# ANALYSIS OF GA-INDUCED ENZYMES

# OTHER THAN α-AMYLASE FROM BARLEY ALEURONES

by

TIMOTHY VERSCHELDEN

B.E., Kansas State University, 1982

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A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Graduate Biochemistry Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1986

Approved by:

Major Professor
Department of Biochemistry

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ACKNOWLEDGMENTS

First of all, I must acknowledge God as my Creator, Savior, and Provider. My relationship with Him is my source of motivation and strength for any accomplishment. I must also recognize my parents, George and Peggy Verschelden, for their faithful love, support, and example throughout my education.

I would like to thank Dr. S. Muthukrishnan for his generous help, patience, and understanding. I also want to express my appreciation for the opportunity to work in his lab with his post-doctoral research associates: Cheryl Knox and Lolita Corpuz; his graduate students: Lauren Kam-Morgan, Grace and Steven Lee, Rownak Rahmatullah, Burachai Sonthayanon, and Mark Swegle; and his research assistant, Susan Ward.

I am also very grateful to Dr. Jerry Reeck and Dr. Delbert Mueller for serving on my committee, and for their generous advice.

Finally, I'd like to thank Dr. Tom Roche, my present employer, and his research assistants and student: Mohammed Rahmatullah, Gary Radke, and Gopalakrishnan Subramanian, for their tolerance with me while I complete my Master's requirements.

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Even before the turn of the century it was discovered that the aleurone layers of barley seeds are responsible for the production of a substance, or substances, which cause(s) the breakdown of the starchy portion of the endosperm(1). In 1960 it was reported that gibberellic acid acid (GA) induces the production of the enzymes responsible for this starch breakdown(2). In 1963, D. E. Briggs reported that GA induced the aleurone cells to produce or activate enzymes that hydrolyzed β-glucan, 1,6-α-linked dextran, sucrose, glycyl-L-tyrosine, glycyl-DL-leucine, hordein, and hordealin(3).

Since that time, many different aspects of the induction of these enzymes have been discussed in the literature. The activities of numerous hydrolytic enzymes have been reported to be increased by GA. The extent of induction by GA, whether GA induces de novo synthesis or promotes the release of these enzymes, and the time-course of GA induction and release have been the subjects of study by several investigators.

Most of this work has been carried out with the Himalayan variety of barley because it does not contain a seed coat. Among the GA-inducible enzymes, only α-amylase(s) has been purified to homogeneity from cultivar Himalaya. β-glucanases have been isolated from another cultivar, Clipper. However, there is only scant data available on the GA-inducibility of hydrolases in

Clipper.

In addition to the study of the extent, physiological manifestation, and time-course of GA induction, another approach has been to study the pattern of newly-synthesized proteins in the presence or absence of GA, using primarily sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Several protein bands of defined molecular sizes have been concluded to be GA-inducible, but only α-amylase bands have been identified among the several GA-inducible proteins.

The aim of this research is to attempt to purify GA-induced enzymes, other than α-amylases, which may allow the clear association of given enzyme activities with given protein band(s) and facilitate studies on the effects of plant hormones on these enzymes.

## Morphological Changes in GA-Treated Aleurone Cells

The GA-induced synthesis or activation of hydrolytic enzymes follows a lag period of 6 to 8 h after the start of GA treatment, during which pronounced morphological changes occur in the aleurone cells. These have been studied extensively with light and electron microscopy(4). One main set of structures which has drawn the greatest attention because of the dramatic GA-induced changes that they undergo are the aleurone grains. These aleurone grains are

membrane-enclosed structures containing one or two globoids having a crystalline appearance, and one to several transparent, possibly air-containing, internal cavities. These globoids and internal cavities are surrounded by a proteinaceous matrix. The aleurone grains, in dry barley seeds, are generally surrounded by lipid-containing bodies called spherosomes.

It has been suggested that the crystalline appearance of the globoids is due to phytin, a calcium-magnesium salt of inositol hexaphosphoric acid(5). Phytin is the major storage form of phosphate, calcium, and magnesium in seeds. Calcium ions are reputed to play an important role in the release, synthesis, and/or maintenance of several of the GA-induced hydrolases. In many seeds, it is found within globoids in protein bodies, as appears to be the case with barley(6). The proteinaceous matrix has been proposed to consist of storage proteins which will be hydrolyzed to provide amino acids for GA-induced de novo synthesis of enzymes.

During the lag phase of GA induction the aleurone grains swell from the second to the tenth hours, this swelling being due to changes in the proteinaceous matrix. During this same time, the formation of stacks of rough endoplasmic reticula can be observed. Associated with this formation of rough endoplasmic reticula is the loss of the crystalline appearance of the phytin globoids as they

become filled with vesicle-like structures. Also at this time, about 10 to 12 h after the start of the GA incubation, significant α-amylase activity can first be detected in the medium surrounding isolated aleurone layers.

This proliferation of endoplasmic reticula peaks at 14 h after GA addition, after which the aleurone grain size and the number of spherosomes per cell decrease. At the same time, vesicles are formed, being pinched off from these endoplasmic reticula, as well as from dictyosomes(4c).

After 24 h of GA treatment, the aleurone cell begins to terminate its enzyme production. The continuous network of endoplasmic reticula, along with the empty aleurone grains, fuse to form a large central vacuole. The vesicles formed from the dictyosome cisternae, which are larger than those derived from the endoplasmic reticula, are suspected to disappear in association with the secretion of ribonuclease from the aleurone cells(4d).

Another very pronounced aspect of the secretion process is the deterioration of the aleurone cell walls. These cell walls originally have a noticeable middle lamella, a primary wall, and a secondary wall which is fibrillary in nature. After ten or more hours of incubation in the presence of GA, this secondary wall loses its fibrillar appearance as it is digested away.

Gibberellic acid has been reported to be responsible for the stimulation of enzymes that may be responsible for this cell wall degradation. Aleurone cell walls have been found to be mainly made up of arabinoxylan, this carbohydrate accounting for possibly 85% of the total cell wall composition(19). It has also been found that GA is responsible for the enhancement of activity of three enzymes that work in the breakdown of arabinoxylan. These enzymes include endo-$\beta$-1,4-xylanase, $\alpha$-arabinofuranosidase, and xylopyranosidase(19). Apparently this GA-induced breakdown of the aleurone cell walls is necessary for the release of other GA-induced hydrolases, including phosphatase. This cell wall degradation also provides soluble carbohydrates as energy for the growing plant.

GA Action Involves De Novo Synthesis of Hydrolases

In 1967, Chrispeels and Varner reported that GA enhances the de novo synthesis of $\alpha$-amylase and ribonuclease(RNAse) in isolated aleurone layers(7). The synthesis of $\alpha$-amylase begins about 8 h after addition of the hormone, and continues for 24 to 36 h after hormone addition. RNAse synthesis starts immediately after GA treatment and continues for up to 48 h. However, the synthesis of RNAse is much less dependent on GA than is that of $\alpha$-amylase in that lower concentrations of GA are

required, and the level of RNAse is increased only 2-fold by GA, compared to the level in aleurones incubated without GA. It was concluded that these enzymes are synthesized de novo because their formation was found to be affected by inhibitors of protein and RNA synthesis.

In the same year, Jacobsen and Varner reported that GA induced the de novo synthesis of a protease that was active on gliadin, suggesting that this protease is produced for the hydrolysis of barley endosperm proteins, hordeins, which are similar to wheat gliadins(8). The induction of protease followed a time-course and GA-requirement similar to that of α-amylase induction. The question of the de novo synthesis of this enzyme was investigated by the labeling of newly-synthesized protein with $^{18}O$ from $H_2{}^{18}O$ and the subsequent determination of the density of the protein peak after cesium chloride density gradient centrifugation. The density shift of the protein peak containing protease activity demonstrated de novo synthesis.

Bennett and Chrispeels, in 1972, used similar density-labeling experiments to show that ribonuclease and β-glucanase are also synthesized de novo during imbibition, and incubation in GA medium(9). Previously it had been reported that the synthesis of these two enzymes was only slightly increased by GA, but that their release was GA-dependent. These authors confirmed the de novo synthesis

of these enzymes, but, in fact, they also presented evidence that this synthesis occurred primarily before the incubation in the presence of GA.

In 1974, Jacobsen and Knox carried out the incubation of aleurone layers in a mixture of tritiated amino acids in order to label all the newly-synthesized proteins(10). Sodium dodecyl sulfate polyacrylamide gels and autoradiography were used to detect newly-synthesized proteins. Ten different GA-induced, newly-synthesized, proteins ranging in size from 15.5 to 81 kDa, were observed in the autoradiograms. Of these radio-labelled proteins, the 49 kDa protein, most likely α-amylase (although the molecular weight is different from the currently accepted value of 44 kDa), contained 30% of the total radioactivity incorporated into protein.

In 1980, Mozer reported on proteins, other than α-amylase, whose synthesis was induced by GA(11). In extracts of aleurone tissue induced by GA he found that two proteins with molecular weights of 16 and 23 kDa appeared concurrently with α-amylase. In extracts from later times in the incubation, proteins with molecular weights of 52, 28, and 14 kDa were found and these proteins were concluded to be GA-induced. The identities of these proteins were only speculated upon.

The pulse-labelling studies of Higgins, Jacobsen, and Zwar allowed the discovery that proteins with molecular

weights of 44, 42, 30, 27, and 26 kDA were newly synthesized and released at distinctly higher levels in GA-treated cells(12). Some proteins were synthesized and released in greater quantities by aleurones incubated without GA, than by GA-treated aleurones. These included polypeptides of molecular weights 23, 20, and 15 kDa.

## Secretion of GA-Induced Enzymes

The effect of GA on enzyme release has not yet been well established. Most likely the release of the GA-induced enzymes involves other factors as well. Certainly in the case of α-amylase, most of this GA-induced enzyme is synthesized for action outside of the aleurone cell, and thus most of it is secreted soon after it is synthesized. Even though the synthesis of RNAse is also stimulated much earlier in the incubation, the release of this enzyme has been reported not to occur at a measurable level until 24 hours after the beginning of GA treatment(7). It does appear, though, that this delayed release of RNAse is GA-dependent.

Jacobsen and Varner reported that the synthesis of a protease is induced 12-fold while its release is induced 70-fold by GA(8). They reported that 79% of this protease is secreted from the aleurone cells. Therefore it is apparent that this protease is responsible for the

hydrolysis of endosperm reserve proteins, and/or, as has been suggested by some, the hydrolysis of secreted aleurone storage proteins. The time-course of the release of this protease is very similar to that of α-amylase.

Hammerton and Ho, in a 1986 paper, discussed the GA regulation of protease and carboxypeptidase activities secreted from barley aleurone layers(30). They found proteolytic activity that was increased significantly by incubation with GA, and that seemed to be accounted for by proteins that incorporated radiolabel when the aleurones were incubated in the presence of $^{35}$S-methionine. Their two methods of molecular weight determination: gel filtration chromatography and SDS-PAGE with molecular weight standards, gave varying results, 28 and 37 kDa, respectively. However, they concluded that the 37 kDa value, obtained from SDS-PAGE, was the more reliable one because of the sensitivity of gel filtration to molecular shape. Tests of pI values indicated three different proteases varying in pI but sharing the same size.

