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During barley germination, α-amylase and other hydrolases are synthesized in the aleurone layer and secreted into the starchy endosperm. As starch is degraded by amylases, lysophospholipids are released from inclusion complexes with the starch and undergo hydrolysis by a lysophospholipase (LPL) activity.

Studies with embryo-free half seeds and isolated aleurone layers from barley (Hordeum vulgare L. cv Himalaya) show that LPL activity appears in the aleurone layer of imbibed half-seeds. Secretion, on the other hand, requires the continued presence of gibberellic acid (GA_3) and commences after a 10 to 14 hour lag period. Ca^{2+} alone has very little effect on the level of LPL activity in the aleurone layer or that secreted into the surrounding medium. However, 50 mM Ca^{2+} together with GA_3 dramatically increases the total activity. Density labeling experiments with deuterium oxide indicates that GA_3-stimulated LPL is synthesized de novo.

Two forms of LPL activity are distinguished by ion exchange chromatography on carboxymethyl cellulose. A predominantly basic activity is observed in the starchy endosperm of germinated barley and the incubation medium of GA_3-stimulated half-seeds. In contrast a predominantly acidic activity appears in the incubation medium of GA_3-treated aleurone layers. The predominantly acidic activity from the medium of GA_3-stimulated aleurone layers is converted to the basic form in the presence of starchy endosperm, amylase-digests of barley starch or EDTA. The factor from starch is thermally stable. Divalent metal ions do not shift the basic activity to the acidic form. On gel filtration chromatography the acidic LPL elutes at a high molecular weight position (M_r ~ 160000) distinct from the basic activity (M_r 40000). The elution volume of the acidic activity treated with EDTA shifts to that of the basic activity.

Anti-serum prepared against a purified basic LPL generates similar enzyme inactivation profiles against extracts of both the predominantly basic and acidic LPLs.

Characterization of Different Forms of Lysophospholipase in Barley

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CHARACTERIZATION OF DIFFERENT FORMS OF LYSOPHOSPHOLIPASE IN BARLEY

CHAPTER I. INTRODUCTION

Early History of Barley Research

Dating back to the early 1800's the barley seed has been the focus of extensive research. Much of the emphasis on barley is due largely to mankind's preoccupation with alcoholic beverages so that the brewing industry is linked to many significant findings. Also barley has provided an important focus for studies on the physiology of seed germination. In particular, the elucidation of the mechanism of action of the plant hormone, gibberellic acid (GA_3), has relied on the barley aleurone experimental system.

Early investigations used visual observation to study the process of starch breakdown. In 1866 Tangl (1) and later in 1890 Brown and Morris (2) noted that the scutellar region adjacent to the embryo was the initial site of starch breakdown during germination. In contrast Haberlandt (3) considered the aleurone layer to be the most important source of amylolytic action. Clearly, the embryo end of the cereal grain is the initial site of starch degradation (4,8,25), but the relative role of the scutellum and aleurone remains controversial. Recent evidence suggests that in rice the scutellum is more important (4,25) while in barley the aleurone layer appears to contribute more α-amylase (4,5,6).

Barley Structure

Barley is a cereal belonging to the taxonomic family Gramineae and genus Hordeum (7). It is closely allied with other cereals such as oats, wheat, & rye and less so to corn, rice and sorghum. The barley seed is enclosed by a persistent husk which is absent in naked varieties such as Himalaya, or that is easily removed from wheat and rye upon threshing. Covering the entire seed is two layers of dead, compressed cells, the testa and pericarp (Fig. I-1). These tissues have no function during germination. At the base of the grain is the embryo which gives rise to the shoots and roots upon germination. Separating the embryo from starchy endosperm the scutellum acts as an absorptive tissue during germination, but may also secrete certain proteins as in rice (25). The endosperm is made up of two tissues which are important during germination. The starchy endosperm is a dead tissue consisting mainly of starch grains and protein that serve as the storage reserves for the growing embryo during germination. The outermost structure surrounding the starchy endosperm, the aleurone layer, is a living, respiring tissue, 3-5 cells thick, but does not grow or divide. The germination of the grain is initiated by the uptake of water and synthesis of the group of plant hormones known as gibberellins (GA). Once in contact with the aleurone layer GA causes a dramatic series of events leading to the secretion of α-amylase (8,9), lysophospholipase (LPL) (10,11) and many other hydrolases (8,9) into the starchy endosperm resulting in digestion of

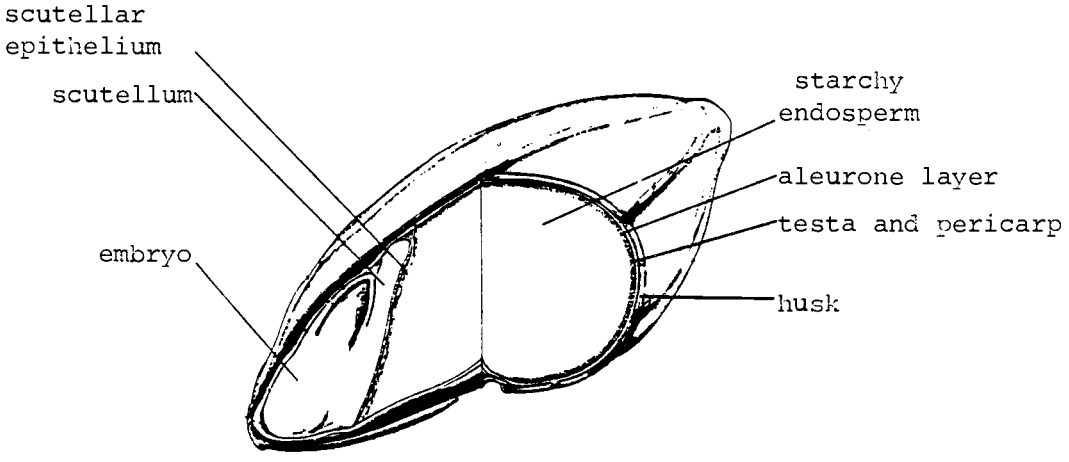

Fig. I-1 Diagramatic representation of a barley seed.

storage reserves.

Gibberellins

Over fifty types of gibberellins have been identified, all of which have a characteristic 4-membered ring ent-gibberellane structure (Fig. I-2). Gibberellic acid (GA_3) is a very active, commercially available gibberellin used in the classical studies of plant hormone action in the barley aleurone layer. The long-standing interpretation of GA appearance involves synthesis in the embryo axis and scutellum, diffusion through the starchy endosperm and stimulation of the aleurone tissue (8,9). More recently, Atzorn and Weiler (12) have shown that a factor emanating from the embryo is capable of stimulating the synthesis of a gibberellin (GA_4) in the aleurone tissue. They suggested that the production of α-amylase requires the de novo synthesis of GA_4 in the aleurone layer. However, even more recently, contradictory results have been observed by Gilmour and MacMillan (13). Using the same barley cultivar, Himalaya, they could not detect GA_4 in 3-day germinated barley and, furthermore, GA biosynthesis inhibitors did not prevent α-amylase formation by the aleurone layer. The inconsistency of the two research groups may be due to variability in the same cultivar as well as in batches of grain (13). These recent investigations on the precise nature of GA biosynthesis, however, do not change the interpretation of the GA_3 activation of hydrolases in aleurone layers.

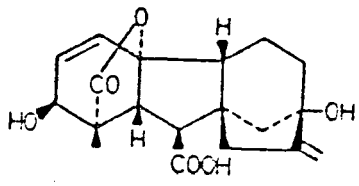

Fig. I-2 Structure of gibberellic acid (GA_3).

Whatever the precise mechanism for the appearance of gibberellins, once in contact with the aleurone tissue, transcription and translation events are initiated which result in the production and release of a variety of hydrolases to the starchy endosperm.

Starch and Starch-lipid

The degradation of starch during germination relies on two different types of amylases. α-Amylase hydrolyzes α-1,4-glucosidic linkages between glucose residues of the linear amylose and branched chain amylopectin components of starch. Its synthesis in the aleurone layer and secretion to the starchy endosperm requires GA_3 (14). On the other hand, β-amylase cleaves successive maltose units from the non-reducing end of either amylose or amylopectin and occurs as a latent form in the starchy endosperm (14,15).

The cereal starches, are unique among plant starches in that they contain about 1% (w/w) of monoacyl lipid in association with the amylose component of starch (16,17). The presence of high concentrations of lysophospholipids in nature is unusual because of their cytolytic and membrane perturbing properties (18). The acyl chain of lysophosphatidylcholine (LPC), the predominant monoacyl starch-lipid, forms a very stable association with the hydrophobic core of the amylose helix (16) (Fig. I-3). The tightly bound starch-lipids cannot be removed by the usual organic solvents but require repeated extraction with hot water-saturated butanol (19). Studies

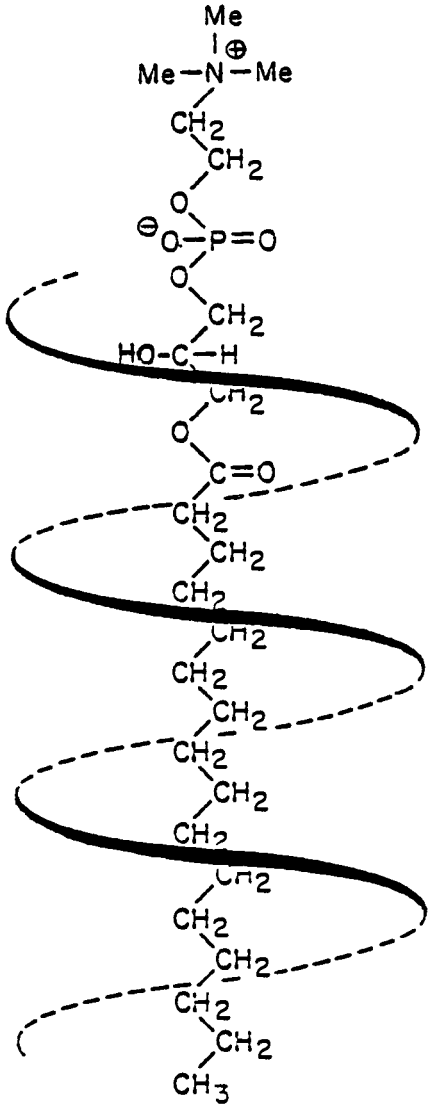

Fig. I-3 Representation of an inclusion complex of lysophosphatidylcholine in an amylose helix (22).

examining the susceptibility of the included lipid to enzymatic attack by phospholipases (16) together with X-ray diffraction data (20) reveals the lipid polar head-group to be exposed.

The biogenesis and function of starch-bound lipids is not known (16,17). The formation of starch-lipid during development could occur by either acylation of glycerylphosphorylcholine or deacylation of phosphatidylcholine (16). During starch synthesis the lipid may promote helix formation of amylose chains or regulate the ratio of amylose to amylopectin (21). The protruding lipid polar head-groups also may provide binding sites for proteins (17).

During germination of barley, amylases digest the starch thus releasing the included lysophospholipids (22). The lipids do not accumulate however, due to the appearance of a lysophospholipase (LPL) activity (22,23). As with other hydrolases (24,25) this activity first appears in the embryo half of the germinating seed (26) and is stimulated by GA_3 (11,26). Although the majority of the lysophospholipids released by amylolysis are degraded, a residual level remaining in the seed may be sufficient to interact with the aleurone cell membrane and thereby promote the secretion of hydrolases (11,17).

Early Response of Cereal Aleurone Layers to GA_3

A consistently recognized feature of the response of aleurone layers to GA_3 is a characteristic lag phase between GA_3 contact of the

aleurone and the formation and secretion of hydrolases. The lag period is typically 8-12 hours for most hydrolases including α-amylase (8), protease (8), and LPL (11) but may be as short as 4 hours with β-1,3-glucanase (27) or up to 20 hours in the case of ribonuclease (28). The biochemical changes occurring during this lag period have been the subject of extensive study. Electron microscope morphological studies of hormone-treated aleurones (29-32) indicate a proliferation of rough endoplasmic reticulum (RER), and development of intracellular membrane systems. Also observed is the enhanced formation of membrane-bound polysomes soon after GA_3 treatment yet before the appearance of hydrolases (33). Although the specific requirement of GA_3 in the induction of some membrane systems has been disputed (34) the overall picture involves induction of ribosomes, membrane synthesis and RER proliferation, and enhancement of polysome formation and protein synthesis.

GA_3 and Lipid Metabolism

Studies of lipid metabolism in aleurone layers during the lag phase after exposure to GA_3 are consistent with the ultrastructural work showing proliferation of endoplasmic reticulum (ER). Varner and colleagues have described the enhanced incorporation of radioactive choline (35,36) and orthophosphate (37) into phospholipids of membrane fractions from GA_3-stimulated aleurone layers. Another early effect of GA_3 on the lipid metabolism of aleurone tissue is the increased

activity of two enzymes participating in phospholipid biosynthesis, phosphorylcholine cytidyl transferase and phophorylcholine glyceride transferase. However, other evidence suggests that GA_3 may not be required for changes in phospholipid metabolism. For example, Koehler & Varner (37) found that GA_3 did not increase the incorporation of radioactive acetate into membrane lipids of barley aleurone. Also, Firn and Kende (38) were unable to show any GA_3-stimulation of lipid synthesis. Working with wheat aleurone Laidman and colleagues (34) found that phospholipid metabolism occurs independently of the presence of GA_3. However, wheat aleurone studies are complicated by the involvement of unidentified factors from the embryo and starchy endosperm which profoundly affect lipid metabolism. The suggestion by Jelsema and colleagues (39) that wheat membrane phospholipid synthesis occurs from storage lipids in spherosomes also contributes to the puzzle of lipid metabolism during early stages of cereal seed germination (34).

Taking into account the data from morphological studies with electron microscopy and from lipid metabolism the most significant early events of the GA_3 response are membrane formation and membrane-bound protein synthesis.

