heterogeneous pattern of the N-terminal residues of peptides was noted in 'peak II' after a 72 hour incubation period. For example, glutamine and glutamate represented more than 50% of the estimated N-terminal residues. And proline, which was barely detectable as an N-terminal residue of peptides in 'peak I' at 24 hours, was detected in very high levels when incubations were performed in the presence of GA_3 for 72 hours. The levels of other N-terminal residues, e.g., serine, glycine/threonine, arginine, alanine, valine, were also high compared to those recorded after a 24 hour incubation period.

3. Initial Hydrolysis of Hordeins:

The initial hydrolysis of insoluble hordeins must involve steps, which result in soluble products. These initial products are then further hydrolyzed to small peptides and amino acids. A clear-cut distinction has never been made between the two, and it has been generally assumed that both reactions involve a single enzyme and that this enzyme is induced by GA_3. The results summarized above show unambiguously that later stages in the overall hydrolysis are dependent on the presence of GA_3. However, it remains to be seen whether the initial steps of the hordein solubilization

are also under the same control mechanism. This point was examined in the following set of experiments.

3.1. Preparation of the antibodies against hordeins and enrichment of crude serum for hordein IgG's: Subsequent to the incubation of endosperms in the presence of GA_3, an increase in total nitrogen was noted in the TCA-precipitable material. The electrophoretic separation of the latter showed no significant change, except for an increase in the -amylase polypeptide. To further investigate the possibility that the hordein-derived polypeptides may also account for an increase in total nitrogen in the soluble fraction, an immunological approach was adopted.

Hordeins are immunogenic proteins (28, 66). If the early polypeptide fragments released from the hydrolysis of hordeins retain antigenicity, then the antibodies raised against hordein proteins could be used in the identification of the hordein-derived products. As a first step, therefore, antibodies were prepared against hordeins. The presence of hordein IgG's in the crude serum was tested following the Ouchterlony's double immunodiffusion assay. The crude serum was tested with the hordein and non-hordein proteins (albumins + globulins). A precipitin line was observed against both the types of proteins (Fig. 24A and 24B), suggesting that IgG's against both the types of proteins were present in the crude serum. After pre-incubation of the

Figure 24. Double immunodiffusion assay using crude serum and hordein -IgG enriched sera against hordeins and soluble proteins (albumins + globulins). A, B, and C represent three immunodiffusion sets. A. Center well - hordeins; Outer well - crude serum (1-6). B. Center well - soluble proteins; Outer well - serum pre-treated with 125 (1), 250 (2), 500 (3), 1000 (4), 2000 (5), and 4000 (6) µg of soluble proteins. C. Center well - pre-treated serum (with 4000 µg of soluble proteins); Outer well - soluble proteins (1, 3, 5), hordeins (2, 4, 6). Proteins were measured by Biorad reagent as described in the Materials and Methods.

The assay was performed several times and similar results were obtained.


A
B
C
1
2
3
4
5
6

crude serum with soluble proteins, the resultant serum was again tested with both the types of proteins. As seen in Figure 24C, a precipitin line was noted against hordeins only, showing that the contaminating IgG's had been removed from the crude serum. The resultant serum, enriched for hordein IgG's, was used as the source of primary antibody in the following experiments.

3.2. Western immunoblotting: The serum enriched for hordein-IgG's was used to detect the polypeptide fragments released from the hydrolysis of hordeins. Soluble fractions were prepared from dry endosperms and half-kernels incubated for 8, 24, and 72 hours in the presence and absence of GA_3. A number of immunoreactive bands were seen in the control samples (lane 0; Figure 25). The presence of hordein-related polypeptides in this sample could be due to: a) sharing of common epitopes by soluble proteins and hordeins; b) prematurely aborted hordein polypeptides during seed development, which are extractable with the aqueous buffer; and finally c) partial hydrolysis of hordeins, which may have begun during the later stages of seed development itself.

No significant change compared to the controls (0) was noted in the samples incubated for 8 hours in the presence and absence of GA_3. In the samples incubated for 24 hours in the absence of GA_3, however, a major band at 40 kDa was more prominent. Also, additional bands in the size range

Figure 25. Western immunoblot analysis of hordein-derived polypeptides in the soluble protein fractions of barley (cv. Perth) endosperms. Thirty embyro-less endosperms were incubated for 8, 24, and 72 hours in 10 ml media in the presence (+) and absence (-) of gibberellic acid (5 µM). Soluble proteins from dry kernels (0) were run as controls. Approximately 75-100 ul samples containing ~ 10-20 µg proteins were loaded in each well. The polypeptides were electrophoresed through 15% gel. Electrophoresis and electroblotting were performed as described in the Materials and Methods. Protein mole mass (MW) standards were BSA (67 kDa), ovalbumin (45 kDa), and cytochrome C (12 kDa). The western immunoblot was performed several times and similar results were obtained.


+72
-72
+24
+72
-24
+8
-8
0
7
5
12
MW

of 25-30 kDa appeared. On the other hand, in the samples incubated for 24 hours in the presence of GA_3, some of the bands around 45-60 kDa disappeared. In addition, low molecular weight bands of less than 15 kDa were apparent. In the samples incubated for 72 hours in the absence of GA_3, there was no significant difference in the banding pattern of immunoreactive polypeptides compared to those incubated for 24 hours. Most of the bands were lost when the samples were incubated in the presence of GA_3 for 72 hours. This result showed that the appearance of some larger hydrolytic products is independent of GA_3. Complete hydrolysis, however, was dependent on the presence of this growth regulator.

Two controls were performed to confirm that: a) the immunoreactive bands are specific to hordein IgG's, and b) the antibodies are specific to hordeins. In the first control, the membrane was reacted with different solutions in normal manner (see Materials and Methods) with the exception that instead of hordein IgG's, a pre-immune serum was used as a source of primary antibody. In the other control, soluble proteins from barley roots and shoots were transferred on the nitrocellulose membrane and then reacted with different solutions in a normal manner. No discrete bands were seen in either of the controls (Fig. 26 and 27), confirming: a) that the antibodies are specific to hordeins, and b) that the immunoreactive bands on the western blots are specific to hordein IgG's.

Figure 26. A western immunoblot control. An absence of any band shows the specificity of immunoreactive polypeptides to hordein IgG's. The soluble protein fractions were extracted from 30 endosperms incubated in 10 ml media in the presence (+) and absence (-) of gibberellic acid (5 μM). Approximately 90 - 100 μl samples containing 10 - 20 μg proteins were loaded in each well. Electrophoresis and electroblotting were performed as described in the Materials and Methods. The nitrocellulose membrane was first reacted with the pre-immune serum (primary antibody). The other treatments were same as in Figure 25. Po and SPo = prolamins and soluble proteins from dry kernels; SPo (IS) = soluble proteins from dry kernels, which after electrophoresis and transblotting were reacted with the immune serum (positive test); -24 and +24 = soluble proteins from endosperms incubated for 24 hours in the absence and presence of gibberellic acid; NIS = pre-immune serum; GAR-HRP = goat anti rabbit IgG horseradish peroxidase conjugate; 1^{o} and 2^{o} = primary and secondary antibody, respectively.
The experiment was performed once.