Their conclusions on the GA regulation of carboxypeptidase activity were similar to those offered previously by Schroeder and Burger in 1978, and by Mikola in 1983(31,32). Mikola found five different carboxypeptidases with slightly different substrate specificities, and Hammerton and Ho distinguished three different carboxypeptidases on the basis of size, as

determined by gel filtration. All three of these research groups found that only the release, and not the synthesis, of these carboxypeptidases was induced by GA.

In 1972, Bennett and Chrispeels reported RNAse and β-glucanase as being in the same class of GA-induction(9). The synthesis of these two enzymes is only slightly induced by GA, but their release is much more strongly GA-dependent. The time-course of their release is not discussed by these authors.

Jones and Jacobsen, in 1983, reported that $Ca^{2+}$ is required for the release from aleurone cells of many of the GA-induced hydrolases(13). This $Ca^{2+}$-dependence was specific for the release of group B α-amylase isoenzymes, as opposed to the group A α-amylase isoenzymes. (The α-amylase isoenzymes with pI's from 4.5 to 5.1 have been classified as group A. The higher pI isoenzymes, with pI's ranging from 5.9 to 6.6, have been placed in group B(20).) Previously the calcium ion contribution to the measured value of α-amylase activity had been explained by the proposal that $Ca^{2+}$ somehow stabilized the enzyme against proteolytic degradation.

In 1984, Jones and Carbonell extended the investigation of the influence of calcium on α-amylase production and secretion(14). They concluded that $Ca^{2+}$, in the presence of GA, induced the synthesis and release of α-amylases 3 and 4, the group B isoenzymes, at the expense of α-amylase

1(group A enzyme) production. These writers acknowledged the possibility that the calcium ion influence on α-amylase synthesis could be a result of its effect on enzyme release, so that the calcium-regulation of α-amylase synthesis is an indirect result.

In 1986, Deikman and Jones reported further on the influence of calcium ions on α-amylase synthesis and release(29). They used group A and group B α-amylase clones to hybridize with mRNA produced in aleurones treated with GA, with or without $Ca^{2+}$. Their results indicated that the amount of mRNA hybridizing to either the low or high pI α-amylase clones was not affected by the presence of $Ca^{2+}$. The precise mode of action involved in calcium-regulation of the appearance of α-amylase in the aleurone incubation medium was not pinpointed, but its effect on transcription was ruled out.

Jones and Carbonell also reported the $Ca^{2+}$ induction of the synthesis and release of acid phosphatase. The $Ca^{2+}$-induced increase in acid phosphatase was measurable, but much less pronounced than with α-amylase.

GA-Induced Release of Pre-existing Proteins

A paper by Melcher and Varner, published in 1972, reported the release of large amounts of reserve protein from isolated barley aleurone layers, this release being only partially dependent upon GA(15). The released proteins were of consistently lower molecular weight when GA was present, as compared to incubation without GA. Also an obvious change in the N-terminal profile of the released proteins was brought about by the presence of GA. These two observations, as well as the finding that the release of these proteins was reduced by the addition of protease inhibitors, pointed to the conclusion that the release of these proteins was at least partially dependent upon GA-induced proteolysis.

In 1974, Jacobsen and Knox reported some discoveries that allowed for much clearer discernment of the extent of GA induction of protein synthesis(10). They had found that the washing of aleurone cells, with or without GA, caused the release of water-soluble endosperm proteins. These proteins were plentiful enough that they contributed one-third of the total protein in the medium surrounding GA-induced aleurones.

At the same time, these authors reported that twelve different GA-induced proteins were found in the medium surrounding aleurones that had been incubated in

GA-containing medium. They used radioactive amino acids in the incubation medium to distinguish pre-existing proteins from those that were newly synthesized. Of these twelve GA-induced proteins, two of them, of molecular weights 64 and 43 kDa, were found to not be newly synthesized. Tests with protease inhibitors led to the conclusion that the release of these two proteins was dependent upon GA-induced proteolytic activity. These two proteins made up 40% of the GA-induced protein.

In a 1984 paper, Jones and Carbonell reported a complex of proteins in the 38 to 39 kDa range, the release of which was dependent upon GA(14). These proteins did not incorporate radioactive label when the aleurones were incubated in GA medium containing $^{35}$S-methionine or a $^{3}$H-amino acid mixture. The authors did not attempt to identify these proteins, but did suggest that they may be proteolysis products or sub-aleurone proteins.

## GA-Regulation of Gene Activity

Certainly the greatest amount of research on this area of GA-regulation of gene activity, even more pronounced than the study of GA-induced proteins, has been focused on α-amylase. As early as 1976, Higgins, Zwar, and Jacobsen had reported that the GA induction of the de novo synthesis of α-amylase involved an increase in the levels of

translatable mRNA for α-amylase(17). At this point they were hesitant to make any definite conclusions, but their evidence strongly supported their suspicion of GA-induction of transcription. Their evidence for the GA-induction of α-amylase gene transcription was from cell-free translation of the mRNA of GA-treated, and untreated, aleurone cells. Increases in polypeptides of molecular weights 45 and 35 kDa were seen as the major effect of GA. The 45 kDa protein was identified as α-amylase.

In 1979, Muthukrishnan, Chandra, and Maxwell further supported the suggestion of transcriptional control by GA(18). They, like Higgins et al., found that GA caused an increase in the levels of translatable mRNA, with the mRNA level peaking at 12 h after GA-induction. Their research also ruled out the possibility of GA-induced activation of previously existing mRNA.

In 1980, Mozer, in studying the regulation of protein synthesis in barley aleurones by GA and abscisic acid (ABA), concluded that both GA and ABA induce the formation of new translatable mRNA's and cause new proteins to be synthesized(11). The mRNA's induced by GA are those coding for α-amylase, and for a few other GA-induced proteins, and thus the patterns of protein synthesis are entirely redirected. The mRNA's induced by ABA code for a different set of proteins, and like GA, but to a lesser extent, ABA also redirects protein synthesis patterns. Mozer then

reported that ABA acts as an antagonist to GA in that it prevents the overall change in protein synthesis that is seen in aleurones treated with GA alone. However, he found that the transcriptional changes induced by GA were not reversed by ABA, but rather the translation of GA-induced mRNA's was inhibited by ABA. He concluded, then, that one or both of the hormones are involved in the control of *in vivo* protein synthesis at the level of translation.

In 1982, Higgins, Jacobsen, and Zwar gave a report focusing on the same phenomena as studied by Mozer(12). Their conclusions differed from Mozer's in that they found ABA to have a strong effect on GA-induced transcription as well as on GA-induced translation. They concluded that both GA and ABA act as regulators of protein synthesis, primarily by regulating transcription, and secondarily by regulating the translation efficiency of the new mRNA's.

Rogers, Dean, and Heck, in 1985, described a cDNA clone that they had made from GA-induced mRNA, and had subsequently sequenced(16). This cDNA was made from an mRNA transcript that was found to increase seven-fold in aleurone cells in response to 18 h of incubation in the presence of 1 μM GA. The hormonal response of this mRNA, increasing with GA, and decreasing with ABA, resembles that of the α-amylase mRNA's. The deduced amino acid sequence of this clone has shown significant similarity to that of a rat lysosomal thiol protease in the C-terminal two-thirds

of the protein. In the introduction of the paper by Rogers, et al, they refer to a report by Hammerton and Ho of a thiol protease which may be "the major endoprotease synthesized in response to GA". As mentioned in the discussion of de novo synthesis of hydrolases, this protease is newly synthesized during the incubation of barley aleurones with GA, and its synthesis is dependent upon the presence of GA. Rogers, et al, offer the suggestion that their cDNA clone may well code for this protease.

As concluded by Jacobsen and Knox in 1974, and as still may be the case, the available evidence is sufficient to justify the conclusion of GA-induction of transcription in the case of α-amylase and protease only(10). The effect of GA on RNAse and β-glucanase activity was reported to be restricted to the release of enzymes that were synthesized during the imbibition of water, although some minor increase in synthesis may occur in the presence of GA. A recent report by Stuart, et al, provides more solid evidence for de novo synthesis of β-glucanase induced by GA to a significant degree(26). As for other enzyme activities increased in the aleurone incubation medium by GA, including phosphatase, pentosanase, peroxidase, esterase, and glucosidase, the mechanisms of GA-induction are yet to be established.

## EXPERIMENTAL PROCEDURES

| Materials | Source |
|---|---|
| **Enzyme Assay Substrates:** | |
| β-glucan (from barley) | |
| p-nitrophenyl-β-D-xylopyranoside | |
| N-CBZ-L-Phenylalanyl-L-alanine (Z-Phe-Ala): | |
| azocasein: | Sigma Chemical Co. |
| cibachrome blue: | gift from Dr. Paul Mathewson, Grain Marketing Research |
| **Equipment** | |
| capillary viscometer: | Thomas Scientific Co. |
| metabolic shaker: | Dubnoff |
| peristaltic pump: | LKB 2120 Varioperpex II Pump |
| spectrophotometer: | Cary 219 Spectrophotometer, (Varian) |
| Mighty Small Slab Gel: | Hoefer Scientific Instruments |
| scintillation counter: | Beckman LS 3801, (Beckman Instruments, Inc.) |
| conductivity meter: | ElectroMark Analyzer, (Markson Science Inc.) |
| **Chemicals, reagents, etc.:** | |
| 5% sodium hypochlorite | |
| sodium thiosulfate | |
| cupric sulfate | |
| sodium chloride | |
| silver nitrate | |
| ammonium hydroxide | |
| 38% formaldehyde | |
| concentrated hydrochloric acid | |
| toluene | |
| methanol | |
| glycerol | |
| bromphenol blue | |
| sodium phosphate dibasic | |
| ammonium chloride | |
| glucose | |
| Triton X-100: | Fisher Scientific Co. |

| | |
|---|---|
| chloramphenicol | |
| gibberellic acid, grade III | |
| herring sperm DNA | |
| tris-hydrochloride | |
| glycine | |
| ammonium persulfate | |
| casein enzymatic hydrolysate | |
| calcium chloride | |
| N,N,N,N tetramethylethylenediamine (TEMED) | |
| MES (2(N-morpholino)ethanesulfonic acid) | |
| POPOP (1,4-bis(2-(5-phenyloxazolyl))benzene; 2,2′-p-phenylenebis(5-phenyloxazole)) | |
| sodium acetate | |
| trichloroacetic acid (TCA) | |
| β-mercaptoethanol | |
| cysteine | |
| pepsin | |
| papain: | Sigma Chemical Company |
| acrylamide | |
| Coomassie Blue R-250: | Bio-Rad Laboratories |
| bis-acrylamide: | International Biotechnologies |
| glacial acetic acid | |
| potassium phosphate monobasic | |
| citric acid: | Mallinckrodt, Inc. |
| magnesium sulfate: | J.T. Baker Chemical Co. |
| sodium dodecyl sulfate (SDS): | Polysciences, Inc. |
| sodium phosphate | |
| disodium ethylenedinitrilo-tetraacetic acid (EDTA) | |
| sodium hydroxide: | MCB Manufacturing Chemists |
| carboxymethyl (CM)-cellulose (Whatman CM-52) | |
| Whatman 3MM chromatography paper: | Whatman, Inc. |
| PBO (1,4-bis(2-(5-phenyloxazole))) | |
| $^{35}$S-methionine (10 μCi/μl solution: | New England Nuclear |

| | |
|---|---|
| Kodak X-Omat AR film - 13x8 cm for small gels 20.3x25.4 cm for large gels | |
| Kodak hypoclearing agent: | Eastman Kodak Company |
| Millipore filters: | Millipore Corporation |
| Ethanol: | Midwest Solvents |
| 50% glutaraldehyde: | Fluka |
| uridine (5,6-$^{3}$H): | ICN Radiochemicals |
| Himalayan barley seeds (*Hordeum vulgare*) 1981 crop: | Washington State Univ. Pullman, Washington |
| sodium tetraborate decahydrate: | Aldrich Chemical Company, Inc. |