GA_3 and Gene Expression

In parallel with the morphological and phospholipid studies has been the investigation of GA_3 action on gene expression. Early

studies on the mechanism of action of GA_3 in aleurone tissue by Varner and colleagues (40,41,42) showed that protein synthesis was required for the appearance of α-amylase activity. They used radioactive amino acids and the translation inhibition cycloheximide. The de novo synthesis of α-amylase (43) and other hydrolases (8,11) including lysophospholipase (11) has been definitively demonstrated using an elegant technique worked out by Varner (43). By imbibing the aleurone tissue in $H_2{}^{18}O$ the amino acids resulting from hydrolysis of reserve proteins incorporate ^{18}O, and consequently de novo synthesized proteins become density labeled. The small density difference of a $H_2{}^{18}O$ labeled protein versus the $H_2{}^{16}O$ labeled protein can be detected with equilibrium centrifugation on cesium chloride gradients. A similar density labeling occurs with D_2O (44).

The first indication for mRNA involvement in the formation of α-amylase and other hydrolases was with the use of transcription inhibitors such as actinomycin D, cordycepin and 5-bromouracil (8,9). Independent researchers (45,46) have found a GA_3-dependent increase in poly(A)-mRNA. Direct evidence for GA_3-induced mRNA specific for α-amylase was shown by Higgins and colleagues (47). Using a cell-free translation system they showed that the level of mRNA from GA_3-stimulated aleurone layers increased in parallel with α-amylase activity in vivo.

It has long been recognized that two groups of α-amylase isozymes exist, one with pI 4.6-5.2 (Group A containing isozymes 1 and 2) and another with pI 5.9-6.6 (Group B containing isozymes 3 and 4).

Jacobsen and associates found that the two groups generate different peptide maps (48) and that the loci of the two isozyme groups are located on different chromosomes (49). Recently, Rogers and his colleagues (50,51,52) have applied molecular biology techniques of cloning and hybridization probes to examine the regulation of barley aleurone α-amylase genes by GA_3. They have found a total of about seven α-amylase genes or pseudogenes and the two pI groups of isozymes are regulated differently by GA_3. There exist two major mRNA species in aleurone tissue which corresponds to the low pI α-amylase group and which increase coordinately with GA_3. The mRNA giving the low pI α-amylase group occurs in the aleurone cells in the absence of GA_3 and increases 20-fold with GA_3. The high pI isozyme group shows one major mRNA species which is present in very small levels in unstimulated aleurone layers but increases about 100-fold after GA_3 treatment.

The general observation of a large increase in mRNA coincident with α-amylase activity strongly suggests that GA_3 acts to increase mRNA synthesis. However, alternative explanations could be a decreased rate of mRNA degradation or activation of latent mRNA (47). As noted by Rogers (52), definitive proof that GA_3 acts at the transcriptional level will now be possible with runoff transcription experiments which directly measure DNA template activity.

Cereal Hydrolase Processing and Secretion

Another aspect of the GA_3-stimulation of cereal aleurone layers

which has received much attention is the mechanism by which α-amylase and other hydrolases are secreted. In particular, in the last few years research has provided a clearer understanding of the secretion process in barley.

As mentioned previously the endoplasmic reticulum (ER) proliferates soon after GA_3 action. By incorporation of radioactive leucine into an α-amylase associated with the ER membrane fraction, Locy & Kende (53) suggested that the ER is the site of synthesis and packaging of the enzyme. Detergent and sonic disruption of the ER suggested the activity was contained within the lumen of the organelle.

Recently Jones and coworkers (54) have confirmed more rigorously the involvement of the ER in barley aleurone α-amylase transport. They used a purified ER fraction obtained by gel filtration chromatography on Sepharose 4B followed by sucrose density gradient centrifugation. A ^{35}S-methionine labelled α-amylase, identified by immunoprecipitation and SDS-polyacrylamide electrophoresis, was sequestered within the ER and could be chased from the organelle with unlabelled methionine. This and other data indicate that the ER is involved in the processing of α-amylase as it moves to the cell exterior.

A recent method of preparing GA_3-responsive protoplasts from mature barley aleurones provides a less disruptive means of cell fractionation and should be helpful for elucidating organelle involvement in the secretion process (55). Such cells lacking cell

wall material will also clarify the involvement of the cell wall in the production and secretion of hydrolases.

The most detailed study revealing the processing of newly synthesized α-amylase has been accomplished by Akazawa and colleagues using rice seed scutellar epithelium. Although the scutellar tissue is distinct from the aleurone layer and α-amylase is more extensively glycosylated in rice than in barley the results provide intriguing insight into the glycosylation and processing of a secreted protein in plants.

The ^{35}S-labelled α-amylase obtained from cell free translation of poly(A)-mRNA of rice scutellum gave an M_r of 42,900 on SDS-gel electrophoresis. However, the mature α-amylase molecule secreted by the scutellum has a larger M_r of 44,000 suggesting addition of a carbohydrate unit during processing. To dissect the steps involved, Akazawa used the antibiotic tunicamycin which specifically inhibits the first enzyme step of the dolichol pathway and thereby prevents N-linked glycosylation of polypeptides at asparagine residues. Studies with tunicamycin combined with ^{35}S-methionine and ^{3}H-mannose labelling allowed the detection of a non-glycosylated intermediate. From these experiments it was concluded that the 42,900 translation product undergoes proteolytic cleavage of an 1800 signal peptide. The resulting intermediate precursor, M_r 41,000, is glycosylated producing the mature, secreted α-amylase, M_r 44,000 (56,57).

Further studies with isolated polysomes from rice scutellum using the classic translation "readout" system of Blobel and Dobberstein

(58) revealed the temporal relationship of signal sequence cleavage and glycosylation of the α-amylase polypeptide during translation. Direct evidence was provided showing that proteolytically-processed and already glycosylated segments of α-amylase exist on the polysome. Furthermore, protein glycosylation follows the removal of the NH_2-terminal signal peptide (59). The signal sequence on the α-amylase molecule prevents conversion of the unprocessed polypeptide to the enzymatically active form (60). This work directly proved for the first time in plants or animals the temporal sequence of processing and glycosylation of polypeptides during translation on the ER.

The precursor form of α-amylase obtained from cell-free translation of poly(A)-mRNA has also been characterized for barley aleurone (47,61,62). In contrast to rice scutellum many independent studies with barley indicate the α-amylase precursor to be 2500 daltons larger than the mature, secreted α-amylase. This suggests peptide processing because the mature enzyme contains little or no carbohydrate. In addition, there has been no report of a carbohydrate-containing precursor-form of the enzyme.

Ca^{2+} and Cereal Hydrolases

The influence of Ca^{2+} on the production and secretion of aleurone hydrolase has received much attention. Chrispeels and Varner (28) originally showed that Ca^{2+} was required in the GA_3 incubation medium

of barley aleurone layers in order to maintain a high level of α-amylase formation in the absence of starchy endosperm. They suggested Ca^{2+} was required to maintain enzyme activity. However, recently Jones (63,64) has provided convincing evidence indicating that the Ca^{2+} enhancement of α-amylase is not due to an enzyme-stabilizing effect. Ca^{2+} has also been implicated in the release of α-amylase as well as other hydrolases (64) from the aleurone layer including lysophospholipase (11). Initially Varner and Mense (65) using single aleurones suggested that cations such as Ca^{2+} acted on the cell wall to facilitate movement of already secreted proteins to the exterior of the cell. In contrast, Moll and Jones (66) in an elegant study using a flow-through apparatus with single aleurones, argued that the kinetics of Ca^{2+} regulated α-amylase secretion involved a transport mechanism that was controlled by the plasma membrane and not diffusion across the cell wall. Strontium and barium were partially effective while other cations were without effect in eliciting a rapid on-off secretion response.

Interestingly, Ca^{2+} causes a selective induction of the previously described α-amylase isozymes. In the original work of Jacobsen (67) it was found that the low pI group (isozymes 1 and 2, pI 4.6-5.2) was present in the GA_3 incubation medium of barley aleurones and a high pI group (isozymes 3 and 4, pI 5.9-6.6) also appeared in the medium when Ca^{2+} was present. The recent work of Jones and colleagues (63,64) leads to a more complicated picture of control of α-amylase isozymes by GA_3 and Ca^{2+}. Appearance in the aleurone and incubation medium of

one of the two isozymes of the low pI group strictly requires GA_3 while the other isozyme is produced under all conditions with or without GA_3 and Ca^{2+}. The two α-amylase isozymes of the high pI group require both GA_3 and Ca^{2+}, the minimum Ca^{2+} concentration being 0.5 mM and a maximum enhancement occurred with 10 mM Ca^{2+}. The stimulation of several other hydrolase activities and total protein by Ca^{2+} was also observed although to a lesser extent.

Although Ca^{2+} is required for the synthesis of the high pI α-amylase isozymes 3 and 4 it was not possible to distinguish between Ca^{2+} regulation at the transcriptional or the post-transcriptional level. Very recently Deikman and Jones (68) provided evidence indicating that Ca^{2+} regulation of the synthesis of the high pI isozymes occurs after mRNA accumulation and processing. Similar to conclusions on the mechanism of gene activation by GA_3 discussed above, the precise level of control by Ca^{2+} requires further studies using runoff translation techniques with isolated aleurone ribosomes.

Ca^{2+} also influences the production and secretion of hydrolases from other cereals. Working with rice seed scutellar epithelium Akazawa and coworkers (69) have demonstrated a similar requirement for Ca^{2+} in the biosynthesis and secretion of α-amylase. Using three hour pulses of ^{35}S-methionine, the rate of synthesis of α-amylase was saturated at 0.5 mM Ca^{2+} while the secretory rate increases up to 10 mM Ca^{2+}. Furthermore, they showed that calmodulin anatagonists inhibited Ca^{2+}-regulated secretion of α-amylase. Jones reportedly has been unable to detect a requirement for calmodulin for α-amylase

secretion from barley aleurones (personal communication). Interestingly, in preliminary results presented by Bush and Jones (70) radioactive Ca^{2+} presented to barley aleurone layers incubated in GA_3 had little influence on internal Ca^{2+} levels suggesting that Ca^{2+} activity is tightly regulated.

Isozymes of Barley Hydrolases

A common feature of GA_3-stimulated hydrolases in barley is the presence of multiple molecular forms of a given enzyme activity. Most often barley isozymes have been distinguished by surface charge based on electrophoretic techniques or ion exchange chromatography. The well studied α-amylase of cereals is a classic example. As discussed previously, two major isozyme groups of α-amylase are observed. Independent research groups have confirmed the presence of at least two separate genes for barley α-amylase (49,52,71). As noted by Jacobsen and Higgins (48) the number of α-amylases reported over the years ranges from 2 to 18 depending on the barley cultivar, type of tissue examined and method of extraction and analysis. Differences occur in pH optima, serological type and sensitivity to sulfhydryl reagents, chelators and low pH. Enzyme heterogeneity is observed with other hydrolases as well. Acid phosphatase from GA_3-treated barley half-seeds and aleurones shows charge heterogeneity (72). A carboxypeptidase purified from malted barley separated into two acidic components on isoelectric focusing, although the N-terminal sequence

analysis was identical (73). An extreme example of isozyme multiplicity occurs with GA_3-treated oat aleurones and isolated protoplasts (74). Careful isoelectric focusing methods revealed thirty-six isozymes of acid phosphatase with isoelectric points ranging from 4.35 to 7.50. β-Amylase, a latent enzyme found in the starchy endosperm of cereals, is heterogeneous with respect to molecular size and charge (15,75) and provides an interesting example of protein association. The enzyme exists in two forms based on solubility characteristics. One is covalently attached to storage proteins by disulfide bonds and requires reducing agents or mild proteolysis for extraction. The other form is extracted with salt. Interestingly, the heterogeneity of barley β-amylase has been partially explained by the specific heterodimer association with a (non-enzymatic) protein Z (75,76,77). Protein Z is a latent storage protein in the starchy endosperm containing many hydrophobic amino acids with a molecular weight of 40,000 and isoelectric point range from 5.55 to 5.8 (77).

Multiple molecular species of enzymes may arise by a variety of mechanisms (78). It may involve independent gene loci producing unique amino acid sequences. Alternatively, a single gene may give different polypeptides through processing of the primary transcript to give heterogenous mRNA. Isozymes of oligomeric enzymes may originate from the association of similar but non-identical subunits. Post-translational modification of enzymes involves numerous covalent and non-covalent changes in the protein. These include glycosylation,

phosphorylation, de-amidation, methylation or acetylation of lysine and arginine residues, sulfhydryl oxidation, proteolytic cleavage, equal stability conformational sub-states, association with cofactors or other proteins, and self-aggregation.

Examples of post-translational alteration with regard to cereals include methylation of lysine residues in wheat α-amylase (79) and bound protein inhibitor of α-amylase from germinating barley (80,81). This α-amylase inhibitor of M_r 21,000 and pI 7.2 does not originate from de novo protein synthesis but presumably occurs as a latent form in the starchy endosperm. It has been demonstrated that the high pI (6.6) α-amylase isozyme of barley can originate by association with the inhibitor (pI 7.2) and an α-amylase isozyme of pI 6.2. Interestingly, this α-amylase inhibitor is also a powerful inhibitor of subtilisin, a serine protease (80) and has considerable homologous structure with Kunitz-type protease inhibitors of leguminous seeds (81).

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Chapter II. GIBBERELLIC ACID STIMULATION OF LYSOPHOSPHOLIPASE ACTIVITY IN BARLEY ALEURONE LAYERS

ABSTRACT

A lysophospholipase (LPL) activity appears in the aleurone of barley (Hordeum vulgare L. cv. Himalaya) half seeds during imbibition on moist agar. Secretion of LPL by half seeds is promoted by GA_3; the increase in secretory rate is almost linear from 10^{-10} to 10^{-6} M GA_3. LPL activity is likewise promoted in isolated aleurone layers by GA_3. Its secretion into the incubation medium requires the continued presence of GA_3 and commences after 10 to 14 hour lag period when 10 mM Ca^{2+} is present. In the absence of Ca^{2+}, the lag period remains unchanged but attainment of the maximum secretory rate is delayed. Ca^{2+} alone has very little effect either on LPL activity accumulated in the aleurone layer or in the surrounding medium. However, 50 mM Ca^{+2} together with GA_3 dramatically increases the level of secreted activity and of total (accumulated & secreted) activity.