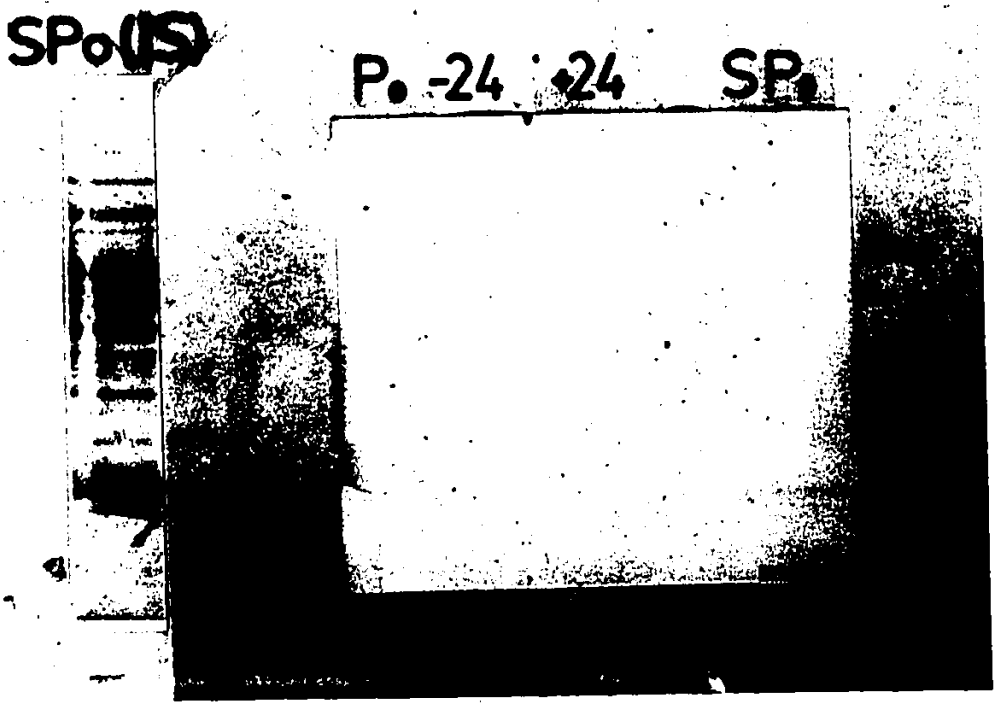
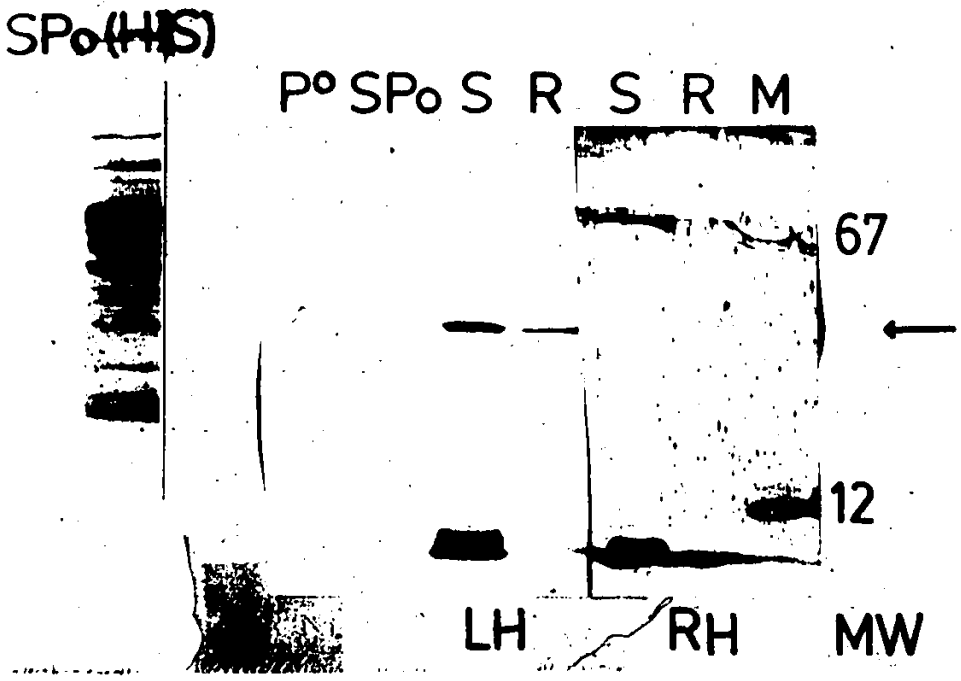
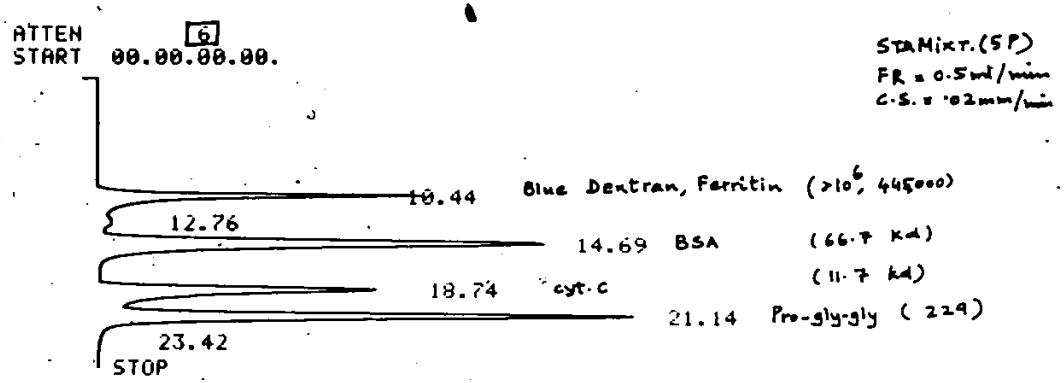

SPo(IS)
P. -24
24
SP.

Figure 27. A western immunoblot control. An absence of bands shows the specificity of hordein IgG's to hordeins and hordein-derived polypeptides. Left half (LH) was treated with the pre-immune serum as primary (1^{o}) antibody. Po and SPo = hordeins and soluble proteins from dry kernels; S and R = extracts from shoots and roots of barley seedlings; SPo (HIS) = soluble protein from dry kernels, which after electrophoresis and transblotting were reacted with the serum containing antibodies against hordeins (positive test). Right half (RH) was treated as in Figure 25, with hordein IgG's as primary antibody. Protein mole mass (MW) standards were BSA (68 kDa) and cytochrome C (12 kDa). Arrow indicates unidentified band in shoot and root extract.
The experiment was performed once.


SPo(H)S)
Pº SPo S R S R M
67
12
LH
RH
MW

3.3. Preliminary characterization of early hydrolytic products: Since the early hydrolysis products do not accumulate in substantial quantities, and since enzymes such as α-amylase and other hydrolases are the major proteins in the soluble fraction, it was necessary to separate the hordein-derived immunoreactive polypeptides from rest of the soluble proteins. To achieve this, the samples were passed through an immunoaffinity column to isolate the immunoreactive polypeptides. The eluted polypeptides were then passed through a size-exclusion Spherogel^{TM}-TSK G2000 SW column. The elution profile of the standard proteins and peptides, e.g. prolyl-glycyl-glycine, cytochrome C, BSA, ferritin, is shown in Figure 28. A linear separation of marker proteins in the size range of about 70-12 kDa is seen in Figure 29. The elution profile of samples prepared from unincubated kernels and endosperms incubated in the presence and absence of GA_3 for 24 hours is shown in Figure 30. A general heterogeneity in the size of immunoreactive polypeptides is clearly visible because of the absence of sharp discrete peaks. Compared to controls (0), in samples incubated in the absence of GA_3, one major broad peak in the size range of about 50 - 70 kDa appeared. The polypeptides in the size range of 15 kDa or less were present in low amounts. In samples incubated in the presence of the GA_3, however, the levels of small polypeptides (15 kDa or less)

Figure 28. Elution profile of marker proteins through a Spherogel^{TM}-TSK G2000 SW column (7.5 mm x 30 cm) using an HPLC. The concentration of proteins was 1.25 mg/ml and 100 µl was injected each time. Proteins were detected at 214 nm using a Beckman Model 160 UV detector. Details of the methodology are described in the Materials and Methods. The standard run was performed several times and a similar elution pattern was observed.