## Methods

### GA Incubation of Barley Aleurones

Solutions:

1% Sodium Hypochlorite: 5% commercial stock solution diluted in deionized, sterilized water

Washing Solution: 1 mg% chloramphenicol in sterilized, deionized, water

1 mM Gibberellic acid stock solution: Made fresh for each incubation in 1 mM sodium acetate, pH 4.8, 10 mM $CaCl_2$, 1 mg% chloramphenicol

1μM GA Incubation Buffer: 1 mM GA stock soln. diluted 1/1000 in 1 mM sodium acetate, pH 4.8, 10 mM $CaCl_2$, 1 mg% chloramphenicol

aqueous scintillation cocktail: 1.82 g. PPO
0.083 g. POPOP
217 ml triton X-100
65.2 ml $H_2O$
435 ml toluene

scintillation cocktail: 4 liters toluene
24 g PPO
0.8 g POPOP

Incubation Buffer: 1 mM sodium acetate, pH 4.8
10 mM calcium chloride
(Mixture is autoclaved and stored at 4°C, brought to 1 mg% chloramphenicol before use.)

The extreme tips of the non-embryo ends of barley seeds were cut off and discarded. Then the seeds were cut in half, discarding the halves containing the embryos. These embryoless half-seeds were then washed in 1% sodium hypochlorite, and stirred 15 minutes in a clean volume of 1% sodium hypochlorite. The half-seeds were then washed

twice in sterile water, and stirred 15 minutes each in two more fresh volumes of sterile water. In a new volume of water, the seeds, in a beaker, were placed into a dessicator which was connected to a vacuum pump for three to four minutes. After closing the dessicator valve, and disconnecting the dessicator from the pump, the beaker of seeds was stirred for one to two minutes while still in a vacuum in the dessicator. The beaker was then removed from the dessicator and the water was poured off.

The washed half-seeds were placed on wet sand in petri dishes, approximately 100 half-seeds per dish. The acid-washed sand, covered with deionized water, had been previously autoclaved in the petri dishes. The petri dishes were then wrapped in aluminum foil and placed in a cold room ($4^{o}C$) for three days to allow the seeds to imbibe water. The soaking of the half-seeds at $4^{o}C$ was a modification made to the procedure in order to maximize the difference in α-amylase activity between the GA and Control incubation media.

After three days the half-seeds were scraped out of the petri dishes and washed several times in Washing Solution. The starchy endosperms were then squeezed out of the aleurone layers using a spatula. The seed-coat was also discarded. For the initial aleurone layer washing the aleurones were put into 13x100 mm disposable culture tubes, 20 aleurones per tube, containing approximately 2 ml

Washing Solution. These aleurones were stirred vigorously with a glass stirring rod, and the Washing Solution was removed. This washing was repeated with two more 3 ml volumes of Washing Solution.

After the third washing, the aleurones were transferred to 25 ml Erlenmyer flasks, 20 aleurones per flask, containing 2 ml Incubation Buffer with 1 mg% chloramphenicol. The flasks were covered with foil and shaken in a shaker bath at 25°C for about 2 h for a pre-wash to removed any bound non-aleurone endosperm. After the two-hour pre-wash, the incubation medium was removed and either discarded, or saved for gel analysis. New 2 ml volumes of Incubation Buffer with 1 mg% chloramphenicol were added to the aleurones. In half the flasks Incubation Buffer was added that also contained 1μM GA. This medium is referred to as GA medium. The medium from the flasks without GA is referred to as Control medium.

After 18 h at 25°C the flasks were removed from the shaker, the medium was removed and pooled into two fractions, 18 h GA medium and 18 h Control medium. The medium was replaced by new volumes of identical Incubation Buffer. To an equal number of GA and Control flasks were added Incubation Buffer containing $^{35}$S-methionine, at a concentration of 25 μCi per flask. All the flasks were re-covered and returned to the shaker.

After the 24 h incubation, again the flasks were

removed from the shaker, and the medium was withdrawn from the aleurones, and pooled in four fractions: 18-24 h GA medium (including $^{35}$S-met), 18-24 h GA medium (no $^{35}$S-met), 18-24 h Control medium (including $^{35}$S-met), and 18-24 h Control medium (no $^{35}$S-met).

The pooled fractions were cooled on ice overnight, then spun at 6,000 x g for 15 minutes. The pellets were discarded. The supernatants were stored at -20°C until later use.

## Test of $^{35}$S-Met Incorporation in the 24-hr. GA Incubation

Six 25-ml Erlenmyer flasks, each containing 20 aleurones isolated from 20 half-seeds, were incubated 18 h at 25°C in 1mM sodium acetate, pH 4.8; 10 mM $CaCl_2$ (Incubation Buffer), 2 ml per flask. Three of these flasks also contained 1µM gibberellic acid (GA) and three did not. These flasks lacking the GA will be referred to as Control flasks.

To 16 ml of Incubation Buffer were added 20 µl of 10 µCi/µl stock solution of $^{35}$S-methionine, to bring the final concentration to 12.5 µCi/ml. 8 ml of this solution was then brought to 1 µM GA by the addition of 8 µl 1 mM GA.

18 h after the start of the incubation, the medium was removed from all the flasks, the medium from the Control flasks was pooled, as was the medium from the GA flasks. 2

ml of the Control buffer, to which the $^{35}$S-met had been added, were added to each of three Control flasks, and 2 ml of the 1 μM GA buffer containing the radiolabelled methionine were added to three of the GA flasks.

After the 24 h had elapsed, the medium from the flasks containing $^{35}$S-met were stored individually in test tubes, as were the aleurones from these same flasks.

The counting procedures:

Fifty μl medium from each of the flasks which contained the labelled methionine were brought to 20% TCA, and left on ice for at least one hour. These were then filtered through Millipore filters, with suction provided by water aspiration. 20 μl each of the medium from these same flasks were spotted onto Millipore filters and dried. 20 μl each of both Control and GA buffer with the same concentration of $^{35}$S-met as had been used in the incubation flasks, were spotted onto Millipore filters and dried. The aleurones from the $^{35}$S-met-containing flasks were ground in a mortar and pestle in 2 ml Incubation Buffer. The pestle was then rinsed with 2 ml more of the Incubation Buffer. These 4 ml were combined as the aleurone extracts from each of these six flasks. 50 μl from each of the extracts were TCA-precipitated, and the radiolabelled proteins were collected on Millipore filters as described above. Also, as with the medium, 20 μl of each of these extracts were spotted on filters. All of these filters were dried,

immersed in 3 ml scintillation cocktail, and counted in the 0-600 window.

Carboxymethyl(CM)-Cellulose Chromatography

The dry, or previously-used, CM-cellulose was activated, or re-activated, by consecutive stirrings in 0.5 M NaOH, $H_2O$, 0.05 M HCl, and $H_2O$. This CM-cellulose was then stirred in 500 mM MES, pH 6.0, this buffer being ten times more concentrated than the buffer to be used in the ion exchange chromatography. After a couple of rinses in 50 mM MES, pH 6.0, the CM-cellulose was packed into a 0.9 cm (i.d.) by 42.5 cm. column at 3-4 psi pressure. This column was then rinsed thoroughly with 50 mM MES buffer, pH 6.0, evaluating equilibration by equal ionic strengths of the buffer going on, and that coming off, the column.

About 20 ml of GA or Control incubation medium was typically used for a single run. As the buffer was, so the incubation medium was loaded onto the column by use of a peristaltic pump. This pump setting was at such a level so as to not be too high to cause leaks or compression of the column packing, but to maintain a flow-rate of about 6 drops per minute.

After the sample was run onto the column, the sample container was then filled with 50 mM MES buffer, pH6.0. During the loading of the sample, and throughout the

elution of the sample, a fraction collector was set to collect approximately 1 ml fractions. After this wash of about 30 ml buffer, a gradient of 70 ml each of 0 M, and 0.3 M, NaCl in 50 mM MES buffer, pH 6.0, was run over the column, followed by a wash of about 30 ml of 2 M NaCl in 50 mM MES buffer, pH 6.0.

Either 50 or 100 µl aliquots of every other fraction were mixed with 3 ml aqueous scintillation cocktail and counted in the 0 to 600 window. Every second or third fraction was read for absorbance at 280 nm. About every sixth and seventh fractions were pooled and read for conductivity. NaCl standards were also made with equilibration buffer and read for conductivity.

A plot was made with fraction number on the abscissa, and $A_{280}$, CPM, and NaCl concentration on the ordinate. (See Figures 3 and 4) The most radioactive fractions were pooled, and 0.5 to 1.0 ml portions were TCA-precipitated for gel samples.

Gel Sample Preparation

Gel Sample Buffer (200 ml):
4.6 g sodium dodecyl sulfate
10 ml β-mercaptoethanol
20 ml glycerol
1.51 g Tris-HCl
1 pinch bromphenol blue
Adjust pH to 6.8

Most often the protein samples were concentrated by TCA precipitation. Equal volumes of the protein samples and 50% TCA were mixed and kept on ice from 15 minutes to several hours. Samples were then spun five minutes at 10,000 x g. The supernatants were discarded and the pellets were washed with diethyl ether, of a volume approximately equal to the original volume of the protein sample.

After the addition of ether, the samples were vortexed and kept on ice from five to fifteen minutes. The samples were then spun four minutes at 8,000 x g. These supernatants were also discarded. This ether wash was then repeated.

After the second ether wash the pellets were allowed to dry. Gel Sample Buffer was added to these final pellets. These were vortexed and immersed in a 100°C water bath for three minutes. These gel samples were often used as soon as they cooled off, or they were stored at 4°C for later use.

Sometimes the protein samples were lyophilized to dryness, usually after being previously dialyzed against a

very low salt buffer, and then prepared for gel samples as described above.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described by Laemmli(33). 4.5 % stacking gels and 12% running gels were typically used. 1% agarose was used to plug the bottom of the small gels.