The metabolic inhibitors cycloheximide and actinomycin D inhibited the accumulation of LPL activity in the aleurone and the secreted activity. Actinomycin D added after the lag period resulted in a much lower inhibition. The increase in LPL activity in response to GA_3 occurs as a result of de novo synthesis; LPL activity from barley half seeds incubated in 80% D_2O in the presence of GA_3 undergoes a shift to higher density compared with the activity from similar controls incubated in H_2O. The characteristics of the GA_3 enhancement of LPL activity are compared specifically with α-amylase and generally with other GA_3-controlled hydrolases.

INTRODUCTION

The aleurone layer of barley is the source of a variety of hydrolytic enzymes which mobilize the endosperm reserves for the growing embryo. During the past two decades many researchers have examined the influence of GA_3 on the aleurone hydrolases such as α-amylase, protease, ribonuclease (20,28,32; reviews), phosphatase (3,4,23,28) and cell wall hydrolases (17,26,27). More recently, Baisted (5,6) reported on the presence of a soluble and particulate lysophospholipase (LPL) activity in the aleurone and starchy endosperm of germinating barley. It was found that a starch-bound LPL reached a peak activity coincident with the most rapid loss of starch-bound lysophosphatidylcholine (LPC); these events occurred one day after amylase activity had reached a maximum. The particulate LPL activity was suggested to be responsible for maintaining a low level of free LPC upon its release from the starch-bound form by amylase. It was also shown (7) that LPL activity originates in the embryo-containing half of the barley seed during germination and that GA_3 enhances both the activity in isolated aleurones and that appearing in the medium surrounding isolated aleurone layers.

Lysophospholipids occur as tightly bound inclusion complexes within the helical segments of the amylose component of cereal starches (1). The disposal of such complexes involves both amylase and LPL activities. A comparison of the characteristics of the stimulation by GA_3 of LPL activity in barley aleurone layers with that of other GA_3-stimulated hydrolases, especially α-amylase, is described in this report.

MATERIALS AND METHODS

GA_3 was from Sigma. Cycoheximide and actinomycin D were from Calbiochem. Barley seeds (Hordeum vulgare L. cv. Himalaya) from the 1979 crop were obtained from B.E. Frazier of Washington State University, Pullman, WA. De-Embryonated half seeds were surface-sterilized with 1% (w/v) NaClO solution and then allowed to imbibe water from 1% (w/v) agar plates for 4 to 5 days. All transfers and incubations were carried out under sterile conditions using sterilized glassware and solutions. Half-seeds or aleurone layers separated from the imbibed half-seeds were incubated in groups of 10 in 25 ml Erlenmeyer flasks in 2 ml acetate buffer (1 mM, pH 4.8) for different time intervals. These procedures were according to those previously described (7). The influence of GA_3 and Ca^{2+}, alone and in combination, on the appearance of LPL activity in the aleurone and its release into the incubation medium was compared with its appearance and release from aleurones incubated in acetate buffer alone. The temperature of imbibition and incubation was 21°.

After incubation, the aleurone layers were washed with the incubation buffer (2 x 1 ml) and homogenates of the layers were made in a pestle and mortar in ice-cold 10 mM phosphate buffer (4 ml, pH 7). For experiments in which Ca^{2+} was present in the incubation medium, the homogenizing medium contained 2 mM $CaCl_2$. The crude homogenates were centrifuged at 1000 g for 10 min and the supernatant used as a source of the aleurone LPL activity. The activity released into the incubation medium was measured on the supernatant resulting from the medium combined with the aleurone washes after centrifugation

at 1000 g for 10 min.

LPL was assayed in Tris-HCl (0.1 M, pH 8) as described by Baisted and Stroud (7). Lysophosphatidylcholine-[1-^{14}C]-palmitoyl (200 nmoles, 10^4 cpm) was dissolved in buffer (0.8 ml) and the reaction started by addition of 0.2 ml enzyme solution. Reactions were run in tilted centrifuge tubes in a shaker bath at 21° for time intervals insuring linear kinetics. Reactions were stopped by addition of 6 ml of a solvent mixture composed of isopropanol-heptane-1 N H_2SO_4 (60:40:1, v/v/v) to extract the liberated palmitic acid-[^{14}C]. One half of the upper phase, separated by centrifugation, was counted in 10 ml of a scintillation fluor of PPO (4 g/liter) in toluene-Triton X-100-water (60:30:10).

The α-amylase assay used β-limit dextrin as substrate. The β-limit dextrin was prepared by digesting 2% (w/v) potato starch with commercial β-amylase for 24 hr at 30°C, according to the procedures described by Lee (19). Any α-amylase present in the β-amylase was first inactivated by acid (pH 3.6) treatment for 20 min at 0°C (18). Cycloheximide (1 µg/ml) and streptomycin sulfate (1 µg/ml) were included in the digestion to prevent microbial growth. The digested starch solution was then dialyzed against 1 mM acetate, pH 5 for 24 hr at 4°C. The dextrin was precipitated with acetone and dried in a vacuum oven.

The α-amylase assay was initiated by adding 0.5 ml of a β-limit dextrin solution (0.6% (w/v) in 50 mM acetate, pH 5, 2 mM Ca^{2+}) to 0.1 ml enzyme solution. After 5 min incubation at 21°C, the liberated reducing sugar was measured by addition of 1 ml of 3,5-dinitrosalicylic acid color reagent and heating the mixture for 5

min in a boiling water bath. After cooling, 5 ml glass distilled water was added and the absorbance read at 540 nm on a Beckman DB spectrophotometer (31). The blank was the same mixture as above, except the color reagent was added prior to the enzyme. The amylase activity was measured as the equivalent of A_{540} x 100 for 0.1 ml enzyme solution incubated 5 min. The assay was linear within the ranges of time and enzyme concentration used.

Two groups, each of 20 sterilized half-seeds, were inbibed in 1 mM acetate buffer, pH 5 (1 ml) dissolved in either water or 80% D_2O. After 3 d, GA_3 (1 μM) in the H_2O-acetate buffer (1 ml) was added to the group imbibed in the H_2O medium, and GA_3 (1 μM) in 80% D_2O-acetate buffer (1 ml) was added to the group imbibed in the medium with 80% D_2O. The half-seeds were incubated for 20 h at 21°C. Aleurone layers were separated from the starchy endosperm, ground in 2 ml 10 mM phosphate, pH 7, and the supernatant (60,000 g for 15 min) was used as a source of enzyme.

The supernatant (0.2 ml), mixed with cytochrome c as an internal marker, was layered on top of 2.8 ml CsCl solution (1.304 g/ml) which was 5 mM in $KBrO_3$ to block protease activity in the supernatant. The samples were overlayed with mineral oil and the gradient established by centrifugation at 45,000 rpm for 68 h at 4°C in a Sorval OTD-2 with an AH-650 swinging bucket rotor. The tubes were punctured in the bottom and 80 three-drop fractions were collected. LPL was assayed and Cyt c measured spectrophotometrically in appropriate fractions. Refractive indices were determined in each fifth to eighth fraction. Density units equivalent to the refractive indices were obtained from tables (25).

In the Figures and Tables where error values are not specified, the data obtained are the averages of at least duplicate runs with ranges about an average $<\pm 8\%$.

RESULTS

Table II-1 reveals that LPL activity is present in the embryoless dry half-seed and increases steadily during the imbibition period. The LPL activity at 3 to 5 days imbibition represents a >10-fold increase over the initial activity in the dry half-seed. Measurement of LPL in the separated starchy endosperm and aleurone of the imbibed half-seed revealed that the activity was confined exclusively to the aleurone (data not shown).

The secretion of LPL from embryoless half-seeds in response to varying concentrations of GA_3 is given in Figure II-1. The GA_3 dose-response curve for LPL is similar to that of α-amylase and protease (9,10,16) with a minimum effective concentration of 10^{-10} M GA_3 and a maximum at about 10^{-4} M. The increase in the medium activity of LPL is nearly linear between 10^{-10} and 10^{-6} M GA_3.

The time-course of secretion of LPL and α-amylase from half-seed aleurones in the presence and absence of GA_3 with 10 mM Ca^{2+} is shown in Figure II-2. The secretion profile in the presence of GA_3 has the 10 to 14 hr lag period characteristic of other GA_3-controlled hydrolases such as α-amylase and protease (12,16). Comparison of the time course of GA_3-induced secretion of LPL and α-amylase (Figure II-2B and II-2D) reveals that both enzymes have nearly the same relative rate of increase following the lag phase. In the absence of GA_3, only a very small amount of LPL is present in the incubation medium. This indicates that, like α-amylase and other GA_3-controlled hydrolases, there is a requirement for the hormone for secretion of LPL. On the other hand, unlike α-amylase, LPL activity appears in the

Table II-1 Increase in LPL activity in half-seeds during imbibition on moist agar.

Ten sterilized half-seeds were removed from the agar at each time period, ground in homogenizing buffer and assayed for LPL activity as described in the Methods section.

days on agar	nmol palmitate released/min/10 half-seeds
0 (dry half-seeds)	4
1	8
2	25
3	47
5	71

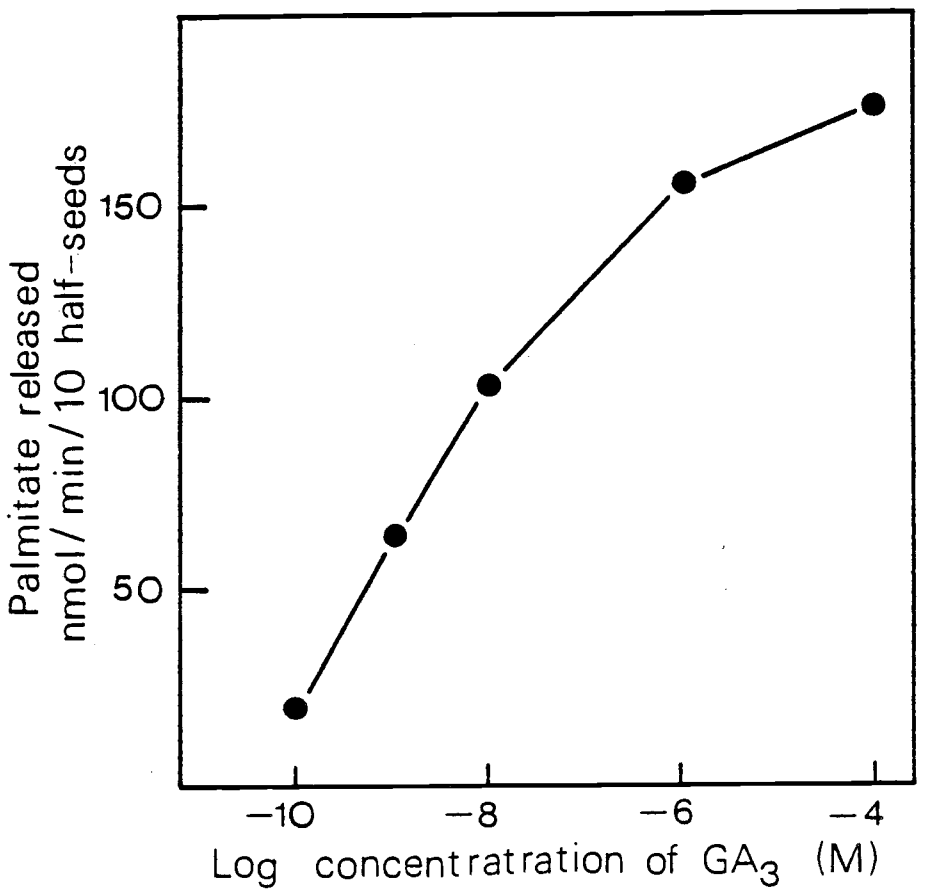

Fig. II-1 Secretion of LPL from half-seeds in response to varying GA_3 concentrations. Medium LPL activity was assayed after a 24 hr incubation with the appropriate GA_3 concentration.

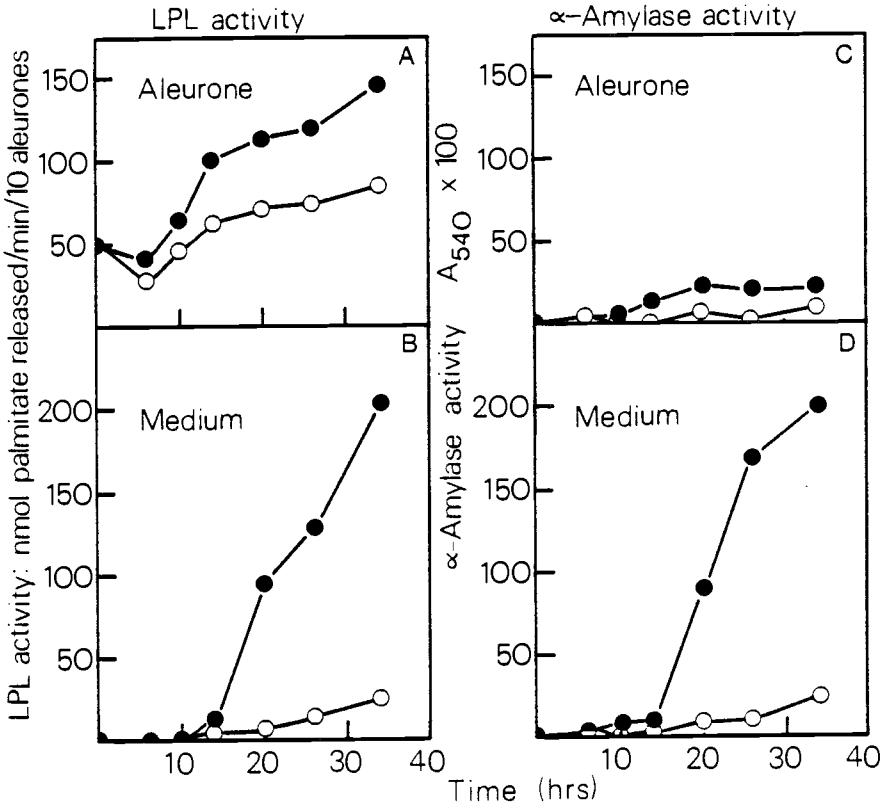

Fig. II-2 Time course of appearance of secreted (medium) and extractable (aleurone) LPL and α-amylase activities from aleurone layers in the presence and absence of 1 μM GA_3. Both control and GA_3 incubations contain 10 mM $CaCl_2$. ○: buffer control; ●: 1 μM GA_3.

aleurone during water imbibition of half-seeds. The increase of LPL activity in the aleurone in the absence of GA_3 during the time course, however, was markedly less (Figure II-2A).