C-R1A
SMPL # 00
FILE # 2
REPT # 3758
METHOD 1041

#	NAME	TIME	CONC	MK	HEIGHT
0		10.44	20.2213		29449
0		12.76	0.9261	V	1348
0		14.69	27.8884	V	40615
0		18.74	17.3066		25204
0		21.14	33.6516	V	49008
0		23.42	0.0058	T	8
	TOTAL		99.9999		145634

Figure 29. Calibration curve for the Spherogel^{TM}-TSK G2000 SW column (7.5 mm x 30 cm). 1 = blue dextran; 2 = ferritin; 3 = BSA; 4 = cytochrome C; 5 = prolyl-glycyl-glycine. The running conditions were same as in Figure 29.

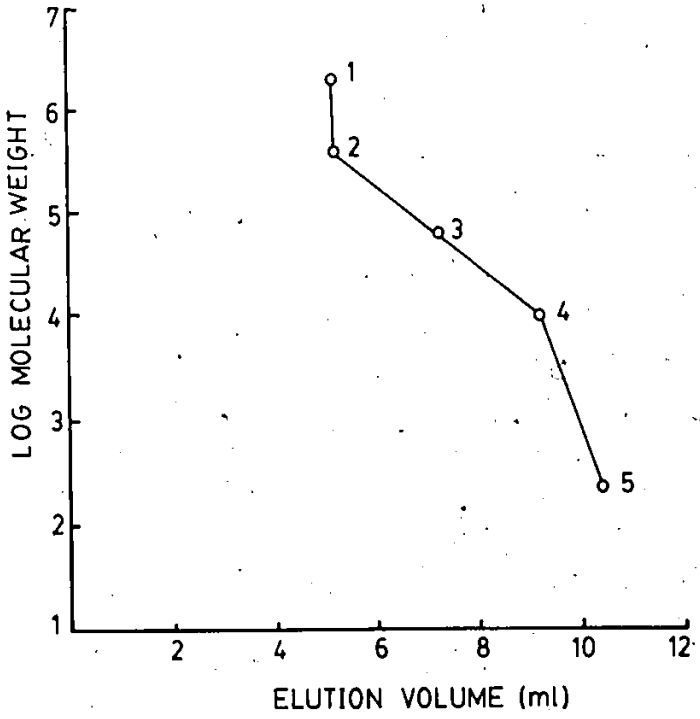
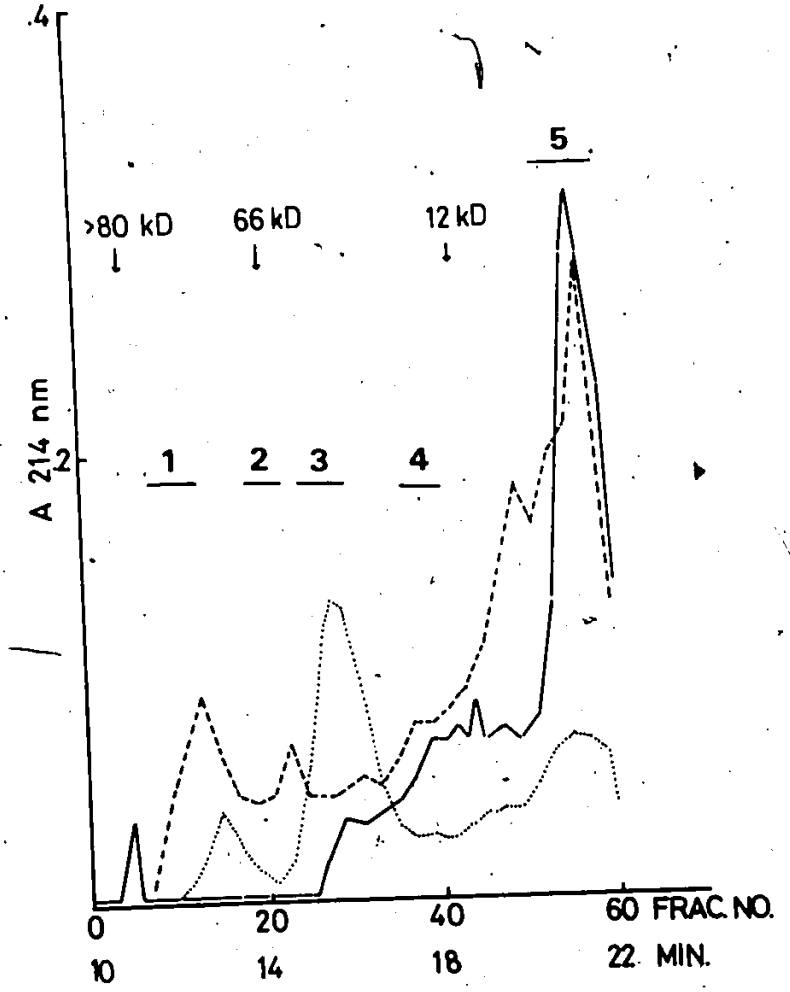

LOG MOLECULAR WEIGHT
7
6
5
4
3
2
1
2
4
6
8
10
12
ELUTION VOLUME (ml)
1
2
3
4
5

Figure 30. Elution profile of soluble proteins through a Spherogel^{TM}-TSK G2000 SW column (7.5 mm x 30 cm) using an HPLC. Water-soluble proteins were extracted from dry kernels (0) and embryo-less endosperm pieces incubated in the presence (+24) and absence (-24) of gibberellic acid (5 µM). One hundred ul containing 50 - 100 µg proteins were injected (25 - 30 injections). A flow rate of 1 ml/min was used. Fractions were pooled in five groups as shown in the figure. Solid line, broken line, and dotted line represent respectively the profiles of soluble proteins extracted from dry kernels, endosperms incubated in the presence of gibberellic acid, and endosperms incubated in the absence of gibberellic acid. 80, 66, and 12 kDa with arrow sign indicate the position where marker proteins, i.e. ferritin, BSA, and cytochrome C, elute. Dashes with 1, 2, 3, 4, 5 numbers indicate the fractions pooled in respective groups. Min. = minutes. A = absorbance. Fract. No. = fraction number.


.4
.2
A 214 nm
>80 kD
66 kD
12 kD
1
2
3
4
5
0
20
40
60 FRAC. NO.
10
14
18
22 MIN.

showed a dramatic increase compared to those observed in samples incubated in its absence. Minor changes were also noted for the larger polypeptides. The fractions containing eluted polypeptides were pooled into five groups (1-5; Fig. 30).

The N-terminal residues of polypeptides in different groups were determined and are summarized in Table 11. A number of N-terminal residues were recorded for group 1 (around 70 kDa) polypeptides in the control sample from dry kernels (0), i.e. aspartate plus asparagine, glutamate plus glutamine, serine, glycine, arginine, valine, isoleucine, leucine, and phenylalanine. Concentrations of these residues were dramatically reduced, when half-kernels were incubated in the presence or absence of GA_3. No additional N-terminal residue(s) was detected.

Additional N-terminal residues of polypeptides appearing in group 2 (40-70 kDa) in the absence of GA_3 were aspartate plus asparagine, glycine, arginine, isoleucine, and leucine. There was also a marked increase in the level of valine as an N-terminal residue. In the presence of GA_3, however, glutamate plus glutamine and serine appeared as additional N-terminal residues and levels of aspartate/asparagine increased up to 3-fold. Concentrations of other N-terminal residues decreased.