Coomassie Blue Staining of Acrylamide Gels

Coomassie Blue R-250 stain:
0.2% Coomassie Blue R-250
50% methanol
12% glacial acetic acid

De-stain: 20% ethanol, 10% acetic acid

For Coomassie staining, immediately after electrophoresis, the gel was shaken from 30 to 45 minutes in Coomassie Blue R-250 stain. After the staining, the gel was transferred to de-stain, and was shaken in several volumes of clean de-stain, for about 12 h until the blue background was sufficiently removed. After de-staining, the gel was dried onto Whatman 3MM chromatography paper.

Silver Staining of Acrylamide Gels

Silver-staining solutions:

Stock solutions:

0.36 % NaOH

Solution 1: 1.5M sodium thiosulfate

Solution 2: 0.15M cupric sulfate/0.6M NaCl

Freshly-made solutions:

10% glutaraldehyde: 50% stock solution diluted with deionized water. Stock solution stored at 4 C.

Solution A: 20% silver nitrate in water
0.8 g $AgNO_3$ in 4 ml $H_2O$

Solution B: 21 ml 0.36% NaOH, 1.4 ml ammonium hydroxide

Solution C: Add Solution A, dropwise, to Solution B, while stirring, bring to 100 ml with deionized water.

Solution D: 2.5 ml 1% citric acid, 0.25 ml 38% formaldehyde, bring to 500 ml with deionized water.

De-stain: 6.08 ml 14.8M ammonium hydroxide, 9.392ml Solution 2. Add 100 ml Solution 1.

This procedure for silver-staining was slightly modified and adapted from previously-published procedures(24,25). For silver staining, immediately after electrophoresis the gel was immersed and shaken in 50% methanol(MeOH)/10% acetic acid. Next, the gel was consecutively shaken in 40%, 30%, 20%, and 10% MeOH, all with 10% acetic acid, at least one half hour in each solution. 200 ml volumes of each solution were used. Then the gel was washed, and shaken briefly in three 200 ml volumes of water, after which it was shaken one hour in 150 ml 10% glutaraldehyde. Again the gel was washed and shaken

briefly in several 200 ml volumes of water, finally being shaken overnight in water.

For staining, the gel was shaken 15 minutes in freshly-prepared Solution C, and washed twice with water. In a clean tray, the gel was washed again with water and then shaken 10 to 15 minutes in Solution D until the protein bands appeared strongly. After the development, the gel was rinsed with water, and then was shaken in approximately 200 ml 50% MeOH, or 45% MeOH/10% acetic acid.

If the background was too dark, the gel was shaken in de-stain until the background had faded almost to the desired level. Then the gel was quickly washed with water, and the de-staining was stopped by shaking the gel in 250 ml Kodak Hypoclearing Agent(a 1:5 dilution of a stock solution). After about 5 minutes, the hypoclearing agent was replaced by a clean, 250 ml volume of the same, and the gel was shaken for at least an hour more.

Autoradiography of Gels

For a gel with radiolabelled samples, after the drying of the gel, it was placed in a cassette in contact with X-ray film. The cassette was then shut, covered with aluminum foil, and placed in a $-70^{o}C$ freezer for the chosen time, usually between one and ten days. After the exposure time had elapsed, the film was developed.

Preparation of $^{3}$H-Uridine-labelled RNA

For use in assaying RNAse activity $^{3}$H-uridine-labelled RNA was prepared by a method similar to that described by Bolle, Epstein, and Salser(23). *E. coli* cells were grown in a nutrient medium. Tritiated uridine was added to a small sample of the log-phase culture. After two more hours growth was stopped, the cells were lysed, and the RNA was isolated.

The incorporation of radioactivity into the RNA was evaluated by comparing the radioactivity precipitated by trichloracetic acid (TCA) between two samples, one being first treated with RNAse, and the other being untreated. These tests confirmed that the radiolabel had been incorporated into the RNA. 98.5% of the counts in the RNA sample were made TCA-soluble by the RNAse treatment.

Amylase Assay

Enzyme assay buffer: 50 mM sodium phosphate, pH 6.2

Cibachrome Blue tablets were crushed with a glass stirring rod and suspended in Enzyme Assay Buffer, 1 tablet per 10 ml Enzyme Assay Buffer. Two ml of this suspension were placed in 13x100 mm disposable culture tubes, and these tubes were placed in a water bath at 50°C. Samples to be assayed, from 20 to 100 µl, were brought to 1 ml

total volumes in Enzyme Assay Buffer.

At regular intervals the different assay samples were vigorously pipetted into separate tubes containing the Cibachrome Blue suspension. Immediately after this mixing, 1 ml of the mixture was withdrawn through a cotton filter into a syringe for the zero time sample. Again at five minutes 1 ml samples were withdrawn in the same way. A zero-time blank was also run using 2 ml of the Cibachrome Blue suspension to which 1 ml of Enzyme Assay Buffer had been added, and 1 ml of the mixture had been withdrawn like the assay samples.

All of the 1 ml fractions were spun 4 minutes in an Eppendorf centrifuge, the supernatants were drawn off and read for absorbance at 620 nm against the zero-time blank. The amylase activity value assigned to each sample was obtained by subtracting the zero time absorbance from the five-minute absorbance for each sample.

β-Glucanase Assay

β-Glucanase assay buffer: 3M sodium acetate buffer, pH 5.0

2.41 ml 4.15 mg/ml β-glucan, in water, and 41.7 µl 3M sodium acetate, pH 5.0 were mixed in a 13x100 mm test tube and equilibrated in a 30°C water bath. After ca. 15 minutes to achieve temperature equilibrium, 50 µl of the sample to be assayed, or $H_2O$, were added. (For more dilute

samples, the substrate concentration was increased to 4.24 mg/ml so that only 2.36 ml was needed to give a final substrate concentration of 4 mg/ml. This then allowed the use of 100 µl enzyme sample.) The mixture was vortexed and transferred to the viscometer for the initial viscosity measurement. After this initial reading, the mixture was left in the viscometer, the viscometer being kept in the 30°C water bath throughout the reaction time. Timings were done at consecutive intervals, of usually 10 or 15 minutes.

Azocasein Protease Assay

Azocasein assay incubation buffer:
0.15 M sodium acetate buffer, pH 4.2
2 mM EDTA
6 mM cysteine

The assay was preceded with a preincubation of at least 5 minutes at 40°C of 0.125 ml of the enzyme sample with 0.25 ml incubation buffer. The reaction was started by the addition of 0.125 ml 6% (w/v) azocasein, in $H_2O$, and was allowed to proceed for some set amount of time, usually 10, 20, or 30 minutes. At the end of this time interval, the reaction was stopped by the addition of 2.5 ml of 3% (w/v) trichloroacetic acid.

This mixture was then spun 5 minutes at 8,000 x g. The supernatant was drawn off with a Pasteur pipette and

was read for absorbance at 366 nm, against $H_2O$. One sample, the blank, was treated the same way, but the enzyme sample was replaced by $H_2O$.

Ribonuclease Assay

Tubes containing 50 µl 1.0 M Tris-HCl, pH 7.2, 50 µl 500 µg/ml herring sperm DNA, and 50 µl of the sample to be assayed for RNAse activity, were equilibrated ten minutes at 37°C.

The reaction was started by the addition of 5 µl of RNA labelled with tritiated uridine (containing ca. $6 \times 10^5$ counts/µl). After 30 minutes incubation, the reaction was stopped by the addition of 2.5 ml 10% trichloroacetic acid.

After cooling on ice for 15 minutes, the reaction mixture was filtered through a Millipore filter. This filter was then washed with three ca. 1.5 ml volumes of cold 10% TCA, which were also used to rinse the reaction tube, and two similar volumes of cold 95% ethanol. The filters were then dried, immersed in 3 ml scintillation cocktail, and counted.

Xylanase Assay

This assay was adapted from that described in a 1976 paper by Taiz and Honigman(19). For each sample to be assayed, 0.3 ml of p-nitrophenyl-β-D-xylopyranoside, the substrate, was equilibrated at 27°C, in a test tube rack in a shaker bath. The reactions were started by the addition of 0.3 ml of the enzyme sample.

After 60 minutes, the reactions were stopped by the addition of 0.3 ml 1 M $NH_4OH$/2mM EDTA. This base also allowed the development of the yellow color of the liberated p-nitrophenol. These mixtures were then read for absorbance at 400 nm against a sample treated in the same manner, but with water replacing the enzyme sample.

Carboxypeptidase Assay

| | |
|---|---|
| Assay buffer: | 50 mM sodium acetate, pH 5.2, 0.5 mM EDTA |
| Substrate: | 2mM Z-Phe-Ala in assay buffer |
| TNBS Reagent: | 3 volumes 5% sodium tetraborate to 1 volume 0.2% trinitrobenzenesulfonate(TNBS) |

The carboxypeptidase assay described by Mikola and Kohlemainen was followed exactly except that the volumes were all proportionately reduced(28). After the substrate solution had been equilibrated at 30°C, the assay reactions were started by the addition of 50 µl enzyme sample to 250 µl substrate. This mixture was incubated 100 minutes at

$30^{\circ}C$, the reaction being stopped by the addition of 0.5 ml TNBS reagent. Sixty minutes at $30^{\circ}C$ was allowed for color development, being concluded by the addition of 250 µl 1N acetic acid. Absorbance at 340 nm was read within 60 minutes of the addition of acetic acid against a blank using water as the enzyme sample.

## RESULTS AND DISCUSSION

### GA-Induced Release of Pre-existing Proteins

As previously stated, the intention of this research was to find enzyme activities induced by GA, and to associate these activities with gel electrophoresis bands. Therefore initial evaluations of the task were made by comparing the gel electrophoresis patterns of the incubation media from aleurones treated with GA, with those incubated without GA.

Comparing the patterns obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins secreted by barley aleurones incubated in the presence or absence of GA revealed more of what they shared in common than differences. (Figure 2) These gel patterns do provide clear evidence that GA causes many changes in the relative amounts of the secreted proteins. However, these gel patterns were not to be entirely explained by the simple induction by GA, or lack thereof, of protein synthesis and secretion.

In fact, much of the early research done for this paper was devoted to pursuing the isolation of two proteins which appeared to be the most abundant protein components found in the aleurone incubation medium, and of which at least one was consistently found at higher levels as a

result of the presence of GA.

Looking at the protein gel pattern of 24 h GA incubation medium (Fig 1), these two protein bands stand out very strongly. One appears just below the α-amylase band, (Lane 7 contains purified α-amylase.), at about 43 kDa, and the other is in the 27kDa range.

Both of these proteins were evidently present in the incubation before 18 h. (Fig 2) When incubations were done using medium containing $^{35}$S-methionine from 18 to 24 h, neither of these two proteins incorporated the radio-label. These same two proteins are present in the -GA medium, but clearly at lower levels than in the +GA medium. (Fig 2)

As discussed in the Introduction section, under GA-induced Release of Pre-existing Proteins, GA was indirectly responsible for the release of significant quantities of pre-existing, non-enzymic, proteins. Jacobsen and Knox had suggested that these proteins made up as much as 40% of the GA-induced protein(10). As was reported, and recommended, in a paper by Melcher and Varner, a 2 h pre-incubation of the aleurone layers, without GA, removed significant amounts of water-soluble endosperm proteins that were apparently bound to the aleurone cells(15). Awareness of these two reports allowed clearer discernment, and even some cleaning up, of the GA-induced enzymes.