The rate of GA_3-induced LPL secretion into the medium becomes maximal after the 14 hr lag period in the presence of 10 mM Ca^{2+} (Figure II-2B). Without Ca^{2+}, the lag period remains unchanged but the rate at which LPL appears in the medium is much slower (Figure II-3); the rapid rise of activity in the medium under these conditions is delayed about to 6 to 9 hr. The profile for accumulation of GA_3-induced LPL activity in the aleurone is similar whether Ca^{2+} is present (Figure II-2A) or absent (Figure II-3).

The response of the aleurone to the removal of GA_3 from the incubation medium after a 12 hr exposure to the hormone is shown in Figure II-4. Both control and experiment flasks were washed free of GA_3 after 12 hr, but the hormone was then added back to the control flask. It is clear that there is a requirement for the continued presence of GA_3 for the release of LPL into the medium. This parallels the observation made for α-amylase (10,17) and is in contrast to the findings with β-1,3-glucanase, the activity of which is maintained after GA_3 removal (17).

Table II-2 shows the influence of Ca^{2+} on the appearance of LPL and α-amylase activities in the aleurone and the incubation medium. The control flasks without GA_3 show the typical high LPL activity and low α-amylase activity in the aleurone, and very small activities in the incubation media. The presence of 10 mM Ca^{2+} alone has only slight effect on the accumulated and secreted activities. Total activities (A+M) and secreted activities (M) are dramatically

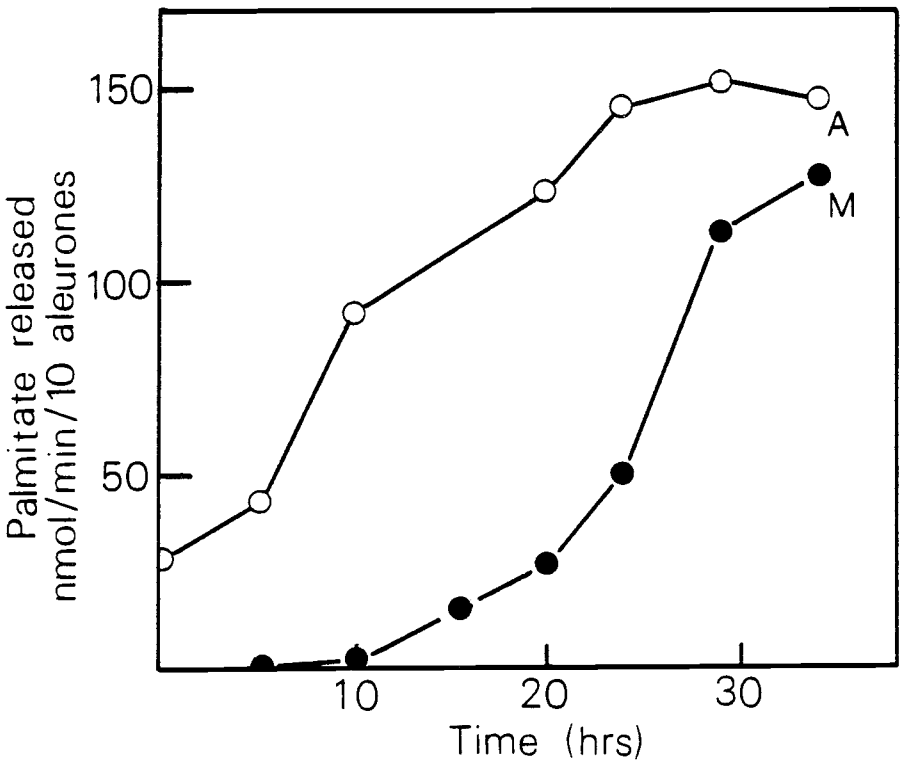

Fig. II-3 Time course of LPL activity in aleurone (A) and medium (M) with 1 μM GA_3 but lacking 10 mM $CaCl_2$. Data shown is representative of replicate experiments over the same time interval.

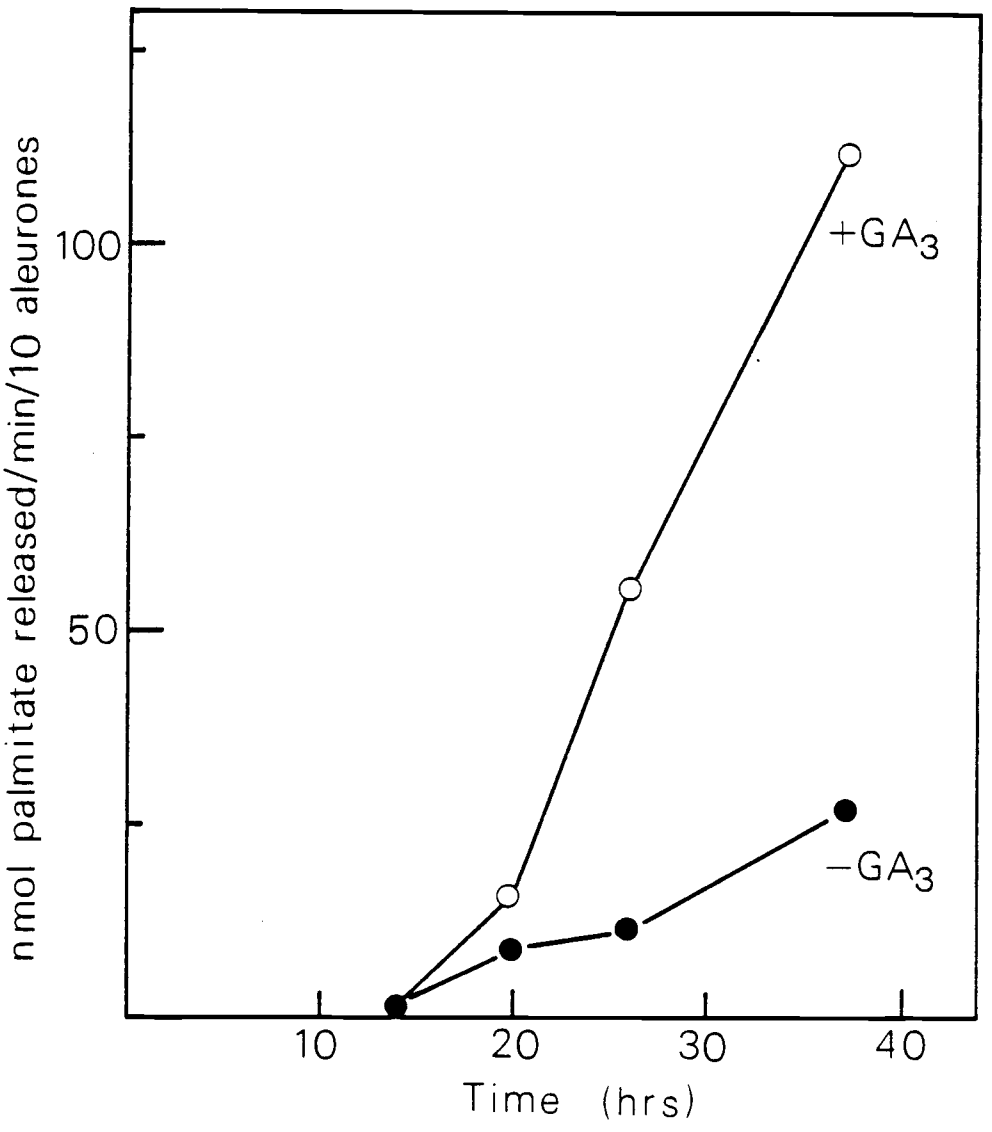

Fig. II-4 Secretion response to the removal of GA_3 at the end of the lag period. Aleurone layers were incubated with 2 x 10^{-8} M GA_3, 10 mM $CaCl_2$ for 12 hr, washed several times during 2 hr with 1 mM acetate, 10 mM $CaCl_2$ (pH 4.8) to remove GA_3. GA_3 (2 x 10^{-8} M, 10 mM $CaCl_2$) was added back or withheld for the subsequent time period. ○: GA_3 added back at 14 hr; ●: GA_3 withheld (buffer, 10 mM Ca^{2+} added back).

Table II-2 Influence of Ca^{2+} on the production of LPL and α-amylase in the aleurone (A) and medium (M).

Aleurone layers were incubated for 24 hrs with the given amount of $CaCl_2$. GA_3 concentration was 1 μM. Data is representative of replicate experiments.

	LPL activity nmol palmitate released/min/10 aleurone			α-amylase activity A_{540} x 100		
	A	M	A+M	A	M	A+M
Buffer only	103	6	109	6	10	16
10 mM $CaCl_2$	80	12	92	6	13	19
GA_3 (1 μM)	119	89	208	14	54	68
GA_3 (1 μM)	141	95	235	15	48	63
GA_3 + 1 mM $CaCl_2$	142	78	220	20	54	74
GA_3 + 10 mM $CaCl_2$	144	108	252	33	120	153
GA_3 + 50 mM $CaCl_2$	76	216	291	27	130	157

increased in the presence of GA_3 as expected. Total and secreted α-amylase activities are greatly enhanced by 10 mM Ca^{2+}, however, at 50 mM Ca^{2+} only a small further increase in secreted amylase occurs. In contrast, the total and secreted LPL activities are only moderately increased by 10 mM Ca^{2+} but at 50 mM Ca^{2+} we see a marked increase in total LPL activity and a sharp increase in secreted activity. Neither Ca^{2+} nor the calcium chelator, EGTA, had any effect on an LPL activity isolated from aleurone tissue (data not shown).

Table II-3 shows the effect of the metabolic inhibitors cycloheximide and actinomycin D on the appearance of aleurone and medium LPL activity in the presence of GA_3. Cycloheximide (1 μg/ml) added simultaneously with GA_3 produced 80% inhibition of aleurone activity and 100% inhibition of secretion as compared with the GA_3 control. Actinomycin D (100 μg/ml) added with GA_3 produced a 20% and 84% inhibition of aleurone and medium activity, respectively. However, actinomycin D added 10 hr after GA_3 treatment showed no inhibition of activity in the aleurone and only a 50% decrease in secretion. The inability of actinomycin D to inhibit the formation of aleurone enzyme activity after the lag period has also been observed with α-amylase (10,14).

Results of the density labeling of LPL in D_2O are given in Figure II-5 and Table II-4. There is a density shift in GA_3-stimulated LPL activity from the aleurone of half-seeds imbibed in 80% D_2O compared with the activity of seeds imbibed in H_2O. Figure II-5 shows a profile for the density distribution of H_2O-LPL and D_2O-LPL using Cyt c as an internal marker. Table II-4 gives the actual density values for two separate experiments. The D_2O-LPL has an average density

Table II-3 Effect of metabolic inhibitors on the appearance of GA_3 enhanced LPL activity.

Aleurones were incubated for 20 hrs with 1 μM GA_3 and cycloheximide (1 μg/ml) or actinomycin D (100 μg/ml). In one case actinomycin D was added after 10 hr GA_3 incubation. The aleurone activity represents the sum of the activities from the soluble and particulate fractions of a 105 kg spin (see Baisted, Ref. 7). LPL activity expressed as nmol palmitate released/min/10 aleurones.