In the absence of GA_3, glycine appeared as an additional N-terminal residue for polypeptides in group 3

Table 11. Estimation of N-Terminal Residues of Hordein-Related Polypeptides in Soluble Fractions of Barley Half-kernels

pMOLES PER ENDOSPERM

	GROUP 1 (70-80 kDa)			GROUP 2 (40-70 kDa)			GROUP 3 (20-40 kDa)			GROUP 4 (12-20 kDa)			GROUP 5 (7-12 kDa)		
Amino Acid	0	-24	+24	0	-24	+24	0	-24	+24	0	-24	+24	0	-24	+24
Asp	542	120	0	0	175	422	233	637	192	255	200	472	0	440	348
Glu	187	69	0	0	0	86	0	0	144	0	22	90	0	254	218
Asn	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gln	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ser	541	0	565	0	0	595	0	0	0	0	551	0	0	0	0
Gly	671	308	0	0	785	0	0	983	0	224	543	0	1060	0	0
Thr	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arg	532	14	0	0	254	0	343	291	0	384	0	0	364	0	0
Ala	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pro	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Val	369	42	86	16	197	0	298	291	162	360	94	52	292	0	193
Met	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ile	223	13	34	0	110	52	147	85	71	163	28	18	158	66	62
Leu	296	19	58	0	152	56	243	85	111	278	45	34	242	83	89
Phe	174	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
diLys	924	230	532	957	554	378	781	672	685	898	243	216	718	541	594
diTyr	179	23	34	62	168	48	205	191	178	295	85	45	279	102	76

Thirty half-kernels were incubated for 24 hours in $\pm$ GA_3 as

described in the Materials and Methods. Soluble proteins were extracted from incubated and dry (o) endosperms. Hordein-related polypeptides were prepared after immunoaffinity column chromatography. They were separated into different size groups after high performance-size exclusion chromatography. N-terminal residues were determined by dansylating the polypeptides. The separation of dansylated products was achieved as described in the Materials and Methods.

(20-40). Levels of aspartate plus asparagine as N-terminal residues increased by about 3-fold. Concentrations of other N-terminal residues, i.e. arginine, valine, leucine, and isoleucine, decreased by as much as two-thirds. In the presence of GA_3, glutamate plus glutamine appeared as additional N-terminal residues. Levels of other N-terminal residues, i.e., aspartate plus asparagine, valine, isoleucine, and leucine, showed minor changes. Glycine and arginine were not detectable as N-terminal residues.

Glutamate plus glutamine and serine appeared as additional N-terminal residues of group 4 (12-20 kDa) polypeptides in the absence of GA_3. A 2-fold increase in the level of glycine as N-terminal residue was noted. The level of aspartate plus asparagine as N-terminal residues showed a minor reduction. Concentrations of other N-terminal residues, i.e., glutamate plus glutamine, valine, isoleucine, and leucine, decreased drastically. In the presence of GA_3, concentrations of both aspartate plus asparagine and glutamate plus glutamine showed appreciable increases as N-termini. Glycine and arginine were not detectable as N-terminal residues. Levels of other N-terminal residues, e.g. valine, isoleucine, and leucine, showed only minor changes.

Both aspartate plus asparagine and glutamate plus glutamine appeared as additional N-terminal residues for group 5 (less than 12 kDa) polypeptides released in the presence and absence of GA_3. While glycine and arginine were

not detectable as N-terminal residues, concentrations of other N-terminal residues, e.g., valine, isoleucine, and leucine, showed only minor changes.

4. Control of Initial Hydrolysis:

The starch and protein reserves stored in the barley endosperms are hydrolyzed following imbibition of the caryopsis (whole kernel) or incubation of the half-kernels in the presence of GA_3. This growth regulator induces synthesis of a number of hydrolases, for example -amylases and proteases (24, 25, 56, 102). The appearance of -amylase is dependent on the new synthesis of both proteins and mRNAs (49, 50, 51, 82). In this set of experiments, a dependence of the appearance of proteases and release of early hydrolytic products from protein reserves on protein and mRNA synthesis was tested.

4.1. Development of hydrolases: In the presence of GA_3, a 54-fold and 7-fold increase in the levels of -amylase and protease activities, respectively, was observed (Table 12). Additions of cycloheximide and cordycepin prevented the GA_3-dependent increase in the levels of both activities (Table 12). This result suggested that the appearance of -amylase and the major protease is dependent on synthesis of both proteins and mRNAs.

4.2. Release of polypeptide fragments: Results summarized in the earlier sections indicated that in the

Table 12. Effect of Cycloheximide and Cordycepin on α-Amylase and Protease Activities in Barley (cv. Perth) Half-kernels.

Treatment	α-Amylase (μg starch/min. endosperm)	Protease (μg tryptophan/min. endosperm)
Control	6.1 ± 0.2	0.1 ± 0.0
GA_3	329.0 ± 22	0.6 ± 0.1
GA_3 + CHX	5.0 ± 0.4	0.1 ± 0.0
GA_3 + COR	6.9 ± 0.3	0.1 ± 0.0

Barley half-kernels were incubated for 4 days in a buffered media containing GA_3 (5 μM), CHX (Cycloheximide, 20 μM), and COR (Cordycepin, 250 μM), as indicated. Standard starch and hemoglobin hydrolysis assays were used to test the enzyme activities as described in the Materials and Methods. The experiment was performed in triplicate and the mean values ± standard deviation are represented.

absence of GA_3, fragments in the size range of 40 kDa and 25-30 kDa are released within a 24 hour incubation period. In the presence of GA_3, however, fragments in the size range of 15 kDa or less were more dominant. A GA_3-independent protease(s) is thus apparently active in the initial hydrolysis. The appearance of a GA_3-independent protease activity may be due to: a) activation of an inactive zymogen, or b) synthesis of the enzyme from a stored mRNA. The following experiment was performed to distinguish between the two possibilities.

The endosperm pieces were incubated in the presence and absence of GA_3 and cycloheximide. In the presence of cycloheximide and the presence or absence of GA_3, fragments in the size range of 40 kDa do not appear to accumulate to the same degree as in the control (Figure 31). The fragments in the size range of 25-30 kDa were not visible. The fragments in the size range of less than 15 kDa were also not prominent. This result indicated that the appearance of the initial protease(s), which mediated the release of large fragments (40 and 25-30 kDa), is dependent on new protein synthesis.

The fragments in the size range of 40, 25-30, and less than 15 kDa, were more prominent in the samples incubated in the presence of both GA_3 and cordycepin. This result indicated that when new mRNA synthesis is blocked, the appearance of proteases mediating the further hydrolysis of

Figure 31. Western immunoblot analysis of soluble proteins. Soluble proteins were extracted from dry kernels (T_0) and embryo-less endosperm pieces incubated for 24 hours in the presence (+GA_3 , 5 μM) and absence (-GA_3) of gibberellic acid, cycloheximide (±GA_3+CHX), and cordycepin (+GA_3+COR). Cycloheximide and cordycepin were used at 20 uM and 250 μM concentrations, respectively. Arrows 1 and 2 indicate approximate positions of BSA (67 kDa) and cytochrome C (12 kDa).

The whole experiment was performed once.