Figure 1: SDS-PAGE Released Pre-Existing Proteins

20 ml incubation medium from aleurones incubated for 24 h in 1 μM GA were loaded onto a CM-cellulose column of volume 125 cubic centimeters, equilibrated with 50 mM MES, pH 6.0. After a 40 ml wash with the equilibration buffer, a salt gradient of 75 ml each of 0 M and 0.3 M NaCl, in the same buffer, was used to elute the proteins.

The fractions collected were read for absorbance at 280 nm. Numerous representative peak fractions were analyzed by SDS-PAGE. Fractions giving similar gel electrophoresis patterns were pooled, dialyzed against a dilute buffer, and were concentrated by lyophilization.

Lane 1 contains 0-24 h +GA incubation medium. Lane 3 is the pattern from a pooled sample of fractions eluted from the CM-cellulose at 0 to 0.05 M NaCl in the salt gradient. Lane 4: 0.05 to 0.07 M NaCl; Lane 5: 0.13 to 0.16 M NaCl.

Lane 7 contains purified α-amylase. The gel was stained with Coomassie Blue, as described in Methods.

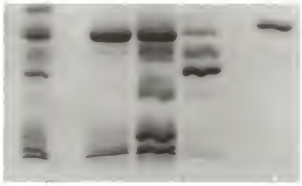
Figure 2: SDS-PAGE of Aleurone Incubation Media

50 μl samples of aleurone pre-wash medium and 0-18 h and 18-24 h, +GA and -GA, incubation media were TCA-precipitated and prepared for gel samples, as described in Methods.

Lane 4 contains purified α-amylase. The gel was stained with Coomassie Blue.

| Lane | Sample |
|---|---|
| 1 | 0-18 h -GA incubation medium |
| 2 | empty |
| 3 | 18-24 h -GA incubation medium |
| 4 | α-amylase |
| 5 | 18-24 h +GA incubation medium |
| 6 | 18-24 h +GA incubation medium |
| 7 | 0-18 h +GA incubation medium |
| 8 | Aleurone pre-wash medium |

FIG 1



1 3 4 5 7

FIG 2


1 2 3 4 5 6 7 8

## GA-Induced Enzyme Activities

All of these aleurone incubations were done in the presence of calcium, so that its effect was held constant throughout these experiments. The induction of α-amylase activity in the +GA, relative to the -GA, medium was typically used to monitor what appears to be an overall shift in seed function towards germination, GA playing an important role in this shift.

In the early period of this research, obtaining a significant difference in amylase activity between the +GA, and -GA, media was turning out to be a very difficult task. Many different modifications were employed to maximize this difference, but none of these modifications clearly and consistently reduced the apparent GA-induction in the aleurones incubated without GA. This dilemma was finally overcome by soaking the seeds on wet sand at $4^{o}C$, instead of at room temperature, or higher, as had previously had been done. All of the comparisons of other enzyme activities between +GA and -GA media were then made with media showing significant difference in α-amylase activity.

With the use of various synthetic and natural substrates, six different enzyme activities were detected in the GA incubation medium. All six of these enzyme activities were reported to be induced by GA, and this research confirmed that incubation with 1 μM GA increased

the enzyme activities in the incubation medium of a protease, xylopyranosidase, α-amylase, β-glucanase, and a carboxypeptidase. (See Table I.)

Table I: GA-Induced Enzyme Activities

| Incubation Medium | Protease Units/ ml | Amylase Units/ ml | Carboxy-peptidase Units/ml | Xylanase Units/ ml | β-Glucanase Units |
|---|---|---|---|---|---|
| 18 hr +GA | 2.552 | 26.50 | 52.6 | 14.25 | 29.1 |
| 18 hr -GA | 0.288 | 2.00 | 26.6 | 1.38 | 8.9 |
| 18-24 hr +GA | 1.096 | 52.35 | 51.2 | 11.80 | 27.8 |
| 18-24 hr -GA | 0.224 | 4.10 | 2.7 | 0.20 | 7.3 |

Protease Units: amount of enzyme giving difference in $A_{366}$ of 1.0 between: 15 min. reaction of enzyme sample with Azocasein substrate, and 15 min. reaction of assay buffer with substrate.

Amylase Units: amount of enzyme giving difference in $A_{620}$ of 1.0 between: 0 min . and 5 min. readings of enzyme sample with Cibachrome Blue substrate.

Carboxypeptidase Units: amount of enzyme giving difference in $A_{340}$ of 1.0 between: 60 min. reaction of enzyme sample with substrate and 60 min. reaction of assay buffer with substrate.

Xylanase Units: amount of enzyme giving difference in $A_{400}$ of 1.0 between: 60 min. reaction of enzyme sample with substrate and 60 min. reaction of assay buffer with substrate.

β-Glucanase Units: amount of enzyme giving a one minute decrease in flow-through time for β-glucan solution viscometric assays done at 1 and 20 min. after mixing of enzyme sample with substrate.

The protease and xylopyranisodase activities were found to be strongest in the 0-18 h medium. The α-amylase activity was higher in the 18-24 h medium than in the 0-18 h medium. The β-glucanase and carboxypeptidase activities were roughly equal in the 0-18 h and 18-24 h media. The ribonuclease activity was not detectable in the 0-18 h, or 18-24 h, media, but was significant in the 24-30 h medium.

The GA-induction of α-amylase activity is very significant. Perceptible, yet very weak, amylase activity was always found in the -GA aleurone medium but the +GA medium would range from five to thirty times higher in amylase activity. This wide variation resulted from slight differences obtained in the relatively low activity values of the different -GA media assayed. The +GA media were consistently strong in amylase activity.

Even more extreme than the amylase activity, the xylanase activity was found to be from about nine to one hundred times higher in the +GA, than the -GA, medium. Again this wide variation in calculated hormonal induction resulted from small variations in the very low activities in the -GA medium.

The assay for xylanase activity, adapted from one of the assays described by Taiz and Honigman, was designed to measure the activity of β-xylopyranosidase, an exoxylanase(19). The results of other assays that they used led them to conclude that this enzyme is the first of

the aleurone's xylanases to be found secreted into the incubation medium. Neither arabinofuranosidase nor endoxylanase are secreted to a significant extent until more than 24 h after the start of incubation with GA.

The β-glucanase activity, as measured by viscosity reduction of the β-glucan substrate, generally was two to three times higher in the +GA, than in the -GA, medium. Definite, significant activity was always seen in the -GA medium, but the activity in the +GA medium was always much higher.

Protease activity was also found to be consistently induced by GA. This induction of protease activity was generally eight to ten-fold.

The azocasein protease assay, used by Schwartz and Barrett, was designed for measuring proteolytic activity of cathepsin H, a mammalian lysosomal thiol protease(22). However, Rogers, Dean, and Heck reported 63% amino acid sequence identity between rat cathepsin H and the GA-induced barley aleurone protease, this identity holding strongly in the proposed active site regions(16). Therefore, this assay was chosen for use in locating and quantifying the barley aleurone protease activity.

Some standard proteases were run in this assay, in order to test its usefulness in detecting proteolytic activity. Papain, a plant thiol protease, in Tris buffer at pH 7.5, showed the most significant activity. Less, but

still significant, activity was seen with pepsin as the enzyme. Pepsin was assayed in glycine-HCl buffer at pH 1.5.

Medium from an 18 h GA incubation of barley aleurones was assayed in acetate buffer at pH 4.0, and protease activity was consistently seen. pH optima experiments were run with this 18 h GA incubation medium, and a pH optimum of 4.23 was found.

From a single incubation, 18 h and 18-24 h GA and Control, media were assayed for protease activity using the azocasein substrate and pH 4.23 sodium acetate buffer. Practically no protease activity was seen in the 18, or 18-24, h Control medium. Both of the GA media showed significant protease activity, again verifying GA-induction of enzyme activity.

In the few carboxypeptidase assays done, it appeared that the activity in the +GA medium was from ten to twenty times higher than that found in the -GA medium. These assays tended to give very high blank values, so that it was not conclusively determined whether or not the -GA medium was showing significant activity.

GA-induction of RNAse activity was not established because no assays were done on medium from aleurones incubated for 36 h in medium without GA. Enzyme assays were done with media from aleurones incubated with and without GA for 0-18 h and 18-24 h, and with GA for

24-36 h.

No significant RNAse activity was found in the 18-24 h media from aleurones incubated either with or without GA. However, between 24 and 30 h after GA addition, significant RNAse activity had been released into the incubation medium surrounding the aleurones that had been incubated with GA.

## Investigation of α-Amylase Loss

Throughout this research, incubation media, for which significant α-amylase activity and gel patterns had been previously seen, were appearing later without any measurable α-amylase activity or gel pattern. Numerous inquiries were made with little understanding gained. The preservation of other protein bands in the gel patterns of the GA medium serves as reason to doubt proteolytic degradation of α-amylase. This protein loss appeared to be specific for α-amylase.

Typically the incubation medium was treated in the following manner. After the 24 h incubation at 25°C the incubation medium was drawn off the aleurones with a Pasteur pipette and pooled. The pooled medium, which contained many suspended starch granules, was spun 15 minutes at 15,000 x g to remove this starch. Enzyme activity was retained beyond this centrifugation. The

supernatant from this spin was then stored at -20°C for later use.

Oftentimes, upon thawing, this incubation medium again contained some insoluble material at the bottom of the tube. Apparently the bulk of α-amylase was bound to this precipitate, because enzyme assays and gel electrophoresis showed very little α-amylase in the soluble portion.

After much trial and error it was found that significant α-amylase activity could be preserved if the medium was not centrifuged after the incubation until it was first kept on ice overnight. If the incubation medium was spun while it was still cold, most of the α-amylase remained in solution, although some was bound to the cold, insoluble starch grains. Although the solubility of starch granules would certainly be decreased at lower temperatures, apparently the affinity of α-amylase for these starch granules also decreases at the lower temperature. Therefore, the centrifugation of cold incubation medium left more of the α-amylase in solution.

Even these measures allowed the preservation of the α-amylase for only a little extra time. Typically after two to three weeks storage at -20°C, the thawed incubation medium contained still more insoluble, starch-like, material, and no amylase activity.

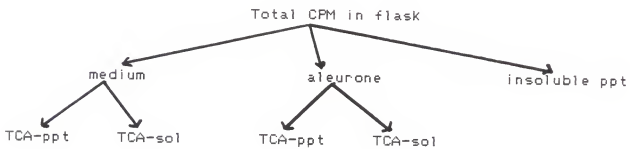
## Test of $^{35}$S-Met Incorporation in the 24-hr. GA Incubation

Desiring to obtain aleurone incubation medium with as little distracting protein material as possible, so as to enable the identification of proteins whose de novo syntheses were induced by GA, the medium was removed from the aleurones 18 h after the pre-wash. New medium was then added, this medium containing $^{35}$S-methionine. This method then allowed the specific detection of proteins that were synthesized between 18 and 24 h after the beginning of the incubation of the aleurones. It was already well-substantiated that this was a peak interval for the GA-induced synthesis of α-amylase, the most well-studied GA-induced enzyme.