	Aleurone		Medium	
	LPL activity	% inhibition	LPL activity	% inhibition
control (1 μM GA_3)	139		63	
cyclohexamide	29	79	<1	99
actinomycin D	111	20	10	84
actinomycin D >10 hrs	146	0	32	50

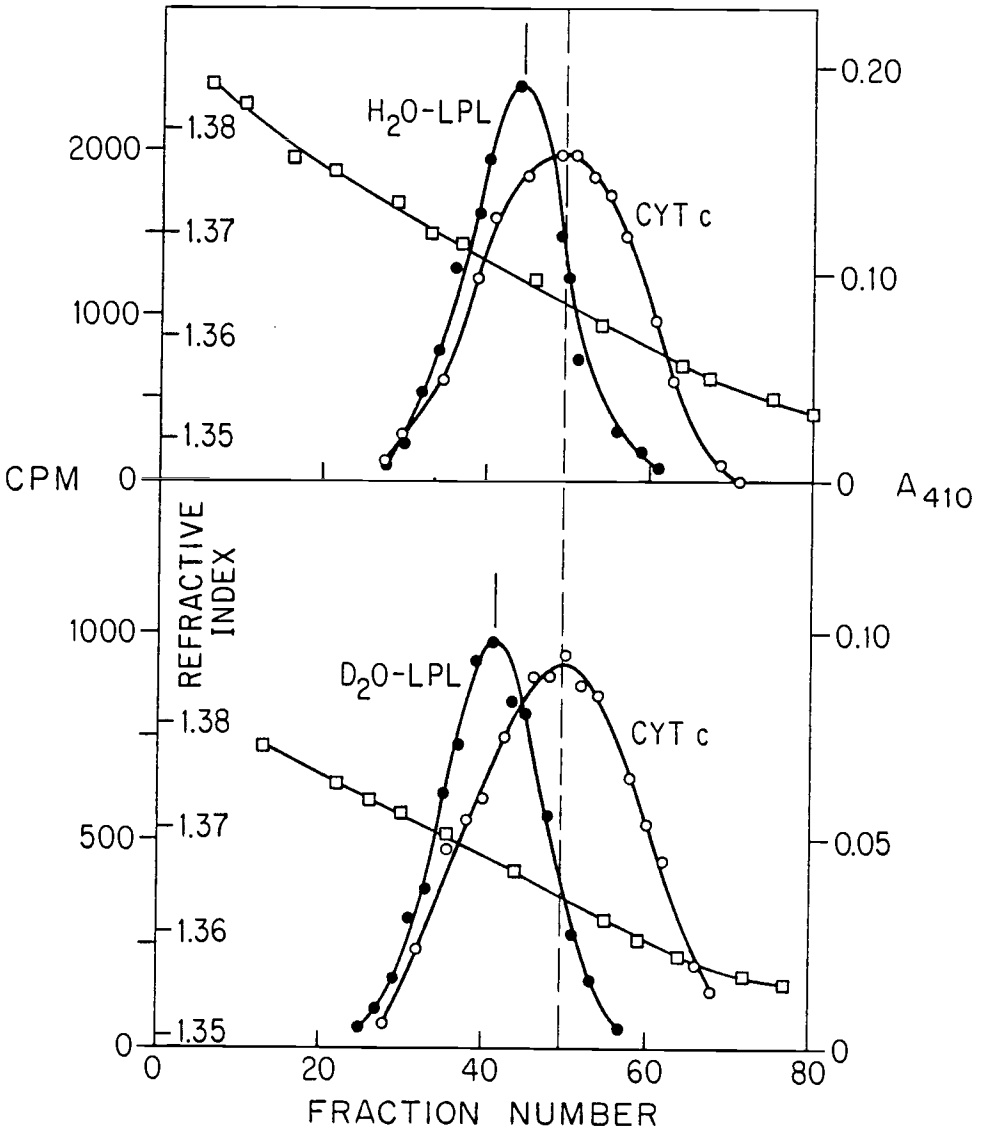

Fig. II-5. Equilibrium distribution in cesium chloride gradients of aleurone LPL labeled in either H_2O or 80% D_2O. O: cytochrome C (A_{410}) internal marker protein; ●: LPL, relative activity in cpm; □: refractive index.

Table II-4 Deuterium Labeling of Lysophospholipase

Twenty barley half-seeds were imbibed for 3 d with either H_2O or 80% D_2O in NaOAc (1 mM, pH 5). The half-seeds were then transferred to flasks containing GA_3 for further 20 hr. LPL was isolated from the separated aleurones of the half-seeds and the buoyant density of the enzyme activity relative to a Cyt c internal marker was measured by centrifugation in a CsCl density gradient. The data from two separate experiments are shown.

Experiment	Density of LPL		Density Shift
	H_2O	D_2O	
	g/ml		%
1	1.298 ± 0.002	1.320 ± 0.002	0.022	1.67
2	1.333 ± 0.002	1.350 ± 0.002	0.017	1.30

shift of 0.020±.003 density units or about 1.49%. The de novo synthesis of α-amylase (13), protease (16), peroxidase isozymes (2), and glucanase and ribonuclease (8) have been similarly demonstrated by density shifts of the enzyme labeled either with D or ^{18}O.

DISCUSSION

The increase in LPL activity during imbibition of half-seeds indicates that there is not an absolute requirement for GA_3 for the appearance of the aleurone activity (Table II-1). This is also the situation with ribonuclease (9), phosphatases (3,4,23), β-1,3-glucanase (27) and other cell wall hydrolases (26).

The secretion of LPL from aleurone layers, like other barley aleurone hydrolases, is dependent upon GA_3 (Figure II-2B). The rapid increases in the activity appearing in the medium immediately following the 10 to 14 hour lag phase is characteristic of other aleurone hydrolases. Protease (16) and α-amylase (12,16) have 10 to 12 hour lag periods while β-1,3-glucanase (17,27) and ribonuclease (9,18) have lag times of 4 and 18 to 24 hours, respectively. Although the time course of secretion of LPL is similar to that of α-amylase the activity profile in the aleurone more closely resembles that of β-1,3-glucanase (17,27) the activity appears during water imbibition and increases during GA_3 incubation. With β-1,3-glucanase and most notably ribonuclease (9), the aleurone activity declines concomitant with an increase in the activity of the medium. On the other hand, LPL activity in the aleurone is maintained at a high level while the activity in the medium is increasing. This indicates that active synthesis or activation of LPL in the aleurone occurs during secretion.

The presence of a low concentration of Ca^{2+} (1 mM) with GA_3 in the incubation medium had little effect on the aleurone and medium LPL activities as compared with GA_3 alone (Table II-2). However, with

high concentrations of Ca^{2+} (50 mM), a dramatically enhanced secretion and also total LPL activity (A+M, Table II-2) is apparent. Neither Ca^{2+} nor EGTA affect the isolated LPL activity so that Ca^{2+} must affect the synthesis and secretion of the enzyme. The influence of Ca^{2+} on GA_3-induced α-amylase shows a different sensitivity to Ca^{2+}. At 1 mM, small increases in synthesis and secretion are evident. Comparison of secreted and total activity at 10 mM and 50 mM indicate that maximal effect of Ca^{2+} appears to be at 10 mM. This is comparable with the data of Chrispeels and Varner (9) who found that Ca^{2+} was required for the maintenance of α-amylase activity against protease attack and that Ca^{2+} concentrations above 20 mM had no further effect on the secreted α-amylase level. The stimulation of secretion by Ca^{2+} is well documented in animal cells (24) and, more recently, in an elegant study by Moll and Jones (22) it was shown that the influence of Ca^{2+} on the release of α-amylase from single barley aleurone layers is more readily explained through an effect on secretion at the plasma membrane rather than an ion exchange phenomenon in the cell wall. The involvement of Ca^{2+} in the secretory process is evident in Figure II-3 where the absence of Ca^{2+} in the GA_3 incubation medium results in an overall reduction in LPL secretion through the 30 hour time-course of incubation. Secretion in the absence of added Ca^{2+} is not unusual; in the single aleurone secretion study of Moll and Jones (22) it was shown that under these conditions low levels of α-amylase were still secreted. Furthermore, small amounts of Ca^{2+} released from the aleurone in response to GA_3 (11) are available to maintain a moderate secretion response.

The protein synthesis inhibitor cycloheximide was particularly

effective in inhibiting both aleurone and incubation medium activities indicating a requirement for protein synthesis (Table II-3). Actinomycin D added with GA_3 also markedly inhibited the medium activity and also the aleurone activity but to a lesser extent. In parallel with the influence of these inhibitors on α-amylase production actinomycin D added after the lag period results in a much lower inhibition, indicating translation occurs on a stable mRNA (15). As noted by previous researchers (9,10,17), actinomycin D may not be the most effective inhibitor of mRNA synthesis, but the results presented here indicate the similarity of the response of both LPL and α-amylase to the inhibitor. This suggests that both enzymes may have a similar mode of activation by GA. Also similar to α-amylase and several other GA_3 stimulated hydrolases, LPL is synthesized de novo. Inhibition of LPL activity by the translation blocker cycloheximide suggested that LPL occurred by de novo synthesis. This was confirmed by the density labeling experiments with D_2O (Fig. II-5 and Table II-4). The D_2O-labeled LPL shows a noticeable shift to increasing density (Fig. II-5). The magnitude of the density shift compares with that for ribonuclease, 1.30% (8) and peroxidase, 1.90% (2) isolated from barley tissues incubated in 80% D_2O.

We have been unable to demonstrate the presence of phospholipase A activity in the incubation medium of GA_3-stimulated aleurone layers. Such an activity is usually accompanied by LPL to dispose of lysophospholipids generated in the course of phospholipid metabolism (29). It is evident that a major role for LPL secreted into the endosperm from barley aleurone layers is to dispose of the lysophospholipids released from the degraded amylose. LPC is a

fusogen (30) and in this capacity the low levels of LPC maintained in the starchy endosperm during germination may be sufficient to enhance the secretion of other GA_3-induced hydrolases.

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CHAPTER III. ACIDIC AND BASIC FORMS OF LYSOPHOSPHOLIPASE ACTIVITY FROM GA_3-STIMULATED BARLEY ALEURONE LAYERS

ABSTRACT

Lysophospholipase activity has been measured in the starchy endosperm of 3-6 day germinated barley and in the incubation media from GA_3-stimulated embryo-free half-seeds and aleurone layers. Two forms of the activity are distinguishable by ion-exchange chromatography on carboxymethyl cellulose. A basic activity (77-90% of total activity) is the predominant form from the starchy endosperm of germinated barley and from the media of hormone-stimulated half-seeds. The incubation medium of isolated aleurone layers, on the other hand, is predominantly acidic (60-95%). After removal of the starchy endosperm from hormone-stimulated half-seeds the secreted activity, which was initially basic, becomes acidic. The predominantly acidic activity from the medium of hormone-stimulated aleurone layers can be converted to the basic form in the presence of starchy endosperm or EDTA. Divalent metal ions do not shift the basic activity back to the acidic form. A mouse IgG fraction made against the basic activity inactivates the acidic and basic activities equally. On Bio Gel P100, the acidic activity elutes as a high molecular weight complex of several proteins. Two other barley hydrolases, phosphatase and α-amylase elute at their expected volumes. In the presence of EDTA the elution volume of the applied acidic activity increases to that for the basic activity. The physiological

role of the acidic form of lysophospholipase in barley germination is discussed.

INTRODUCTION

The germination of barley involves the degradation of starch by α- and β-amylases and the disposal of starch-included lysophospholipids by lysophospholipase, LPL (1-5). We have shown that the LPL, like many other barley hydrolases, is secreted by barley aleurone layers stimulated by GA_3 and that it is synthesized de novo. The enzyme has recently been purified to homogeneity and has been shown to be a basic (pI 8.8), hydrophobic glycoprotein with M_r 40000. During the purification from germinating barley an acidic LPL was found to be present by its ready elution from carboxymethyl cellulose columns; the basic enzyme, the major LPL, eluted in a NaCl gradient. This report describes experiments which show that an acidic LPL consists mostly of the basic activity aggregated with several other proteins. The role of such a high molecular weight complex in the secretion of LPL is discussed.

MATERIALS AND METHODS

Materials

Barley seeds (Hordeum vulgare L. cv Himalaya) from the 1979 crop were supplied by B. E. Frazier of Washington State University, Pullman, WA. GA_3, ρ-nitrophenylphosphate, LPC, bovine serum albumin (BSA), carboxymethyl cellulose, EDTA, pronase, crude α-amylase, streptomycin sulfate and gel filtration molecular weight standards were from Sigma. Chloroamphenicol was from CalBiochem. The SDS-gel electrophoresis markers were from Pharmacia.

Preparation of LPL activity

For experiments involving germinating whole Himalaya barley, batches of dry seeds were surfaced-sterilized with 1% (v/v) NaClO solution for 15 min and rinsed thoroughly with glass distilled water. The seeds were soaked overnight in water and then germinated on moist filter paper discs in petri dishes. At daily time intervals 20 starchy endosperms were carefully removed to avoid contamination with any scutellar or aleurone tissue. They were ground with a pestle and mortar in 2 ml ice-cold 10 mM phosphate buffer pH 7 containing 0.1 M NaCl. The homogenate was centrifuged at 5,000 x g for 15 min at 4°C and the resulting supernatant assayed for LPL activity before dialysis. Dialysis was carried out overnight against 1 l of 20 mM sodium phosphate, 20 mM β-mercaptoethanol and 10% (v/v) glycerol pH 7.2 (buffer A).

Preparation of half-seeds and aleurone layers is described in the Materials and Methods section of Chapter II. Aleurone layers peeled from half-seeds imbibed for 5 days on moist agar were washed in the standard "GA_3 buffer" (1 µM GA_3 in 2 mM sodium acetate pH 5) to remove small amounts of starch adhering to the tissue. The aleurone layers or non-embryo half-seeds were then incubated (21°C) under sterile conditions for different time intervals in the GA_3 buffer with or without $CaCl_2$ and other factors.

For analysis of acidic and basic LPLs on ion exchange chromatography the incubation media were removed with a Pasteur pipet and centrifuged at 1200 x g for 5 min at 4°C. The supernatant was dialyzed in buffer A and chromatographed on carboxymethyl cellulose (CMC).

Enzyme assays

Lysophospholipase (LPL) and α-amylase were assayed as described in the Materials and Methods section of Chapter II. Acid phosphatase activity was measured by incubating at 21°C 1 ml of 4 mM ρ-nitrophenyl phosphate in 0.1 M sodium acetate (pH 5) with 50 µl of enzyme solution for 15 to 30 minutes. The reaction was stopped with 1.5 ml of 1 M glycine (pH 9.5) and the absorbance immediately measured at A_{400} (6).

Ion exchange chromatography

The proportion of LPL activity with acidic and basic character was determined by separation of the two activities on carboxymethyl

cellulose (CMC) at pH 7.2. A slurry of CMC in dialysis buffer A was used to prepare the columns (0.8 cm x 4 cm). Aliquots (1-2 ml) of LPL samples dialyzed in buffer A were applied to the column. Non-binding LPL activity, referred to as acidic LPL, was washed through with 8 ml of the buffer. LPL activity bound to CMC, referred to as basic LPL, was eluted with 8 ml of 0.5 M NaCl in buffer A. LPL activity in the fractions was measured using 0.2 or 0.3 ml of solution and an incubation time of 10 to 45 min.

The stabilities of the two activities to ion exchange chromatography was assessed by subjecting each separated activity again to the processing procedure on the carboxymethyl cellulose columns. The acidic LPL activity suffers a small but significant loss of approximately 8% during the separation, while the basic activity suffers variable losses from 30-60%. Consequently, in mixtures of the two activities, the recovered acidic activity was corrected for the loss of 8%. The basic LPL was calculated by the difference between the LPL applied to the ion exchange column and the corrected acidic activity.