1 - controls (dry kernels)

2 - + GA_3 + COR

3 - - GA_3 + CHX

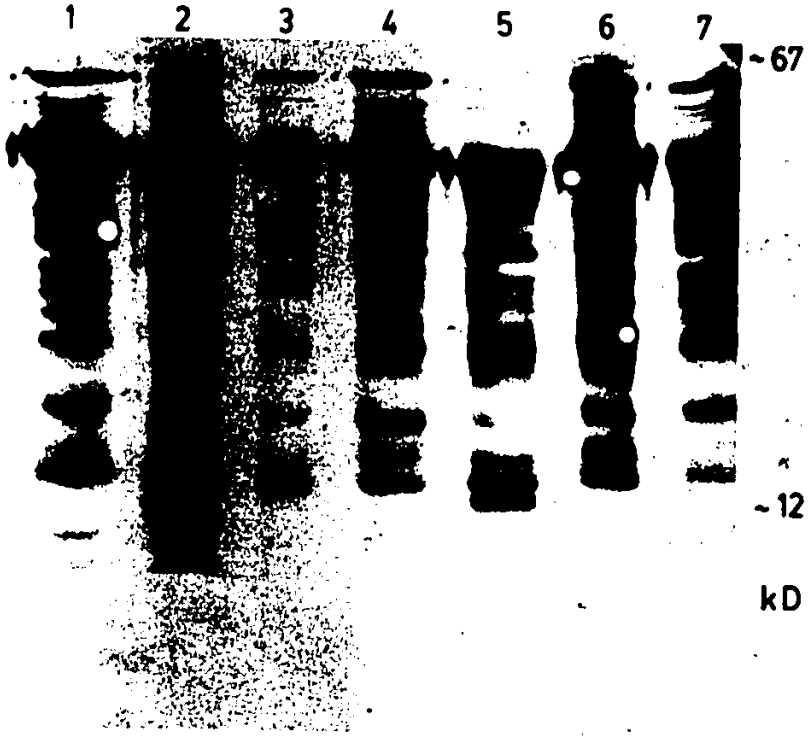
4 - + GA_3 + CHX

5 - + GA_3

6 - - GA_3

7 - controls (dry kernels)

Approximately 50-75 ul samples containing 15-25 ug proteins were loaded in each well.


1
2
3
4
5
6
7
~67
~12
kD

large size fragments (40, 25-30, less than 15 kDa) into peptides of smaller sizes is inhibited.

It appears, therefore, that the appearance of protease(s) mediating the release of fragments in the size range of 40, 25-30, and less than 15 kDa, is dependent on protein synthesis. However, when mRNA synthesis was blocked, the appearance of proteases mediating the further breakdown of large fragments was also inhibited.

DISCUSSION

Prolamins, the storage proteins in barley endosperms, are insoluble in aqueous buffers. As a result, these proteins can not be used as substrate for assaying the peptide hydrolase activities. Using synthetic dipeptides (blocked at N-terminal) as substrates, Mikola (75, 76, 77) reported presence of five carboxypeptidases in the extracts of barley endosperms germinated for six days. These proteases were purified to homogeneity. Presence of hemoglobin and gelatin-degrading endoproteases in the crude extracts of barley endosperms has also been demonstrated (8, 56). Whether these protease activities are really involved in the initial hydrolysis of hordeins, is not known. Globulins, the major storage proteins in pea, pumpkin, and mung bean, have been used as substrates, and unequivocal evidence in support for the involvement of endoproteases has been provided (5, 6, 38). Since, it is not possible to use the storage proteins of barley as substrates, I decided to analyze the products released from the hydrolysis of hordeins. The results from this analysis permit an interpretation on the mode of hydrolysis of hordeins.

1. GA_3 and Protein Hydrolysis:

The overall hydrolysis of hordein proteins appears to be accomplished in two general steps: a) an initial step, in which the soluble products are released; and b) further breakdown of the soluble products into amino acids and small peptides. In the literature, this distinction has not been made, and in fact it is generally assumed that the protease activities that are induced in the presence of GA_3 mediate both the initial and overall mobilization of the storage proteins (14, 56, 75, 76, 102). The present results, which showed: a) no change in the redistribution of total nitrogen in the soluble and insoluble fractions in the absence of GA_3 (Fig. 9); b) no change in the banding pattern of hordeins in the absence of the growth regulator (Fig. 11); and finally c) no change in the proportion and profile of peptides in 'peak I' and 'peak II' in the absence of GA_3 (Fig. 21), are consistent with the notion that the overall hydrolysis of hordein proteins is dependent on the presence of this growth regulator.

A dependence of the initial step on the presence of GA_3 has however, never been looked for, let alone established. An identification of the immunoreactive products related to the hordein polypeptides in the soluble fraction made it possible to examine whether the initial steps of hydrolysis of hordeins are also dependent on the presence of gibberellic acid. In the initial experiments,

using SDS-PAGE, I analyzed large polypeptides released into the soluble fraction. The different components appearing in the soluble fraction of incubated endosperms are as follows: a) stored amino acids and peptides; b) hydrolytic enzymes induced in the presence of GA_3, such as α-amylase, which comprise up to 50% of the total proteins synthesized (82); and finally c) polypeptides released from the hydrolysis of hordeins. In samples incubated in the presence of GA_3, the appearance of α-amylase polypeptide was noted (Fig. 10). No other significant difference was noted in controls (0) and samples incubated in the presence and absence of GA_3. The inability to detect any change other than the appearance of α-amylase may be due to two reasons: a) no large polypeptide fragments were released from hordeins, and/or b) the electrophoresis technique does not have the fine resolution needed to detect the initial products which may not accumulate in significant amounts. To distinguish between the two possibilities, a highly sensitive probe was needed to detect and identify the products of hordein hydrolysis.

The immunological approach adopted in this project permitted the detection of hordein-related products in the soluble fraction (Fig. 27). Experiments following the same approach have also been performed to detect the products of hydrolysis of castor bean storage proteins (34), and the turnover of ribulose bisphosphate carboxylase enzyme in the

leaves of barley (78).

Some interesting points emerge from my results. Firstly, the antibody sample which was raised against hordeins also contained the hordein unrelated IgG's. A pre-incubation of the crude serum with soluble proteins resulted in the removal of IgG's against soluble protein determinants (Fig. 24). The resultant serum was, therefore, enriched for hordein IgG's. A number of immunoreactive polypeptides were, however, detected in the soluble protein fraction prepared from dry kernels, when such an enriched serum was used in the western blotting experiments (Figure 25). The three interpretations of this result are: a) that soluble proteins (albumins + globulins) and prolamins may be partially similar in their structure, i.e., share common epitopes; b) that incompletely synthesized hordein polypeptides are extractable with aqueous buffers; and/or c) that partial hydrolysis of the hordein polypeptides may have begun during development of the caryopsis itself.

Secondly and perhaps the most interesting point of this result is the increased or new appearance of polypeptide fragments, in the size range of about 40 kDa and between 25-30 kDa, in the soluble protein fraction of endosperm pieces incubated in the absence of GA_3 (Fig. 25). This implied that the initial steps of solubilization are not dependent on the presence of this growth regulator. Since, cleavage(s) within the hordein polypeptides results in the appearance of large

fragments, an endoprotease(s) activity must be mediating the initial hydrolytic steps.

Thirdly, since the fragments released in the presence of GA_3 were in the size range of less than 15 kDa, it appears that the protease activities induced in the presence of this growth regulator are different from those present in its absence. A different set of proteases, induced in the presence of GA_3, may mediate reciprocal changes in the proportion of peptides in 'peak I' and 'peak II'.

2. Specificity of the Initial Step:

The early step(s) resulting in the appearance of polypeptide fragments may be specific. Three possibilities exist. Firstly, the activity of early protease(s) mediating this step may possess some degree of specificity with regards to the amino acid residue(s) around the susceptible peptide bond(s). Secondly, the specificity may reside in the structure and conformation of the hordein proteins packaged in the protein bodies. And, finally, it is possible that the initial protease(s) lacks specificity, and may cleave peptide bonds at random. Whereas only one or few types of amino acids may preferentially appear as a terminal residue of the products released in the case of sequence- or structure-related specificity, products with a random amino acid as terminal residue are expected if the initial protease(s) is non-specific.