Numerous tests, described in detail in the Methods section, were done to enable the proper evaluation of the extent of radiolabelling of proteins in the +GA medium versus the -GA medium. The results of these tests are given in Table II on the following page:

Table II: Test of $^{35}$S-Met Incorporation in the 24 h GA Incubation

| | +GA | | -GA | | +GA/-GA |
|---|---|---|---|---|---|
| | CPM | % of Total | CPM | % of Total | |
| TCA-pptable CPM in medium | 1,063,333 | 8.7 | 216,853 | 1.9 | 4.3 |
| TCA-sol. CPM in medium | 6,629,200 | 54.0 | 541,213 | 4.8 | 10.9 |
| Total sol. CPM in medium | 7,692,533 | 62.7 | 758,066 | 6.7 | 9.0 |
| TCA-pptable CPM in aleurones | 893,920 | 7.3 | 3,547,680 | 31.3 | 0.22 |
| TCA-soluble CPM in aleurones | 3,373,680 | 27.5 | 6,971,520 | 61.6 | 0.43 |
| Total CPM in aleurones | 4,267,600 | 34.8 | 10,519,200 | 92.9 | 0.36 |
| Insoluble CPM in medium | 313,082 | 2.6 | 44,550 | 0.4 | 7.0 |
| Total CPM | 12,273,215 | 100 | 11,321,816 | 100 | 1.1 |
| % of total TCA-pptable CPM found in medium | | 54.3 | | 5.8 | 9.4 |



Upon examination of the results of these tests, the following patterns can be noted:

1) The total counts in the GA medium were consistently about nine times as plentiful as those in the Control medium, while the free counts were eleven times higher in GA than Control. This can be explained by a higher amino acid pool in the GA aleurones as opposed to the Control aleurones. In discussing the incorporation of radio-labelled amino acids in isolated aleurones incubated with or without GA, Varner and Ram Chandra, in 1964, reported that GA-induced proteolytic activity caused the release of large quantities of free amino acids within the aleurone cells(21). A greater amino acid pool in the aleurones, resulting from GA-induced degradation of aleurone storage proteins, would make these cells less likely to take up the added methionine, thus more is left in the medium.

Melcher and Varner, in 1971, reported on the release of reserve protein from isolated barley aleurone layers, this release being at least partially dependent upon GA(15). Their molecular weight and amino-terminal amino acid analyses led them to the conclusion that the effect of GA upon the release of these reserve proteins occurred via the action of GA-induced proteases. These writers concluded that the bulk of the proteolysis responsible for the provision of amino acids to provide precursors for seedling

growth, as well as for hydrolase production within the aleurone cells, occurs outside the aleurone cells. Their evidence supported the hypothesis that reserve proteins are released, after some proteolysis, from the aleurone layers and are degraded to amino acids by the action of proteases also released from the aleurone cells. This report provides some information on the mechanisms that lead to the higher amino acid pool in the GA-treated aleurone layers, as well as to the dilution of the radio-labelled amino acids in the +GA incubation medium.

2) The total counts that were TCA-precipitable in the incubation medium was generally about 4 to 5 times higher for the GA than for the Control. Assuming the higher amino acid pool in the GA over Control, for which evidence has been shown, the GA-induction of the synthesis and release of proteins is certainly greater than 4 or 5.

3) The total counts found in the Control aleurones was generally about two and a half times higher than in the GA aleurones. Higher counts in the Control aleurones, as compared to the GA aleurones would certainly be expected from the data cited in observation 1.

4) The total TCA-precipitable counts retained in the aleurone cells was typically 4 times higher in the Control than GA. This may be interpreted as evidence for the GA-induction of protein secretion. However, the data cited in

observation 1, that the counts in the medium were ten times higher in GA vs. Control, certainly suggest a greater amino acid pool in the GA vs. Control aleurones. Therefore, it would be expected that the newly-synthesized proteins in the Control aleurones would be much more strongly radiolabelled.

The evidence and logical explanation for the increased amino acid pool in the +GA aleurones must be considered when evaluating the induction by GA of de novo synthesis of proteins. This expanded amino acid pool would cause a dilution of the radiolabel in the +GA medium proteins. Therefore any protein bands from the +GA medium that carry the radiolabel are prime candidates for GA-induction unless the radiolabel is much stronger in the same protein in the -GA medium.

5) After being frozen for three days after the incubation, the incubation medium from all six of the flasks that had contained the $^{35}$S-met were thawed and the three fractions of GA medium were pooled, as were the three fractions of Control medium. All this medium was then kept on ice for two hours before spinning at 6,000xg for 15 minutes. 1.0 ml of incubation buffer was added to both of the white, starch-like, pellets. These pellets and incubation buffer were incubated at 50$^{o}$C to see if the bound amylase would hydrolyze these pellets. No signs of hydrolysis were

evident after three hours reaction time. 100 µl of purified barley α-amylase was then added, and this buffer and pellet were incubated at 50°C. The pellets still did not appear to significantly decrease in size after another six hours reaction time. After the attempt to hydrolyze the white precipitate, the tubes were vortexed, and 100 µl of each of these mixtures were counted for both GA and Control.

These counts from 100 µl of the mixture would represent 1/11th (100/1100) of the total counts in the pellet, assuming all the counts were at least temporarily suspended by this treatment. Therefore, these counts would represent 3/11 of the total insoluble counts per flask:

```
Control:  12,150(11/3) =  44,550 CPM/flask
     GA:  85,386(11/3) = 313,082 CPM/flask
GA <=> 7*Control
```

These counts do not represent a very significant percentage of the total counts per flask, about 0.45% in Control and about 3% in GA, but this may well offer a clue as to where the α-amylase is lost. (See calculations on the following page.) It is worthy of noting that the GA counts were about seven times higher than the Control counts, as might be expected if these counts are from newly-synthesized α-amylase.

In the Control flasks, 34% of the counts were found in soluble protein. This 0.45% of the counts in insoluble

material, assumed to be α-amylase, leads to the conclusion that 1.3% of the newly-synthesized protein in the Control flasks is starch-bound α-amylase. Likewise, 15.8% of the newly-synthesized protein in the GA flasks is insoluble, starch-bound α-amylase.

Calculations:

Other $^{35}$S-met-labelled proteins:
Control = 34% GA = 16%

Control: .45/(34 + .45) x 100 = 1.3%
GA: 3/(16+3) x 100 = 15.8%

## CM-Cellulose Column Chromatography of GA and Control Incubation Media

The use of this 18-24 h medium, especially with the newly-synthesized proteins carrying a radioactive tag, allowed much clearer direction for the research. The radioactivity of the CM-Cellulose fractions was plotted to evaluate the resolution accomplished. The 18-24 h medium from incubations with, and without, 1 μM GA were passed through CM-cellulose equilibrated with 50 mM MES, pH 6.0. The pI's of the α-amylase isozymes had already been reported, most of them being below, or close to, 6. The prospect of the bulk of the α-amylase passing through the CM-cellulose unhindered, coming out in the flow-through fraction, offered much hope for the isolation of anything

that did stick to the CM-cellulose at pH 6. It had already been well-established that α-amylase was the major GA-induced enzyme, and its removal appeared to be a high priority in the isolation of any other GA-induced enzyme.

As can be seen in the CM-cellulose elution plots of both +GA and -GA incubation medium (Figure 3 & 4), distinct differences can be observed in the radioactivity patterns of the eluted fractions between the +GA and -GA medium.

Figure 3: Elution Profile of CM-Cellulose Chromatography of +GA Incubation Medium -- p. 57

Figure 4: Elution Profile of CM-Cellulose Chromatography of - GA Incubation Medium -- p. 58

The data for both Figures were obtained as described in Methods: CM-Cellulose Chromatography.

FIG 3

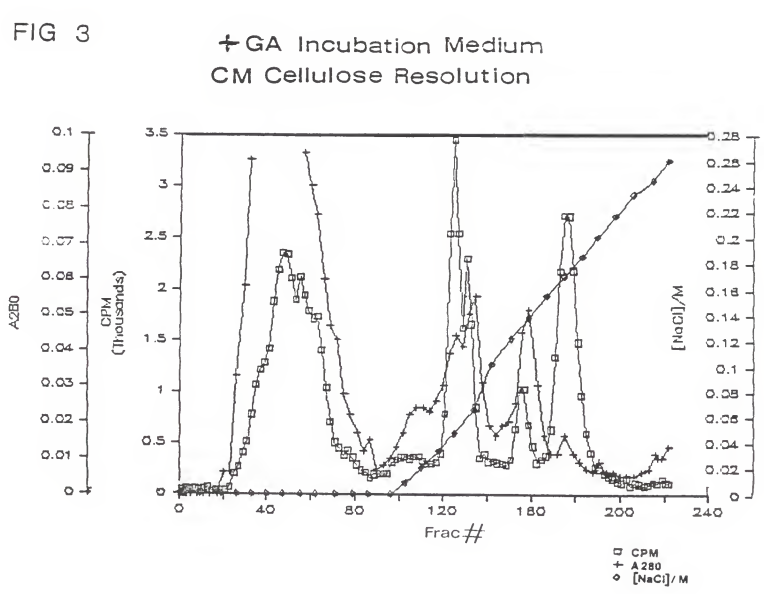

+GA Incubation Medium
CM Cellulose Resolution
A280
CPM (Thousands)
[NaCl]/M
Frac #
CPM
A 280
[NaCl]/M

FIG 4

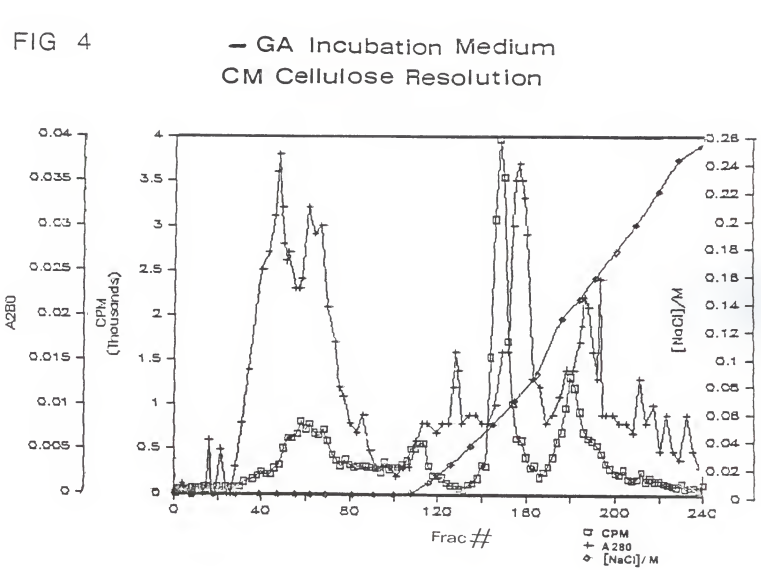

– GA Incubation Medium
CM Cellulose Resolution
A280
CPM (Thousands)
[NaCl]/M
Frac #
CPM
A280
[NaCl]/M