Incubation with Various Factors

All 28 hour incubation media with or without added factors were centrifuged at 1200 x g for 10 minutes at 4°C prior to dialysis in buffer A. The starchy endosperm incubated with aleurone layers or isolated incubation medium were from 5 to 6 day imbibed half-seeds. To check for differential binding of the acidic or basic LPL activities to starch, the starchy endosperm from a 21 hour incubation

with aleurone layers was extracted with 0.1 M NaCl in 10 mM phosphate buffer (pH 7) and analyzed for LPLs. The buffer extract of starchy endosperm was prepared by grinding 40 starchy endosperm (from half-seeds imbibed for 5 days) in 2 ml of 2 mM sodium acetate buffer at pH 5. The slurry was centrifuged at 10,000 x g at 4°C for 15 minutes. 0.5 ml of the supernatant was used in the experiment. The amount of potato starch used (250 mg) was equivalent to the dry weight of 20 starchy endosperm. Aleurone layer incubations with succinate and citrate were lacking the usual 2 mM sodium acetate in the GA_3 buffer. For the 1 M NaCl and 0.4 M maltose treatments, the acidic LPL activity was dialyzed in the solutions for 8 hours and then thoroughly dialyzed back into buffer A. EDTA additions were from a stock solution (0.2 M) at pH 5. LPC was added (100 µl) from a 20 mM solution in 2 mM sodium acetate (pH 5). When a crude hydrolase solution separated from the aleurone layers was incubated with various factors the samples contained the antibiotics chloramphenicol (20 µg/ml) and streptomycin (4 µg/ml). Protein was measured by absorbance at $A_{280/260}$ unless stated.

Starchy Endosperm Digests

Seventy starchy endosperm from 6 day imbibed half-seeds were incubated with either purified α-amylase or the GA_3-induced hydrolases from a GA_3 incubation of aleurone layers. The GA_3-induced hydrolase digest of the starchy endosperm was prepared as follows. A crude hydrolase solution from a 40 hr incubation of 20 aleurone layers in 1 µM GA_3, 10 mM $CaCl_2$ in 2 mM sodium acetate (pH 5) with antibiotics was

added under sterile conditions to 70 starchy endosperm and further incubated at 21°C for 40 hrs. The aleurone layers were removed and the starchy endosperm solution was ground in a mortar and pestle and centrifuged at 40,000 x g for 15 min at 4°C to obtain a clean supernatant. An aliquot of the supernatant equivalent to 10 starchy endosperm was used for experiments involving starchy endosperm digests. The α-amylase digestion of starchy endosperm was accomplished in a similar manner by incubating 70 starchy endosperm from half-seeds for 40 hrs with 0.2 ml of a filter-sterilized, purified malt α-amylase solution (0.5 mg/ml) in 2 mM sodium acetate containing 2 mM $CaCl_2$ (pH 5). The mixture was ground in a mortar and pestle and spun at 40,000 x g for 15 min. An aliquot of the supernatant equivalent to ten starchy endosperm was used in the experiments.

Purification of α-Amylase

The α-amylase was purified according to the method of Silvonovich and Hill (7). A solution of crude malt α-amylase (50 mg) in 5 ml acetate buffer (20 mM sodium acetate, 2 mM $CaCl_2$, pH 5) was heated to 70°C for 15 min to inactivate β-amylase and then centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was passed over a column (4 cm x 0.8 cm) of cyclohepta-amylose epoxy Sepharose 6B (7) and washed with acetate buffer to remove non-binding proteins. The α-amylase eluted with 0.8 mg/ml cyclohepta-amylose in acetate buffer. The eluted α-amylase solution appeared as a single band on 10% SDS-polyacrylamide gel electrophoresis stained with silver.

Pronase Treatment of Starchy Endosperm Digest Solution

An aliquot (1 ml) of the starchy endosperm hydrolase digest described above was incubated for 2 hrs at 37°C with 100 µl of pronase solution (1 mg/ml). The reaction was stopped by heating at 100°C for 15 minutes to inactivate the protease. The pronase treated solution (0.5 ml) equivalent to 10 starchy endosperm was incubated with 1 ml of acidic LPL for 3 hours at 21°C and then 10 hours at 10°C. Boiling inactivated pronase (100 µg in 0.1 ml) was also added to a control solution of acidic LPL. LPL activity was assayed and subjected to dialysis and CMC chromatography.

Influence of Starchy Endosperm and EDTA on Accumulated LPL of Aleurone Layers

Aleurone layers from 6 day imbibed half-seeds incubated for 40 hours in GA_3 buffer containing 20 mM $CaCl_2$ and layers prior to incubation were homogenized in 10 mM phosphate buffer (pH 7) in the presence or absence of 20 mM EDTA. The 100,000 x g (60 minutes 4°C) supernatant was analyzed for LPL activity. Ten starchy endosperm from 6 day half-seeds were also added to the 100,000 x g supernatant of an aleurone homogenate lacking EDTA. The sample was then incubated for 5 hours at 10°C and analyzed for the distribution of LPLs.

LPL Antibody Production

LPL from four day germinated barley (Advance var.) was purified to

near homogeneity by the method of Fujikura (5) using carboxymethyl cellulose ion exchange chromatography, octyl-agarose, conconavalin-A sepharose and Bio-Gel P-100. The anti-serum (IgG) to LPL was prepared according to Fujikura (5). A purified LPL solution (20 μg/ml) dialyzed in phosphate buffered saline (PBS:NaCl 8 g, KCl 0.2 g, KH_2PO_4 0.3 g, $Na_2HPO_4 \cdot 7\ H_2O$ 2.17 g in 1 ℓ H_2O) was mixed thoroughly with Freund's complete adjuvant (1:1) and 0.4 ml injected into the intraperitoneal cavity of a mouse (Balb/c female). Control serum was obtained by injecting a mixture of PBS and adjuvant into a second mouse. After two weeks, injections were repeated with incomplete adjuvant. A third injection was made one week later. Ten days later antibody production was stimulated with an injection of 0.2 ml of SP-2 myeloma cells (ascites tumor cells at 10^6 cells/ml). Two weeks later sera were tapped from the mice at weekly intervals for the next several weeks. The pooled sera were centrifuged at 1200 x g for 15 min. and the supernatant was made to 0.1% with NaN_3 and stored at 4°C.

For the preparation of the immunoglobulin fraction antiserum (5 ml) was made to pH 8.6 with 0.20 M NaOH. Ammonium sulfate (1.46 g) was added to the serum to 50% saturation and centrifuged at 10,000 x g for 10 min. Albumin, transferrin, and other proteins were removed by washing at 4°C with 3 ml 1.75 M ammonium sulfate. The white pellet was dissolved in 10 mM phosphate pH 7, dialyzed overnight in the buffer and precipitated lipoproteins were removed by centrifugation at 10,000 x g for 10 min. The supernatant contained the immunoglobulin (IgG) fraction.

Inactivation of LPL Activity by Anti-LPL IgG.

The susceptibility of LPL activity to inactivation by LPL antibody was determined by incubating crude LPL solutions with varying dilutions of anti-LPL IgG (Fig. III-3). The LPL activity was from three sources: aleurone or half-seed GA_3 incubation medium and aleurone tissue. The secreted activities were from half-seeds or aleurone layers incubated with GA_3 and 20 mM $CaCl_2$ in acetate buffer (pH 5) for 50 hrs at 21°C and then centrifuged at 1000 x g for 5 min. The aleurone tissue LPL activity was obtained from half-seeds incubated in GA_3, 20 mM $CaCl_2$ buffer solution for 24 hrs. The aleurone layers were removed from the starch and ground with sand in a mortar and pestle with phosphate buffered saline (PBS). The clear supernatant from a 60 min 100,000 x g centrifugation at 4°C was used for the enzyme inactivation study.

An equivalent amount of LPL activity (40 nmol/min) from half-seeds, or aleurone GA_3 incubation media and aleurone tissue was combined with PBS for controls and anti-LPL IgG diluted with PBS. The final mixture (0.6 ml) contained 4 mM $KBrO_3$ to inhibit potential barley protease activity (8) and 0.02% NaN_3 to prevent microbial growth. LPL activity was assayed after 18 hr. incubation at 21°C. In a similar manner, a purified LPL (5) was incubated with varying amounts of anti-LPL IgG.

Gel Filtration Chromatography

The approximate molecular size of the acidic and basic forms of

LPL was determined with gel filtration chromatography. BioGel P100 column (2 cm x 34 cm) was equilibrated with a buffer composed of 50 mM phosphate and 20 mM β-mercaptoethanol, pH 7.2. Samples were dialyzed in the same buffer with 5% (v/v) glycerol added and then layered on top of the column underneath the buffer head. Fractions (1.5 ml) were collected at a flow rate of 6 ml/hr. The molecular weight positions were determined with protein markers.

The size of the high molecular weight acidic LPL was determined on a BioGel P300 column (2 cm x 36 cm) equilibrated with 50 mM phosphate, 20 mM β-mercaptoethanol, pH 7.2. The samples were dialyzed in this buffer containing 5% glycerol and applied to the column as above. Fractions (2 ml) were collected at a flow rate of 3.5 ml/hr. Molecular weight standards (Sigma) were run in the phosphate buffer without mercaptoethanol in order to maintain the disulfide linked subunit structure of the high molecular weight protein markers.

SDS-Gel Electrophoresis and Silver Nitrate Staining

Discontinuous polyacrylamide gel extrophoresis was based on the method of Laemmli (9). A 10% separation gel was made by mixing 4 ml of 20% acrylamide (containing 0.4% N,N-bis-methylene acrylamide), 2 ml of pH 8.9 buffer (9.6 ml of 1 N HCl, 7.26 g tris-(hydroxymethyl) aminoethane (Tris) and 46 µl of tetramethylenediamine (TEMED) in a total volume of 20 ml with H_2O) and 2 ml 0.14% ammonium persulfate containing 0.4% SDS. The 5% stacking gel was made by combining 0.6 ml of 20% acrylamide containing 0.4% N,N-bis-methylene acrylamide, 0.3 ml pH 6.8 buffer (9.6 ml of 1 N HCl, 1.2 g Tris, 92 µl TEMED in final

volume of 20 ml with H_2O), 0.3 ml 0.004% riboflavin, 0.3 ml of 0.8% SDS and 0.9 ml of H_2O. The separating gel was polymerized in a slab (9 x 6 x 0.2 cm) between two glass plates. The stacking gel was poured on top with a comb inserted to form the wells and then polymerized under UV light. Protein samples were mixed with sample buffer to give a final concentration of 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.006% bromophenol blue. After heating for 2 minutes in a boiling water bath the samples were cooled and applied (40 µl) to the gel. Electrophoresis buffer was at pH 8.3 (0.025 M Tris, 0.192 M glycine and 0.1% SDS). Electrophoresis was started at 50 V until the tracking dye reached the top of the separating gel and then at 110 V until the dye was 0.5 cm from the bottom.

Following electrophoresis the gel was washed several times in 50% MeOH over two days. Silver nitrate staining followed the method of Wray et al. (10) with no modifications. The washed gel was gently agitated for 20 minutes in silver nitrate solution (2 ml silver nitrate (20%) in 11 ml 0.36% NaOH and 0.7 ml 58% ammonium hydroxide the volume increased to 50 ml). The gel was then washed 3 x 20 min. in deionized water. The protein bands appeared 10-15 min after addition of developer (10 mg citric acid, 10 µl 37% formaldehyde in 200 ml H_2O). Addition of 50% MeOH stopped the staining.

RESULTS AND DISCUSSION

Distribution of Acidic and Basic LPL in the Starchy Endosperm of Germinated Barley and in the Incubation Medium of GA_3-Stimulated Barley Aleurone Layers.

In initial experiments we measured the LPL activities present in the starchy endosperm of whole seeds germinated from 3 to 6 days inclusive. The procedure involved a salt extraction of the tissue followed by dialysis and ion exchange chromatography of the dialyzed extract on carboxymethyl cellulose. The acidic activity eluted in dilute buffer alone and the basic activity appeared after application of a salt gradient. The data are shown in Fig. III-1. It is clear that the major activity is the basic LPL which reaches a peak after 4 days germination. The acidic activity is much smaller as a proportion of the total LPL and peaks at 5 days. The increase in total activity and the later decline agree with the data previously found for the LPL of the starchy endosperm of another barley variety (2). Throughout it is clear that the basic activity is the major one representing 77-90% of the total and reflects the distribution found during the ion exchange step in the previously reported purification of LPL.