In mammalian and microbial systems, sequence-specific protease activities, which cleave either before or after a defined residue are well documented (31, 59). Trypsin, for example, hydrolyzes the lysine/arginine - Y (Y being any amino acid except proline) bonds and results in products, one of which has lysine/arginine at the C-terminal. On the other hand, the neutral protease from Bacillus subtilis cleaves X - leucine (X being any amino acid) bonds and results in products, one of which has leucine at the N-terminal. The N-terminal residues of the hordein-related polypeptides were therefore determined to see whether the initial protease(s) cleaves before a specific residue(s).

In the case of large protein substrate, an additional requirement must be met, namely, the susceptible peptide bonds be accessible to the attacking protease. In other words, the topography of its environment must fit the active sites of the proteolytic enzyme. Conformation or the structure of proteins therefore determines the specificity. Based on this concept, Linderstrom-Lang (67) proposed the idea of limited hydrolysis, or one-by-one process in which the peptide bonds were cleaved sequentially depending on the step-wise unfolding of the polypeptide. This 'topographical specificity' is in fact responsible for the fact that, in general, the compact native proteins are more resistant to proteolytic degradation than the denatured ones. The observation of Harvey and Oaks (43), that the denatured zeins

(corn storage proteins) rather than the native ones were degraded by a sulfhydryl endoprotease, is consistent with this idea.

In my experiments, the polypeptides that either increased in concentration or appeared new in the absence of GA_3 were recovered in the second and third groups (Fig. 30). It should be noted here that more than one polypeptide is present in each group. The precise number of polypeptides was not determined. The N-terminal data show that aspartate/asparagine, arginine, and glycine are the major N-terminal residues of group 2 polypeptides released in the absence of GA_3. Glycine is also a major N-terminal residue of group 3 polypeptides. In addition, valine and aspartate/asparagine show a dramatic increase in their concentration as N-terminal residues of polypeptides in group 2 and 3, respectively. A comparison between the relative abundance of different amino acids in hordein proteins and the proportion of each type of amino acid as N-terminal residue of the hordein-derived fragments may show whether random cleavages result in the appearance of these fragments. The relative proportion of each amino acid in B and C hordeins is shown in Table 13. The fact that aspartate/asparagine, glycine, and arginine are represented in low amounts, e.g. 1.0, 1-1.5, and 2-3.0, % mole, respectively, in the B and C hordein polypeptides, suggests

Table 13. Amino Acid Composition of B- and C- Hordein Polypeptides Derived from the cDNA Sequence of B- and C- Hordein Genes. Adapted from Forde (30).

Amino Acid	% mole (per 100 residues) B-hordein	C-hordein
Glutamine	31.8	36.2
Proline	15.1	28.6
Valine	7.9	2.8
Leucine	7.9	5.7
Serine	6.8	3.8
Isoleucine	4.9	4.8
Phenylalanine	3.8	4.8
Alanine	3.0	1.9
Arginine	3.0	1.9
Cysteine	3.0	0
Threonine	2.6	1.9
Glutamic acid	1.5	1.9
Glycine	1.5	0.9
Histidine	1.5	0.9
Methionine	1.5	0.9
Aspartic acid	1.1	0
Tyrosine	1.1	1.9
Asparagine	0.7	0
Lysine	0.4	0
Tryptophan	0.4	0.9

that a GA_3-independent protease(s) cleaves preferentially before these residues. Such a preferential cleavage may be either because of the specificity of the enzyme and/or due to the specific conformation of the protein substrate. Since other amino acids are represented in significant amounts in the hordeins, their recovery as N-terminal residues of hordein-derived polypeptides may be due to random cleavages. An alternative interpretation may also explain this last observation. The C-terminal residues were not determined. As a result, I can not exclude the possibility that an initial protease(s), with trypsin-like specificity, released early polypeptide fragments, some of which have one or a few types of C-terminal residues.

Partial sequence derived from the cDNA clones suggests that a consensus sequence of an octapeptide, X-Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln-Y (X and Y being any amino acid), is repeated several times (30). The absence of proline as an N-terminal residue of polypeptides released at early times (Table 10) suggests that the protease(s) active at these times do not cleave the X-proline peptide bond. The protease activities induced at later times and in the presence of GA_3, however, appear to cleave this peptide bond, since proline is one of the major N-terminal residues of the peptides appearing 72 hours after incubation in 'peak II' (Table 10).

3. Mode of Hydrolysis:

The major proteases in barley extracts are carboxypeptidases (75, 76, 77, 102, 124), which suggest that amino acids could be the products of initial hydrolysis of hordeins. In contrast to this, my results show that amino acids are not the initial products. The preliminary analysis of the soluble fraction, for example, indicated that: a) amino acids were recovered in the peptide-bound form at early times, i.e. 24 hours (Table 7; Figs. 17, and 22); and b) peptides with amides (glutamine and asparagine), acidic (glutamic and aspartic acid), and neutral + basic amino acid residues at the C-terminal were recovered particularly in the amide and acidic fractions (Table 8; Fig. 18). The conclusive evidence in support of the fact that polypeptides are the initial products came from the immunoblotting experiments. For example, at early times, i.e. 24 hours, release of large fragments in the size range of 40 kDa and between 25-30 kDa was observed (Fig. 25). The fact that polypeptides of several class sizes appear, indicates that an endoprotease(s) activity is involved in the early stages of hydrolysis of hordeins. This protease may cleave around the specific amino acid residues or the specific regions of the proteins that are locally denatured (see Section 2 of discussion).

In the presence of GA_3, small polypeptides (methanol-soluble) of heterogeneous sizes, less than 15 kDa on the gels,

and in the two broads peaks, labelled 'peak I' and 'peak II', appeared in the media (Fig. 22). With longer periods of incubation in the presence of GA_3, the proportion of peptides in 'peak I' decreased and those in 'peak II' increased. This reciprocal relationship between the proportion of peptides in the two peaks seem to suggest that peptides in 'peak II' may be derived from those in 'peak I'. The peptides in 'peak II' may, alternatively, be derived from large fragments observed on the immunoblots.

After 72 hours of incubation, a large proportion of nitrogen was recovered in the form of peptides in 'peak II', and in the fraction containing peptides + amino acids. Since small peptides are present in high levels at 72 hours, it appears that the major proteases, e.g. carboxypeptidases and other peptidases, described by Mikola (75) are of less importance even in the later stages of mobilization of hordeins.

Based on these results, a possible mode of the hydrolysis of hordeins is shown in Figure 32. According to this model, the initial protease (GA_3-independent), 'Endoprotease I' cleaves the hordein polypeptides. These initial cleavages result in the release of large polypeptides, which are soluble in the aqueous media. The possibility that the small polypeptides recovered in 'peak I' may also be released by this protease has not been ruled out

by results of my experiments. The large polypeptide fragments may be substrates for GA_3-dependent 'Endoprotease II', and this step may result in release of the two types of products, a) polypeptides of less than 15 kDa in size, and b) small polypeptides in 'peak II'. The peptides in 'peak II' could, alternatively, be derived from those in 'peak I'. The peptides in 'peak II' could be hydrolyzed to free amino acids and oligopeptides of less than 9 residues, by exopeptidases (carboxypeptidases and aminopeptidases) and dipeptidases.