Figure 5a: SDS-PAGE of Radioactivity Peaks from the CM-Cellulose Resolution of +GA and -GA Incubation Media

Fractions from these peaks of eluted radioactivity were pooled, concentrated by TCA-precipitation (as described in Methods), and run on SDS-PAGE gels. The gel patterns of the gel in Figure 5a were obtained from 250 µl of the respective CM-cellulose pooled fractions. The gel was silver-stained as described in Methods. These gels, after being dried, were placed in contact with X-ray film for the location of the radio-labelled bands. Photographs of the SDS-PAGE pattern and autoradiogram of the CM-cellulose radioactive peaks are shown in Figures 5A and 5B, respectively.

| Lane | Sample |
|---|---|
| 1 | CM-Cell. (-GA med. Peak A) .050-.066 M NaCl |
| 2 | " (+GA med. Peak A) .042-.055 M NaCl |
| 3 | " (-GA med. Peak B) .068-.076 M NaCl |
| 4 | " (+GA med. Peak B) .056-.067 M NaCl |
| 5 | " ( " Peak C) .117-.136 M NaCl |
| 6 | " (-GA med. Peak C) .126-.144 M NaCl |
| 7 | " ( " Peak D) .146-.164 M NaCl |
| 8 | " (+GA med. Peak D) .156-.183 M NaCl |

FIG 5A

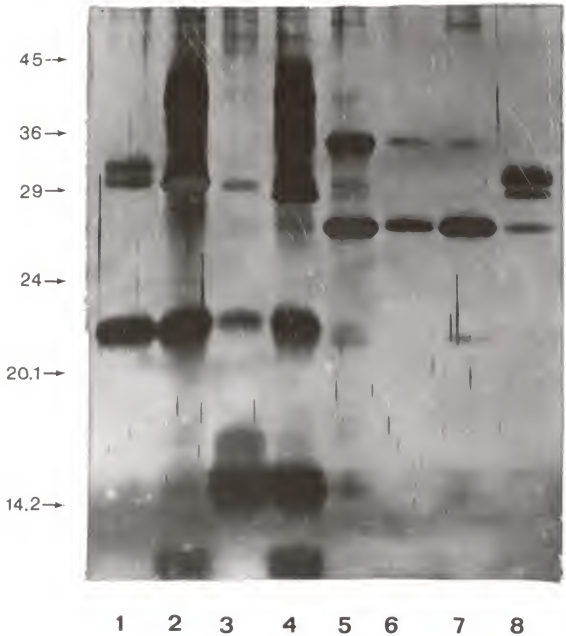


Figure 5b: Autoradiogram of Figure 5a

| Lane | Sample |
|---|---|
| 1 | CM-Cell. (-GA med. Peak A) .050-.066 M NaCl |
| 2 | " (+GA med. Peak A) .042-.055 M NaCl |
| 3 | " (-GA med. Peak B) .068-.076 M NaCl |
| 4 | " (+GA med. Peak B) .056-.067 M NaCl |
| 5 | " ( " Peak C) .117-.136 M NaCl |
| 6 | " (-GA med. Peak C) .126-.144 M NaCl |
| 7 | " ( " Peak D) .146-.164 M NaCl |
| 8 | " (+GA med. Peak D) .156-.183 M NaCl |

FIG 5B

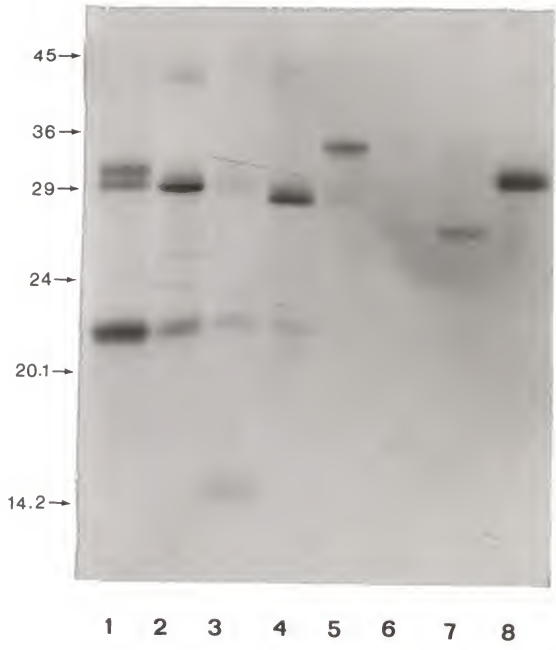


Table III gives a summary of the differences in the radiolabelled, "hot", and non-radiolabelled, "cold", protein gel bands from the CM-cellulose resolution of +GA, and -GA, incubation media.

Table III: Summary of SDS-PAGE Patterns of Radioactive Peak Fractions from CM-Cellulose Chromatography of +GA and -GA Incubation Media

| | | +GA | | -GA | |
|---|---|---|---|---|---|
| Peak | [NaCl] range | MW/kDa "hot" | MW/kDa "cold" | MW/kDa "hot" | MW/kDa "cold" |
| Flow-Through | 0 | 45.0*<br>33.5* | | 11.9#<br>17.7# | |
| A | 0-0.06 | 21.6=<br>31*<br>43* | 39 | 21.6=<br>32.6# | 31 |
| B | 0.06-.08 | 22=<br>30.4*<br>44-45* | | 22=<br>31.5# | 31<br>36<br>40 |
| C | 0.12-.15 | 35* | 28 | 28# | 35 |
| D | 0.15-.18 | 32*<br>62* | 30<br>28 | 28# | 35 |

* indicates that the marked radiolabelled protein is stronger from the +GA medium.

# indicates that the marked radiolabelled protein is stronger from the -GA medium.

= indicates that the marked radiolabelled protein is of equal intensity from the +GA, and -GA, media.

The first noticeable difference in the CM-cellulose patterns for +GA, and -GA, incubation medium is seen in the flow-through and 0 M NaCl wash. This peak is almost three times as high for the +GA medium versus the -GA medium.

The gel patterns from these flow-through fractions did not give a lot of information. In general these pooled flow-through fractions were relatively large, and thus any individual proteins in these fractions were greatly diluted. This dilution made difficult the detection of protein bands, as well as enzyme activities. However, two faint radiolabelled bands from the +GA medium were found, being distinctly different from the two, also faint, radiolabelled bands from the -GA medium. The two +GA proteins were of sizes 45.0 (probably α-amylase), and 33.5 kDa. The proteins unique to the -GA medium were of much smaller size, 11.9 and 17.7 kDa.

Beyond the flow-through fractions, four different consistently-appearing peaks of radioactivity will be described. (See Figures 3 & 4) The first, referred to as peak A, eluting at 0.05 M NaCl, is generally of nearly equal size in +GA versus -GA medium. (See Figures 5 a & b) The SDS-PAGE and autoradiogram of the peak A fractions (lanes 1 & 2) from -GA and +GA, showed a 32.6 kDa radiolabelled protein only from the -GA medium, a 43.0 kDa radiolabelled protein only from the +GA medium, and radiolabelled bands at 31 kDa stronger from +GA medium and

21.6 kDa about equal in intensity between +GA and -GA. Also seen is a strong, not radiolabelled 39 kDa band distinct to +GA peak A. No enzyme activity was detected from any of the peak A fractions.

In these comparisons of intensity of radiolabelled bands, one conclusion discussed in the Test of $^{35}S$-methionine Incorporation in the 24 h GA Incubation Medium should be noted. Because of the greatly increased amino acid pool in the +GA-, versus -GA-, treated aleurones, the $^{35}S$-methionine would be diluted in the GA-treated aleurones. Likewise the radiolabel of newly-synthesized proteins would also be diluted. Therefore protein bands found from both +GA and -GA medium, of equal radiolabelled intensity, might still be induced in their synthesis and release, by GA.

The next radioactive peak, peak B, eluting at about 0.06 M NaCl, appears as a shoulder of peak A in the CM-cellulose run of +GA incubation medium. In the CM-cellulose run of -GA medium peak B is either absent or overshadowed by peak A. Many different protein bands, with and without the radiolabel, are seen in peak B fractions from both +GA and -GA medium. (Figures 5 a & b) One particularly noticeable difference is a 30.4 kDa radiolabelled band that is unique to the peak B of the +GA medium. Enzyme assays of the peak B fractions revealed $\beta$-glucanase activity in the +GA peak B, without any such

activity in the fractions of corresponding NaCl concentration of the CM-Cellulose run of -GA medium.

The next peak of radioactivity, peak C, eluting at about 0.125 M NaCl, is of comparable peak heights from +GA and -GA medium. The SDS-PAGE protein-staining and autoradiogram patterns of the +GA and -GA peak C fractions show that these two peaks of comparable size have significant differences. (Figures 5 a & b) The +GA sample contains a 35 kDa protein that stains very strongly, and also contains the radiolabel. The -GA peak C contains a protein of similar, or slightly larger, size that is not not radiolabelled. The radioactivity of the -GA peak C seems to be due to a radiolabelled protein of about 28 kDa. The +GA peak C contains a protein of similar size, that appears to be of much greater quantity than the -GA 28 kDa protein, but the +GA protein is not radiolabelled. Other, much fainter, non-radiolabelled, bands are seen from the +GA peak C sample. This GA-induced, newly-synthesized, 35 kDa protein is very inviting for further investigation, but no enzyme activity could be assigned to this newly-synthesized, GA-induced, protein.

The final radioactive peak, peak D, eluting at about 0.17 M NaCl, is the most outstanding difference between the +GA and -GA elution plots. (Figures 5 a & b) This peak of radioactivity was invariably strong in the CM-cellulose run of the +GA medium, while it was usually very small, or non-

existent, in the CM-cellulose run of the -GA medium. Like peak C, both the +GA and -GA samples contain 28 kDa proteins, and like peak C, only the -GA sample's 28 kDa protein is radiolabelled. The +GA sample contains a 32 kDa protein that showed up very strongly in both the silver-staining and the autoradiogram. The corresponding -GA sample does not have this protein. The +GA sample also has a fainter, non-radiolabelled, 30 kDa protein not found in the -GA sample. This pooled peak D was found to have strong β-glucanase activity, this activity not being detected at the corresponding NaCl concentration in the CM-Cellulose run of the control medium.

After the radioactive peaks eluted from the CM-cellulose in the NaCl gradient had been thoroughly assayed, and only β-glucanase activities were found, it was decided to further investigate the flow-through and 0 M NaCl fractions. Dialysis of the large pooled flow-through fraction against a low ionic strength buffer, followed by freeze-drying and redissolving of the dried protein in a smaller volume of water, provided a sample concentrated 16-fold. Assays of this sample showed xylanase activity, but no other activities.

Since both α-amylase and protease activities had proven difficult to preserve, it was decided to conduct a quick small-column ion-exchange run of the 18-24 h +GA incubation medium. EDTA and DTT were also added to

prevent degradation and oxidation of the enzymes.