The studies with GA_3-stimulated aleurones described in Chapter II prompted an examination of the incubation medium as a potential source of partially purified LPL activity. Acidic and basic LPL activities were measured in incubation media by replacing the medium with fresh solutions at the time intervals shown (Fig. III-2). After dialysis and ion exchange chromatography of the medium, the distribution of the

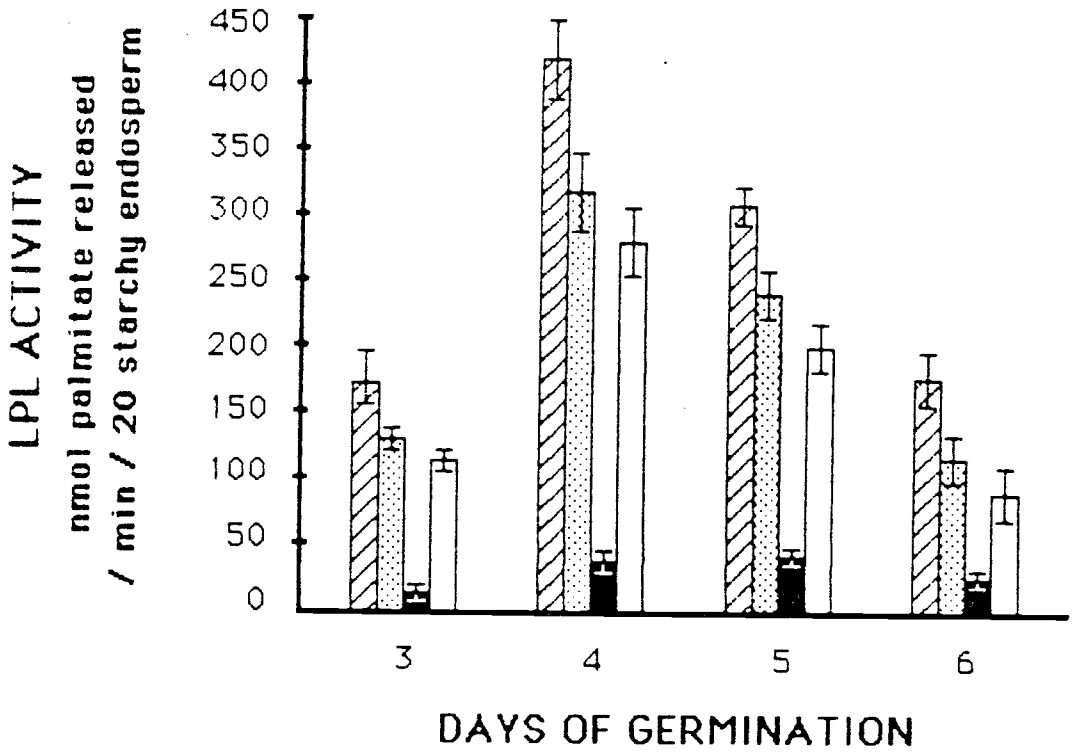

Fig. III-1 Distribution of acidic and basic LPL in the starchy endosperm of whole germinated barley seeds. At each time interval twenty starchy endosperm were removed from the seeds. They were homogenized with 10 mM phosphate buffer (pH 7) containing 0.1 M NaCl as described in Materials and Methods section and analyzed for the distribution of LPL activities. ▨: Activity in starchy endosperm homogenate; ⊡: Activity applied to ion exchange column; Distribution of LPL activity. ■ : Acidic activity; □: Basic activity.

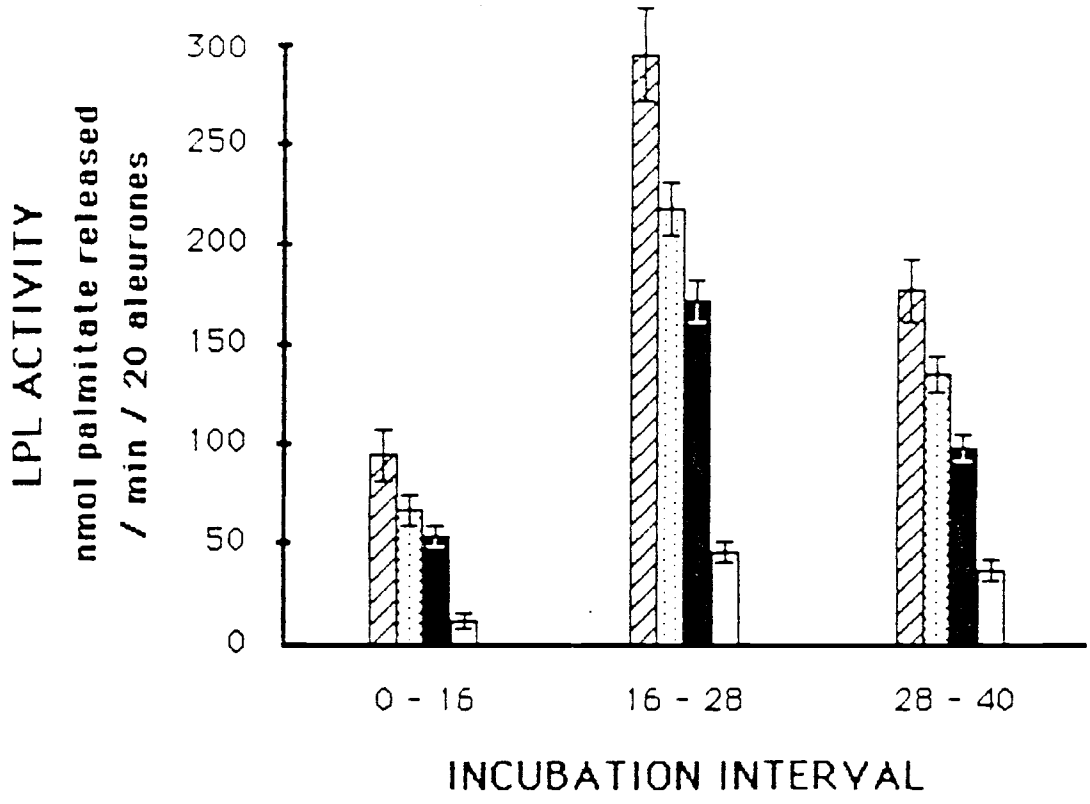

Fig. III-2 Distribution of acidic and basic LPLs secreted from aleurone layers at various time intervals (in hours). Aleurone layers, isolated from 20 half seeds, imbibed for 5 days, were incubated with the GA_3 buffer and 20 mM Ca^{2+}. The incubation medium was replaced with fresh medium at each time interval. Analyses were made on the incubation medium removed at each time interval. ▨ : LPL activity in incubation medium; ⊡ : Activity applied to ion exchange column; ■ : Acidic activity; □ : Basic activity.

two activities surprisingly favored the acidic over the basic LPL. This observation led to a more thorough examination of the nature of the acidic activity and of the factors governing the production of each LPL in barley half seeds and aleurones.

It is evident from Fig. III-1 that there are significant losses in one or both purification steps, dialysis and ion exchange, leading to separation of the two activities. The recoveries are 73, 76, 79 and 66% respectively for the four time intervals. Before proceeding with measurements of the two activities in other experiments, their stabilities in each processing step was assessed.

Stability of Acidic and Basic LPL Activity to Dialysis and to Ion Exchange

Extracts were made of embryo-free half-seeds and aleurone layers incubated with the GA_3 buffer containing 20 mM Ca^{2+} for 28 hours. Acidic and basic activities first separated by ion exchange were subjected again to ion exchange chromatography. The data are shown in Table III-1. The acidic activity with the shorter residence time on the column is clearly the more stable. Importantly, the losses show only small variation ranging from 4-12% of the chromatographed activity in four different trials. The basic activity is much less stable with losses varying from 30-60%. For the remaining experiments the basic activity is calculated by the difference between the LPL applied to the ion exchange column and the recovered acidic activity. The latter is corrected for the small loss during this step (an average of 8% for the four trials).

Table III-1 Stability of acidic and basic LPL activity to ion exchange chromatography on carboxymethyl cellulose

Acidic and basic activities first separated by ion exchange chromatography were subjected again to the same process. LPL activity in nmol palmitate released/min/20 aleurones or half-seeds.

		LPL activity	
	Experiment	Applied to column	Recovered
Acidic LPL activity			
	1	134	125 (93%)
	2	436	400 (90%)
	3	218	210 (96%)
	4	224	197 (88%)
Basic LPL activity			
	1	188	69 (40%)
	2	187	90 (48%)
	3	317	228 (72%)

Dialysis of the media obtained from incubations of different tissues under different conditions gave recoveries of 60 to 92% (Table III-2). Recoveries could be improved by the addition of BSA. However, when these dialyzed extracts were separated on carboxymethyl cellulose, the distribution of LPL activity for a given tissue and incubation conditions showed the acidic enzyme to be essentially a constant proportion of the chromatographed activity. Clearly, the 8 to 40% loss of activity on dialysis in the absence of BSA affected the acidic and basic activities equally as the proportions of the two forms remain the same.

Although BSA stabilizes the activities against losses by dialysis it was not included in subsequent experiments. The possibility that exogenous protein might interfere in the acidic-to-basic "switch" catalyzed by other factors was thereby excluded.

Absence of an Influence of Time of Accumulation Prior to Release on the Acidic/Basic LPL Ratio in Barley Half Seeds

In previous experiments we have shown that an LPL activity accumulates with time in isolated aleurones and is secreted upon addition of GA_3. Synthesis and secretion of LPL is further enhanced when the GA_3 is supplemented with high Ca^{2+} concentrations (4). The possibility that the accumulated activity may be a lysosomal, acidic lypolytic acyl hydrolase suggested a study with embryo-free half seeds. The influence of time prior to stimulation by GA_3 on the distribution of LPL activity between the acidic and basic forms was assessed. The data are shown in Table III-3. It is evident that the

Table III-2 Stability of acidic and basic LPL activity to dialysis.

Embryo-free half-seeds or isolated aleurone layers were incubated for 28 hours with 1 μM GA_3 with or without $CaCl_2$. The incubation media were dialyzed and applied to carboxymethyl cellulose as described in Materials and Methods. LPL activity is expressed as nmol palmitate released/min/20 half-seeds or aleurones.

† The distribution of LPL activity between the acidic and basic forms is determined by assuming an average loss of 8% (see Table III-1) for the acidic activity. The basic activity is calculated by difference between the LPL activity applied to the column and the acidic activity recovered. Numbers in parentheses give percent distribution.

* BSA added before dialysis (2 mg/ml) to stabilize the LPL activity.

	LPL activity for each experiment	Recovery after dialysis (%)	Activity applied to ion exchange column	Distribution of LPL† activity	
				Acidic	Basic
Half-seeds					
	270	60	164	16 (10)	148 (90)
without $CaCl_2$	270	83	224	29 (13)	195 (87)
	270	98*	265	29 (11)	236 (89)
	298	75	223	34 (15)	189 (85)
with 20 mM $CaCl_2$	298	80	238	48 (20)	190 (80)
	298	92	274	41 (15)	233 (85)
Aleurones					
	264	64	169	142 (84)	27 (16)
with 20 mM $CaCl_2$	264	75	198	154 (78)	44 (22)
	264	91*	240	197 (82)	43 (18)

Table III-3 Proportion of acidic to basic LPL from the GA_3 incubation medium of half-seeds imbibed for various times on moist agar.

Twenty half-seeds imbibed for up to 5 days were then incubated with the GA_3 buffer containing 10 mM $CaCl_2$ for 28 hr. The media were dialyzed and applied to carboxymethyl cellulose columns as described in Materials and Methods. LPL activity expressed in nmol palmitate released/min/20 half-seeds. The error values are the range about the mean for the replicates.

† Numbers in parentheses are the number of experiments for each treatment.

Days on agar before GA_3 incubation	LPL activity in incubation medium	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
			Acidic	Basic
0 (2)†	233 ± 28	212 ± 25	57±7 (27)	155±18 (73)
1 (3)	451 ± 68	388 ± 58	81±12 (21)	306±37 (79)
2 (2)	440 ± 35	348 ± 27	101±8 (29)	247±20 (71)
5 (3)	352 ± 53	285 ± 42	34±9 (12)	251±37 (88)

activity appearing in the medium is similar with respect to the acidic/basic ratio of LPL as that previously found for the whole germinated seed (Fig. III-1).

Absence of an Influence of High Ca^{2+} Concentration on the Acidic/Basic LPL Distribution from Barley Half-Seeds

Half-seeds were pre-incubated for 5 days on agar plates before being transferred to the GA_3 buffer supplemented with different levels of Ca^{2+}. The data are shown in Table III-4. Although there is ~3x increase in LPL activity from 0-40 mM Ca^{2+}, the proportions of the two activities remains unchanged. It appears that the basic enzyme activity is the preferred form of LPL for secretion by half-seeds and that the distribution between the two forms is independent of the time allowed for their accumulation before release.

Absence of an Influence of Ca^{2+} on the Acidic/Basic LPL Distribution in the Secreted Activity from Isolated Barley Aleurone Layers

The time course shown in Fig. III-2 in which the incubation medium was replaced with fresh solution at different time intervals was also conducted without Ca^{2+} present in the medium. The data shown in Table III-5 indicates that there is no reversal of the distribution by Ca^{2+} and that there may be a small shift favoring the acidic form when Ca^{2+} is present. To examine this further, increasing concentrations of Ca^{2+} were used in the medium and the analysis was made on the LPL activity accumulated over 28 hours (Table III-6). Clearly, high Ca^{2+}

Table III-4 Influence of Ca^{2+} on the appearance of acidic and basis LPL activities in the incubation media of GA_3 stimulated half-seeds.

Half-seeds imbibed on moist agar for 5 days were incubated with the GA_3 buffer and appropriate concentrations of $CaCl_2$. After 28 hours the media were analyzed for acidic and basic LPL. Activity is in nmol palmitate released/min/20 half-seeds. The error values are the range about the mean for the replicates.

† Numbers in parentheses are the number of experiments for each treatment.

	LPL activity in incubation medium	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
			Acidic	Basic
no Ca^{2+} (2)†	150 ± 8	129 ± 9	26±2 (20)	103±7 (80)
10 mM Ca^{2+} (3)	352 ± 53	285 ± 42	34±9 (12)	251±22 (88)
40 mM Ca^{2+} (2)	425 ± 51	336 ± 40	57±10 (17)	279±33 (83)

Table III-5 Release of acidic and basic LPL from GA_3 stimulated aleurone layers at various time intervals in the presence and absence of Ca^{2+}.

Aleurone layers were incubated in the GA_3 buffer with or without 20 mM $CaCl_2$. The media were decanted at appropriate times and fresh media added back. Analyses were made on incubation media removed at each time interval. LPL activity in nmol palmitate released/min/20 aleurone layers.

† Numbers in parentheses are the number of experiments for each treatment.

	LPL activity in incubation medium	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
			Acidic	Basic
GA_3 buffer only (1)†				
0 to 16 hours	28	25	19 (76)	6 (22)
16 to 28 hours	35	29	19 (67)	10 (33)
28 to 40 hours	68	57	39 (68)	18 (32)
GA_3 plus 20 mM Ca^{2+} (2)				
0 to 16 hours	95 ± 20	68 ± 12	55±11 (81)	13±3 (19)
16 to 28 hours	295 ± 27	218 ± 19	172±15 (79)	46±4 (21)
28 to 40 hours	177 ± 19	135 ± 15	99±10 (73)	35±4 (26)

Table III-6 Influence of Ca^{2+} on the proportion of acidic and basic LPL activities in the incubation media of GA_3-stimulated aleurone layers.