The steps shown in the model and discussed above are by no means definitive, however the results summarized in this thesis do lend some support. The value of this model is that it indicates appropriate next experiments. These are listed below:

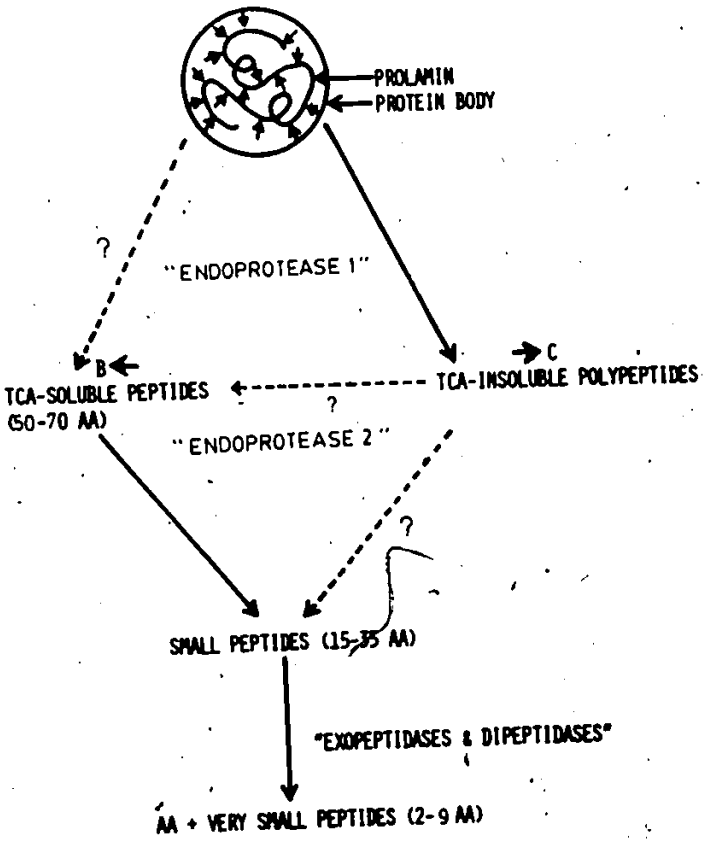
a) Even though, the large polypeptide fragments released in the absence of GA_3 are immunologically similar to hordeins, the former need to be characterized further. For example, the amino acid composition of these fragments and the peptide maps could be analyzed. The data obtained from such an analysis should be comparable to that from hordeins, if hordeins are the true precursors. The precise number of the fragments released by the activity of 'Endoprotease I' is not known. A 2-D analysis should indicate the precise number of hordein-derived polypeptides. Furthermore, the C-terminal residues of these fragments should be determined. This analysis would predict whether or not 'Endoprotease I'

possesses a trypsin-like specificity.

b) The action of the initial protease (GA_3-independent, 'Endoprotease I') needs to be clarified. This can be accomplished by purifying the proteases from barley kernels incubated for 24 hours in the absence of GA_3, and using animal proteins, such as hemoglobin, or small polypeptides of known sequence as substrates, and analyzing the products released. The identification of C- and N-terminal amino acid residues would be of prime importance. Partially denatured hordeins could also be used as substrate to see how similar (or different) the products released from these proteins are to those released from the barley half-kernels.

c) The precise nature of products released by 'Endoprotease II' activity needs to be established. This can be achieved by analyzing the products released when large polypeptide fragments (hordein-derived) are used as substrates for assaying the activity of GA_3-induced protease. A characterization of the products, for example N- and C-terminal residues, should show whether 'Endoprotease II' also possesses some degree of specificity with regards to the residues between which it cleaves the peptide bond(s).

Figure 32. Proposed model for hydrolysis of hordeins. Solid arrows indicate steps for which initial evidence has been presented and dotted arrows indicate steps for which no evidence has been presented. AA = amino acids. "Endoprotease 1" and "Endoprotease 2" are the GA_3-independent and GA_3-dependent proteolytic activities. The small arrows on the prolamin polypeptide within the protein body indicate the possible cleavage sites, if the amino acid residue at the ends of all repeating units were being attacked by the initial protease.


PROLAMIN
PROTEIN BODY
?
"ENDOPROTEASE 1"
B
C
TCA-SOLUBLE PEPTIDES
(50-70 AA)
?
TCA-INSOLUBLE POLYPEPTIDES
"ENDOPROTEASE 2"
?
SMALL PEPTIDES (15-35 AA)
"EXOPEPTIDASES & DIPEPTIDASES"
AA + VERY SMALL PEPTIDES (2-9 AA)

4. In Vivo Significance:

For my experiments, I adopted half-kernel incubation technique, in which the embryonal and the terminal parts were excised and the remaining portion was incubated under sterile conditions in a buffered medium in the presence and absence of GA_3. The products released from the hydrolysis of storage proteins in the soluble media were analyzed. Now, a question may be raised about the relevance of results obtained using this technique with regards to the mode of hydrolysis of hordeins in the normal germination process. In other words, whether a similar picture would emerge if one analyzed the products released during the normal germination of barley grains. The fact that an overall change in total nitrogen in the insoluble and the soluble fraction is observed only in the presence of GA_3, suggests that GA_3 can 'replace the physiological need' for embryo, and as a consequence the changes seen in half-kernel incubations may reflect an in vivo phenomenon. At least one aspect of my results has been examined, and is described below.

In the embryo and endosperm of germinating barley grains, Higgins and Payne (47, 48) reported the presence of a large peptide pool. An increase in the peptide pool was observed up to 3 days after imbibition, after which it started to decline. My results, showing an increase in total nitrogen in the 'peak II' peptides and fraction containing free amino acids and oligopeptides (Fig. 22, Table 9) up to

72 hours, indicates a similar phenomenon. This, therefore, supports the idea that peptides may play an important role in the transfer of stored nitrogen from the endosperm to the growing seedling during germination. Recently, the presence of an active peptide uptake system in the scutellum (the absorptive tissue of barley grain) has been reported (117). It was proposed that the peptide pools appearing in the endosperm are transported across the scutellum to the embryonic axis.

SUMMARY AND CONCLUSIONS

The storage proteins in barley (Hordeum vulgare) endosperms, hordeins, are insoluble in aqueous buffers. Following imbibition, these proteins may be first solubilized and then hydrolyzed into peptides and amino acids during the early seedling growth. A variety of proteolytic activities belonging to the general class of endoproteases, exopeptidases, and dipeptidases, are known to be present in the crude extracts of endosperms collected after germination or after incubation of the embryo-less endosperm pieces (half-kernels) in the presence of gibberellic acid. Since proteins other than hordeins have been used as substrates to assay different proteases, it is not known which enzymes are actively involved in the hydrolysis of the hordein proteins. Hydrolytic products released into the media of incubated half-kernels were analyzed to determine: a) the mode of hydrolysis of hordein proteins; b) the role of gibberellic acid in the hydrolysis; and c) whether the initial steps of hydrolysis are mediated by a specific protease(s). A gibberellic acid-responsive cultivar of barley, Perth, was chosen to examine the role of this growth regulator in the hydrolysis of hordein proteins. The major findings of the present study are summarized below.

1. Appearance of hydrolases during germination: The endosperm reserves, for example starch and proteins, are mobilized during the early seedling growth to provide sugars and amino acids. Alpha- and beta-amylases and a major protease, carboxypeptidase, are thought to be responsible for the breakdown of starch and proteins, respectively. The appearance of alpha-amylase and carboxypeptidase activities after imbibition was followed. Levels of both the hydrolase activities were low for the initial 1-2 days, after which there was a sharp increase.

2. Control of overall protein hydrolysis by gibberellic acid: Total nitrogen in the ungerminated barley endosperms (controls) is distributed in the soluble (buffer extractable) and the insoluble (propanol extractable) fractions. Initiation of the hydrolysis of the hordein proteins should result in a 'transfer' of total nitrogen from the insoluble to the soluble fraction. In the absence of gibberellic acid, the proportion of total nitrogen in the two fractions remained unchanged during a 60 hour incubation period. In the presence of this growth regulator, however, changes in total nitrogen were recorded in both the fractions. For example, in the soluble fraction, the proportion of total nitrogen increased from 12% to 35% and 70% after a 24 and 60 hour incubation period, respectively. And, in the insoluble fraction, a concomitant decrease in the amount of total nitrogen was noted. Increase in the levels

of both TCA or methanol-precipitable material (large polypeptides) and TCA or methanol-soluble (peptides and free amino acids) accounted for an increased total nitrogen in the soluble fraction. The result of this experiment, therefore, established a dependence of overall protein hydrolysis on the presence of gibberellic acid.