Two ml of 18-24 h +GA incubation medium were loaded onto a 1 $cm^3$ CM-cellulose column. 3.0 ml each of 50 mM MES, pH 6.0, 1mM EDTA, 2mM DTT (equilibration buffer) with 0, 0.05, 0.10, 0.15, 0.20, and 0.50 M NaCl were used to elute the proteins in a stepwise gradient. Two fractions were collected at each salt concentration.

Below is shown the results of the enzyme assays done on these fractions:

Table IV: Enzyme Activities in CM-Cellulose Flow-Through

| | Protease Units/ ml | Xylanase Units/ ml | Amylase Units/ ml | Carboxy-peptidase Units/ ml |
|---|---|---|---|---|
| 18-24 h GA Inc. Medium | 1.10 | 7.595 | 22.5 | 51.2 |
| Flow-Through | 0.73 | 0.320 | 1.8 | 32.2 |
| 0 M NaCl (1) | 0.30 | 0.350 | 9.7 | 38.8 |
| 0 M NaCl (2) | 0.24 | 7.920 | 10.7 | 17.5 |
| .05 M NaCl (1) | 0.32 | 0.160 | 5.2 | 15.4 |

The enzyme activity units are the same as described in the legend for Table I.

None of these enzyme activities were found to any significant degree in any of the fractions eluted at higher salt concentrations. Since these enzymes would not bind to CM-cellulose at pH 6, it may be expected that they would bind to DEAE-cellulose at this same pH. The use of the anion-exchanger column could then allow the separation of these different activities, and their association with gel protein bands, giving some information on size and GA-induction. This approach offers much for further valuable study of GA-induced enzymes.

## β-Glucanase

As outlined in the introduction of this paper, the aim of this research was to purify GA-induced enzyme activities, establishing GA-induction, and associating these activities with electrophoretically-separated protein bands. The CM-cellulose resolution of the +GA and -GA incubation media, although most of the enzyme activities passed through unbound at pH 6, revealed two separate peaks of β-glucanase activity, both being strong in the resolution of the +GA medium, and absent in the resolution of the -GA medium.

These two β-glucanase isozymes, synthesized and secreted by the barley aleurone layers during incubation

with GA, are unique in their binding to CM-cellulose. These β-glucanase activities were the only hydrolase activities found in the +GA incubation medium that bound to CM-Cellulose at pH 6.0.

Certainly the results of these resolutions verify the GA-induction of two different, (at least varying somewhat in charge distribution), β-glucanases in the aleurone incubation medium. Woodward and Fincher, in 1982, reported two different β-glucanases arising from germinating barley of the Clipper variety of Hordeum Vulgare. These β-glucanases were reported to be 28 and 33 kDa in size (27). In 1986, Stuart, Loi, and Fincher reported three different β-glucanases arising from germinating barley(26). They concluded from their studies that one of these isozymes was unique to the aleurone layer, one was unique to the scutellum, and the third isozyme was produced by both regions of the barley seed.

The reports up to this point had indicated that the release, but not the synthesis, of β-glucanase is induced by GA. However, tests done by Stuart, et al, with polyclonal antibodies provided evidence that GA, with $Ca^{2+}$, causes a significant increase in both the de novo synthesis and release of β-glucanase(26). Calcium ions appeared to be necessary, more than was GA, for these increases in β-glucanase activity.

They specified that their 30 kDa isoenzyme was mostly

unique to the aleurone, as opposed to scutellar, secretions and homogenates. As mentioned in the previous section, the most noticeable difference between the SDS-PAGE patterns of the +GA and -GA CM-cellulose peak B regions was a 30.4 kDa newly-synthesized protein. This protein may well be the 30 kDa β-glucanase described by Stuart, et al. This peak B sample contains only a very weak, non-radiolabelled band at 28 kDa, and nothing at 32 kDa. (See Figures 5 a & b)

Like peak B, the pooled fraction of peak D contains strong β-glucanase activity that is not found in the corresponding -GA sample. Several attempts were made to further purify this peak D fraction, but no success was achieved. These attempts included a cation-exchanger-HPLC column, DEAE-cellulose, and DEAE-sephadex. The failure to remove the minor 28 and 30 kDa protein impurities from the 32 kDa, radiolabelled, protein makes impossible the indisputable association of any enzyme activity with a specific protein band. (See Figures 5 a & b) However, several lines of reasoning will be offered to support the suggestion that this 32 kDa protein is β-glucanase.

First, and most obvious, is that this peak D contains strong β-glucanase activity, and the 32 kDa protein is by far the major protein in the sample. In some of the gel runs of this peak D sample, only the 32 kDa band could be seen after the gel was stained with Coomassie Blue. Only

upon further silver-staining could the smaller, less-abundant, proteins be seen.

In the 1982 paper by James Woodward and Geoffrey Fincher the purification and characterization of two different β-glucanase enzymes from germinating barley are described(27). Their Enzyme I has a molecular weight of 28 kDa and a pI of 8.5, while Enzyme II has a molecular weight of 33 kDa and a pI >10. The specific site, within the barley kernel, of the production of these enzymes is not discussed in this paper. However, the well-evidenced role of the aleurone layer as an important source of enzymes responsible for endosperm degradation upon germination makes the suggestion that these β-glucanases are produced in the aleurone layers at least reasonable. In fact, the earlier-mentioned paper by Stuart, et al offers evidence that two, or maybe three, different β-glucanases are produced in the aleurone layer(26).

In this 1982 paper by Woodward and Fincher, the purification schemes for these two β-glucanases are outlined, showing sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns for the results of each purification step(27). After several steps of purification, the gel pattern obtained very much resembles the gel pattern of the CM-cellulose peak D fraction, containing a very strong band at 32-33 kDa, with a faint band above this, and two faint bands below it, possibly at

28 and 30 kDa. It is not obvious from the photograph, but the authors acknowledge that even with further purification steps, these impurities were not removed.

If the barley aleurones do produce two β-glucanases, of pI's 8.5 and >10, it would be expected that both would bind to CM-cellulose at pH 6.0, and that they may well be eluted at different stages in the salt gradient. As already described, β-glucanase activity was found from two different CM-cellulose peaks. It could then follow that the peak B β-glucanase is the pI 8.5 enzyme, while the peak D β-glucanase is the pI >10 enzyme, with a molecular weight of 33 kDa. In the attempts to purify the β-glucanase from this peak D fraction, it was found that the β-glucanase activity would not bind to DEAE-cellulose, even at pH 9.0.

In the absence of any evidence to the contrary, the conclusion that the 32 kDa protein from peak D is β-glucanase could be accepted without much reservation. However, as mentioned in the discussion of peak B, the reports of the effect of GA influence on β-glucanase activity in the medium surrounding aleurone layers have mainly focused on its effect on enzyme release. In a 1972 paper by Bennett and Chrispeels, it was reported that the de novo synthesis of β-glucanase occurred, without the influence of GA, during the imbibing of water by the isolated barley aleurone layers(9). In fact, the results of their density-labelling experiments suggested that the

significant majority of the β-glucanase synthesis occurred during the three days of being soaked on wet sand, while no measurable increase in β-glucanase density was found if the aleurones were incubated in the presence of 80% $D_2O$.

However, as mentioned previously, the 1986 paper by Stuart, Loi, and Fincher, does offer evidence for the GA-induction of β-glucanase synthesis(26). In this paper the authors use antibodies raised against β-glucanases for the identification of this enzyme in electrophoresis gels.

As noted previously, the bulk of the work done for both the 1982 and 1986 papers were done using the Clipper variety of H. Vulgare(26,27). All of the research described in this thesis verifies the similar GA-induction of β-glucanase synthesis in the Himalayan variety of H. Vulgare.

In summary, the evidence provided from this research, evaluated in conjunction with the previously-reported information of β-glucanases, could be reasonably interpreted as support of the conclusion that GA, in the presence of calcium, is very much responsible for the induction of β-glucanase synthesis and secretion. It should be reemphasized that the CM-Cellulose profiles of the +GA Peak B and Peak D β-glucanase activities show no corresponding radioactive peak or β-glucanase activity in the corresponding regions of the NaCl gradient used to elute the bound proteins.

As mentioned previously, the use of CM-Cellulose at pH 6 for the resolving of the β-glucanase isoenzymes ideally separated these enzymes from the other GA-induced hydrolases.

Below is shown a table summarizing the purification of the β-glucanase isoenzymes by CM-cellulose chromatography. All of the flow-through times were obtained with 50 µl volumes of the respective enzyme samples, in the β-glucanase assay as described in the Methods section.

Table V: β-Glucanase Purification by CM-Cellulose Chromatography

| Sample | Decrease in Flow-through time/sec | [Protein] (µg/ml) | U / mg protein | Fold Purif. |
|---|---|---|---|---|
| 18-24 h +GA inc. medium | 83.50 | 2006 | 13.9 | --- |
| CM-cellulose (-GA Peak B) | 0 | 450 | 0 | 0 |
| CM-cellulose (+GA Peak B) | 69.08 | 500 | 46.1 | 3.3 |
| CM-cellulose (+GA Peak D) | 64.67 | 55 | 391.9 | 28.2 |

1 Unit activity = amount of enzyme that gives a one minute decrease in flow-through time between 1 20 min reaction.

$$U/mg\ protein = \frac{U}{(\#mg/ml\ protein)(.05\ ml)}$$

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# ANALYSIS OF GA-INDUCED ENZYMES OTHER THAN α-AMYLASE FROM BARLEY ALEURONES

by

TIMOTHY VERSCHELDEN

B.E., Kansas State University, 1982

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Graduate Biochemistry Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1986

Assays of the media containing proteins secreted from aleurone layers isolated from Himalaya barley, incubated with, or without, the plant hormone gibberellic acid (GA) confirmed the previously-reported induction of five different enzyme activities. Comparison of the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) patterns revealed much more similarity than difference between the +GA, and -GA, incubation media.

Addition of radiolabelled amino acids to the incubation media 18 h after the start of the incubation allowed the focusing on the newly-synthesized proteins. The relatively abundant proteins that were common to both the +GA, and -GA, media did not incorporate the radiolabel, and thus their abundance in the incubation medium is at least not directly caused by GA.

Chromatographic fractionation by carboxymethylcellulose, of the proteins secreted by the barley aleurones, revealed some distinct differences between the newly-synthesized proteins of the +GA, and -GA, incubation media. The radioactivity peak in the flow-through fraction of the +GA medium was much higher than that of the -GA medium. This flow-through fraction from the +GA medium contained the GA-induced activities of α-amylase, carboxypeptidase, protease, and xylopyranosidase.

Of the proteins eluted from the CM-cellulose in the salt gradient, two radioactive peaks were distinct to the +GA medium. Both of these peaks contained β-glucanase activity, and in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) both showed strong radio-labelled bands migrating in the size range of previously-reported β-glucanase isoenzymes, of 30.4 and 32.0 kDa. These previously-reported β-glucanase isoenzymes had been isolated from the Clipper cultivar of H. Vulgare.