3. Analysis of the methanol-soluble fraction: The methanol-soluble fraction was analyzed to determine whether amino acids and/or peptides accounted for an increase in the total nitrogen. In the presence of gibberellic acid, at early times, i.e. 24 hours, the principal components in the methanol-soluble fraction were peptides. At later times, i.e. 72 hours, however, the proportion of free amino acids increased. Gel filtration experiments using Sephadex G-10 and G-25 indicated that peptides of heterogeneous sizes appeared in the media. On the basis of size, peptides were pooled in three broad groups, namely: a) oligopeptides of less than 9-10 amino acid residues and free amino acids; b) 'peak I' containing large peptides of 50-70 amino acid residues in length; and c) 'peak II' containing peptides of 15-35 amino acid residues in length. Whereas, the levels of peptides and total nitrogen in 'peak I' decreased with longer periods of incubation in the presence of gibberellic acid, those in 'peak II' showed a concomitant increase. The proportion of total nitrogen in the group containing

oligopeptides and free amino acids also increased dramatically with longer period of incubation. Many types of amino acid residues were recorded as the amino-terminal of peptides in 'peak I' and 'peak II'. The result of this series of experiment established the following points: a) free amino acids are not the principal components even after a 72 hour incubation period; b) peptides of various sizes are present in the media; and c) the proportion of large peptides ('peak I') relative to small peptides ('peak II') showed reciprocal changes over the incubation period.

4. Analysis of the methanol-insoluble fraction: The methanol-insoluble fraction containing large polypeptides was analyzed to determine whether hordein-derived polypeptide fragments appeared following incubation in the presence and absence of gibberellic acid. Electrophoretic separation on polyacrylamide gels in the presence of a denaturing agent, SDS, showed the appearance of a polypeptide of 42 kDa in the presence of gibberellic acid. Using the western immunoblotting technique, the identity of the polypeptide was confirmed to be alpha-amylase.

Antiserum against hordein polypeptides was also used to identify those fragments in the media, which bear the epitopes similar to those on hordein proteins. In the control samples, a number of hordein IgG-reacting polypeptides were detected. At early times, i.e. 24 hours, in the absence of gibberellic acid, additional fragments in

the size range of 40 kDa and 25-30 kDa appeared. Fragments in the size range of less than 15 kDa appeared in the presence of the growth regulator. In addition, fragments of around 60 kDa disappeared. Most of the hordein IgG-reacting polypeptides were lost when the samples were incubated for 72 hours in the presence of gibberellic acid. This result established the following points: a) the initial hydrolysis of hordeins is not dependent on gibberellic acid; and b) protease(s) induced in the presence of gibberellic acid are important for further hydrolysis.

5. Preliminary characterization of hordein-related polypeptides: Immunoaffinity column chromatography of the soluble fraction facilitated separation of hordein-related polypeptides from other water soluble proteins. These polypeptides were fractionated in five groups on the basis of size using size-exclusion column chromatography. The amino-terminal residues of polypeptides in each group were determined. These results showed: a) more than one polypeptide was present in each group. The precise number of polypeptides in each group remains to be determined; b) in the absence of gibberellic acid, new polypeptide fragments appeared in the second and third groups; and c) there was no clear-cut pattern of the amino-terminal residues.

In conclusion, therefore, the present study established following points:

a) the overall hydrolysis of hordeins is dependent on the proteases induced in the presence of gibberellic acid;

b) initial events in the hydrolysis are not dependent on the presence of this growth regulator;

c) large peptides predominate at early times of the hydrolysis, and at later times small peptides are the principal components appearing in the media, suggesting that the action of several endoproteases is involved.

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Appendix I. Amino Acid Concentration in Acidic, Neutral+Basic, and Amide Fractions Before Hydrolysis

	mM/30 endosperms								
	Acidic (I)			Neutral+Basic (II)			Amide (III)		
AA	24	48	72	24	48	72	24	48	72
Asx	0.9	1.3	0.8	-	-	-	3.9	10.0	8.8
Glx	1.3	3.2	5.1	-	-	-	26.4	72.2	70.7
Ser	-	-	-	4.1	15.9	14.3	-	-	-
His	-	-	-	-	1.1	1.5	-	-	-
Gly	0.1	0.1	0.1	5.2	20.6	23.7	-	-	-
Thr	-	-	-	2.0	8.7	10.3	-	-	-
Arg	-	-	-	0.2	2.4	2.6	-	-	-
Ala	0.2	.0.1	0.1	4.8	16.4	19.1	0.2	0.5	0.2
Tyr	0.7	1.5	1.3	1.1	8.1	9.7	0.8	1.3	1.1
Val	0.1	0.1	0.1	4.3	16.0	18.4	0.3	0.2	-
Phe	0.4	0.7	0.5	1.8	11.3	14.7	0.8	0.9	0.6
Ile	0.1	0.2	-	2.1	9.1	10.7	0.3	0.2	-
Leu	0.5	0.5	0.1	6.5	26.1	29.7	1.2	0.8	0.2

Thirty half-kernels were incubated for 24, 48, and 72 h in 10 ml media in the presence of GA_3. The supernatant from crude homogenate was precipitated with 50% methanol. An aliquot from the methanol-soluble fraction was separated into acidic, neutral+basic and amide fractions using ion-exchange chromatography (87).

(-) = less than 25 pmoles levels.

Appendix II. Amino Acid Concentration in Acidic, Neutral+ Basic, and Amide Fractions After Hydrolysis

	mM/30 endosperms								
	Acidic (I)			Neutral+Basic (II)			Amide (III)		
AA	24	48	72	24	48	72	24	48	72
Asx	3.8	10.9	7.6	0.2	0.7	0.5	3.4	8.2	7.3
Glx	12.9	41.8	39.4	0.6	3.7	5.0	29.3	75.3	69.5
Ser	3.1	6.3	5.7	4.0	19.3	28.3	1.3	3.2	2.9
His	0.3	0.8	0.6	0.3	3.4	5.5	5.8	16.2	14.2
Gly	3.4	8.2	8.2	5.3	22.3	43.3	1.3	2.4	2.4
Thr	0.9	3.2	2.5	1.9	14.0	20.1	0.6	1.4	1.6
Arg	0.2	0.7	0.4	0.5	5.7	8.5	-	0.2	0.2
Ala	2.0	4.4	4.0	4.9	17.3	28.9	0.8	1.3	1.0
Tyr	1.4	4.1	0.7	0.5	4.6	7.4	1.3	2.2	1.8
Val	1.6	4.0	2.7	3.4	21.2	22.2	1.7	3.5	3.0
Phe	1.6	5.1	4.7	1.6	11.9	18.3	1.2	2.2	1.7
Ile	1.1	3.1	2.4	1.8	12.6	17.1	1.0	1.8	1.7
Leu	2.6	6.1	4.2	4.5	25.4	34.5	2.3	4.1	3.4

Thirty half-kernels were incubated for 24, 48, and 72-h in 10 ml media in the presence of GA_3. The supernatant from crude homogenate was precipitated with 50% methanol. An aliquot from the methanol-soluble fraction was separated into acidic, neutral+basic fractions using ion-exchange chromatography (87). The fractions were hydrolyzed in presence of 6 N HCl at 110°C for 8 hours under vacuum. The hydrolyzed samples were washed several times to get rid of excess acid (see Materials and Methods). Finally, amino acids were separated using HPLC after OPA-derivatization after Winspear and Oaks (1983). (-) = less than 25 pmoles levels.