Twenty aleurone layers were incubated with the GA_3 buffer with or without $CaCl_2$ for 28 hours at 21°C. LPL activity is in nmol palmitate released/min/20 aleurones. The error values are the range about the mean for the replicates.

† Numbers in parentheses are the number of experiments for each treatment.

	LPL activity in incubation medium	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
			Acidic	Basic
no Ca^{2+} (3)†	175 ± 24	119 ± 16	77±10 (65)	42±6 (35)
10 mM Ca^{2+} (3)	303 ± 61	212 ± 42	131±26 (62)	80±16 (38)
40 mM Ca^{2+} (3)	1054 ± 126	738 ± 90	531±63 (72)	206±25 (28)

concentrations (40 mM) stimulate the appearance 6x of the LPL activity in the medium of aleurone layers incubated in the absence of Ca^{2+}. The distribution however between the acidic and basic form remains essentially unchanged. For these experiments involving long term incubations, it was important to show that LPL is stable through the course of the experiment. A stability study shown in Table III-7 demonstrates that there is only a 12% loss of activity during a 68 hour incubation.

The Starchy Endosperm is Responsible for the Predominantly Basic Form of LPL Activity

Twenty isolated aleurone layers were incubated in a standard medium containing GA_3 and 10 mM $CaCl_2$. A parallel run contained twenty half-seeds incubated under identical conditions. After 22 hours the two media were removed from the flasks for measurement of the acidic and basic LPL activities. The data are shown in rows 1 and 3, respectively, of Table III-8. As anticipated, the predominantly acidic LPL appears from the aleurone layers and predominantly basic LPL from half-seeds. The experiment was continued for an additional 21 hours by adding to the isolated aleurone layers in fresh incubation medium, 20 starchy endosperm from 5 day-imbibed half-seeds. The half seeds from the other incubation were removed and the aleurone layers separated from them. These were incubated alone in fresh medium. The incubation media from these two experiments were analyzed for the acidic and basic LPL activities and the data are shown in rows 2 and 4, respectively, of Table III-8. It is clear from these data that a

Table III-7 Stability of secreted LPL at room temperature in the presence of Ca^{2+}.

The medium from a 24 hour incubation of 20 aleurone layers in 1 μM GA_3, 10 mM $CaCl_2$ was separated from the aleurones and further incubated at 21°C. LPL activity was assayed at the times indicated.

	LPL activity	
	nmol palmitate/min/20 aleurones	Activity remaining (%)
Time (hours)		
0	266	100
5	262	98
20	238	89
45	236	88
68	236	88

Table III-8 The influence of the starchy endosperm in governing the proportion of acidic and basic LPLs.

Twenty aleurone layers or half-seeds were incubated in the GA_3 buffer containing 10 mM $CaCl_2$ for 22 hours. The media were removed and the LPL distribution analyzed (rows 1 and 3, respectively). The experiment was continued by adding twenty starchy endosperm in fresh buffer to the rinsed aleurone layers. The half-seeds were removed and the separated aleurone layers were also incubated in fresh media. After 21 hours the incubation media for the two experiments were analyzed (rows 2 and 4, respectively). LPL activity in nmol palmitate released/min/20.

		LPL activity in incubation medium	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
				Acidic	Basic
1	Aleurone layers incubated 22 hours	189	147	88 (60)	59 (40)
2	Aleurone layers plus 20 starchy endosperm incubated further 21 hours	595	393	94 (24)	299 (76)
3	Half-seeds incubated 22 hours	128	111	27 (24)	84 (76)
4	Separated aleurones incubated further 21 hours	187	120	110 (92)	10 (8)
5	NaCl extract of starchy endosperm (2 above) at 21 hours	123	81	17 (21)	64 (79)

shift from acidic to basic LPL activity is governed by the presence of the starchy endosperm. After removal of the starchy endosperm from half-seeds, the activity secreted is the acidic LPL. The starchy endosperm from the incubation described in row 2 (aleurone layers plus starchy endosperm) showed no differential binding of the LPL activity as evidenced by analysis of a 0.1 M NaCl extract (row 5). Starchy endosperm from 5 day-imbibed half-seeds have a background LPL activity of <5 nmoles/min/20 endosperm.

Influence of Variously Modified Fractions of Starch on Incubated Aleurone Layers

Table III-9 shows the results of control experiments in which the predominantly acidic form of LPL is produced by the isolated aleurone layers and that a shift to the basic form of the activity occurs when the layers are incubated in the presence of added starchy endosperm. The capacity to produce the shift is not possessed by an autoclaved, buffer extract of the starchy endosperm. Soluble digests of starchy endosperm using either endogenous hydrolases or α-amylase did result in aleurone layers producing the basic LPL. Importantly, the factor(s) responsible for the shift was thermally stable as the digests were boiled or autoclaved before use. These data suggest that the factor is either entrapped within the starch granule or is a degraded form of starch. To test the latter, autoclaved potato starch and maltose were used. Under the experimental conditions the potato starch would undergo hydrolysis by the barley endogenous amylases. Thus producing a spectrum of glucans. Although there is some capacity

Table III-9 Influence of variously modified fractions of starch on the distribution of acidic/basic LPLs released from GA_3-stimulated aleurone layers.

Twenty aleurone layers in the GA_3 buffer containing 20 mM $CaCl_2$ buffer were incubated alone or with twenty added starchy endosperm (from half-seeds 6 days on moist agar) for control experiments. Aleurone layers in the same buffer were also incubated with various factors. After 28 hours incubation the incubation media were analyzed for acidic and basic LPL activities. LPL activity is expressed as nmol palmitate released/min/20 aleurone layers.

† Numbers in parentheses are the number of experiments for each treatment.

	LPL activity in incubation medium	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
			Acidic	Basic
Controls				
Aleurone (4)†	354 ± 20	234 ± 12	173±10 (74)	61±3 (26)
Aleurone plus 20 starchy endosperm (3)	520 ± 25	328 ± 16	39±3 (12)	288±14 (88)
Treatment				
Buffer extract of starchy endosperm (1)	533	320	278 (87)	42 (13)
Endogenous hydrolase digest, sterile filtered (1)	573	401	112 (28)	289 (72)
Endogenous hydrolase digest, autoclaved (2)	460 ± 20	354 ± 31	71±6 (20)	283±25 (80)

Table III-9 Continued.

	LPL activity in incubation medium	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
			Acidic	Basic
α-amylase digest of starchy endosperm (1)	469	305	73 (24)	232 (76)
Potato starch (250 mg), autoclaved (1)	204	159	78 (49)	81 (51)
Maltose (0.1 M) (1)	400	284	185 (65)	99 (35)
PIIF (1 mg/ml) (1)	280	168	118 (70)	50 (30)
Succinate 20 mM, pH 5 (1)	256	215	151 (70)	64 (30)
Citrate 20 mM, pH 5 (1)	327	262	188 (71)	76 (29)

to produce the shift by potato starch, the shift is not of the magnitude as that shown by the barley starch. Maltose was ineffective. In addition, proteinase inhibitor-inducing factor (PIIF), which is a cell wall degradation product (11), and succinate and citrate are without effect.

The experiments outlined above do not distinguish between the effect of the starch factor(s) on the aleurone layer itself or on the LPL hydrolases already secreted by the aleurone.

Influence of Factors on the Incubation Medium of GA_3 Stimulated Aleurone Layers

The medium from a 28 hour incubation of 20 aleurone layers in the presence of 1 μM GA_3 and 20 mM $CaCl_2$ was separated from the layers. The influence of the addition of a number of factors on the distribution between the acidic and basic LPLs is shown in Table III-10.

The control shows the medium to be 70 to 95% acidic LPL. In two experiments, β-mercaptoethanol was omitted in order to determine whether the distribution of LPL activities was governed through a SH/S-S redox system. Clearly, it is not. As few as 5 starchy endosperm added to medium equivalent to 20 aleurone layers can bring about a substantial reversal of the distribution to 90% basic LPL. A pronase-treated, hydrolase digest of the starchy endosperm also brings about a reversal, although incomplete. This suggests that surface-adsorbed protein is not responsible for the shift and neither is protein that might be entrapped within the starch granule. High salt

Table III-10 Influence of various factors on LPL activity of the incubation medium from GA_3-stimulated aleurone layers.

LPL activity obtained from a 28 hour incubation of 20 aleurone layers in GA_3 buffer containing 20 mM $CaCl_2$ was separated from the aleurones and incubated alone or with factors and antibiotics for ten hours at 21°C. In two experiments β-mercaptoethanol (β-ME) was omitted from the standard dialysis and ion exchange buffer. All other additions are described in the Materials and Methods section.

	LPL activity in incubation medium	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
			Acidic	Basic
Control incubation medium (3)	433 ± 20	286 ± 14	203±10 (71)	83±4 (29)
β-ME-free processing	360	220	156 (71)	64 (29)
Additions				
5 Starchy endosperm (1)	360	299	30 (10)	269 (90)
20 Starchy endosperm (2)	380 ± 20	285 ± 45	23±3 (8)	262±41 (92)
20 Starchy endosperm (β-ME-free processing)	320	205	24 (11)	181 (89)
Pronase treated hydrolase digest of starchy endosperm (1)	468	300	120 (40)	180 (60)
Heat killed pronase (1)	384	333	280 (84)	53 (16)
2 mM EDTA (1)	450	315	120 (38)	195 (62)

Table III-10 Continued.

	LPL activity in incubation medium	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
			Acidic	Basic
20 mM EDTA (3)	470 ± 10	354 ± 28	35±2 (10)	318±16 (90)
1 mM LPC (1)	438	263	176 (67)	87 (33)
1 M NaCl (1)	400	252	169 (67)	83 (33)
0.4 M Maltose (1)	400	260	182 (70)	78 (30)

concentration and the naturally-occurring detergent substrate for the LPLs, lysophosphatidylcholine, as well as a high maltose concentration are essentially ineffective. EDTA, at 20 mM, clearly mimics the effect of the starchy endosperm and is partially effective as low as 2 mM.

Absence of Influence of Divalent Metal Ions on Conversion of Basic to Acidic LPL

The marked effect of the chelator, EDTA, on the conversion of the acidic LPL to the basic form suggested that a reversal might be effected with divalent metal ions. Mg^{2+}, Mn^{2+} and Cu^{2+} at the 2 mM concentration had no influence on the conversion (Table III-11). Ca^{2+} has previously been shown to have an insignificant effect at the 40 mM level (Table III-4). It is possible that if metal ion is responsible for maintaining the LPL in an acidic form that, once removed, an irreversibly altered and stable conformation of the LPL is produced which displays basic properties.

Nature of LPL Activity in Homogenates of Aleurone Layers from Imbibed Half-Seeds and from GA_3-Stimulated Half-Seeds

The experiments to date concern the distribution between acidic and basic forms of LPL activity secreted by aleurone layers, by half-seeds and by that appearing in the starchy endosperm of germinated seeds. A question to be answered is whether the distribution seen in the LPL activity which accumulates in the aleurone layer in imbibed

Table III-11 Influence of divalent metal ions on the conversion of basic to acidic LPL.

LPL activity from the media of 28 hour incubations of 20 half-seeds in the GA_3 buffer containing 20 mM $CaCl_2$ was dialyzed overnight with various metal ions (2 mM) in buffer A. All samples were dialyzed against buffer A before ion exchange chromatography. LPL activity in nmol palmitate released/min.

† Numbers in parentheses are the distributions expressed as percentages.

	LPL activity for each experiment	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
Half-seed incubation medium				
Control	330	277	30 (11)†	247 (89)
Additions				
Mg^{2+}	274	180	25 (14)	155 (86)
Mn^{2+}	330	248	37 (15)	211 (85)
Cu^{2+}	330	270	30 (11)	240 (89)

half-seeds is the same as that whose synthesis is stimulated by GA_3. A further question is whether the acidic LPL accumulated in the aleurone layer is subject to conversion to a basic form in the presence of starchy endosperm and EDTA. The data are shown in Table III-12. The control experiments show the LPL activity to be very nearly equally distributed between the two forms in the aleurone layers of imbibed half-seeds and also in those of GA_3-stimulated half-seeds. When the homogenizations were conducted in the presence of EDTA and starchy endosperm, the substantial conversion of LPL to the basic form was again evident. It had been anticipated that the acidic and basic LPL distribution in the control experiments would reflect the high proportion of the acidic form secreted by isolated aleurone layers. The larger than expected proportion of basic activity may have resulted from the presence of small amounts of metabolites released on homogenization which function in the same way as EDTA and the starchy endosperm in converting the acidic to the basic LPL. Alternatively, the homogenization process may release a basic LPL which is otherwise retained in the aleurone tissue during its secretory phase.

Inactivation of Different LPL Activities by a Mouse IgG Fraction Obtained Against the Basic LPL

For the production of the immunoglobulin fraction, a highly enriched, basic LPL activity was obtained by carboxymethyl cellulose ion exchange chromatography, octyl agarose, conconavalin A-Sepharose and BioGel P100 from an extract from germinating barley. An

Table III-12 The influence of EDTA and starchy endosperm on the distribution of acidic and basic LPLs from homogenized aleurone layers.

Details of the experiment are described in the Materials and Methods section. LPL activity in nmol palmitate released/min/20 homogenized aleurone layers.

† Numbers in parentheses represent percent distribution of LPL activity.

	LPL activity	Activity applied to ion exchange column	Distribution of LPL activity (% of applied)	
			Acidic	Basic
Aleurone layer homogenate ($-GA_3$)				
control (2)	131 ± 19	97 ± 13	44±6 (45)†	53±7 (55)
20 mM EDTA (1)	131	109	12 (11)	97 (89)
10 starchy endosperm (2)	121 ± 9	101 ± 7	23±1 (23)	77±5 (77)
Aleurone layer homogenate ($+GA_3$)				
control (2)	93 ± 20	71 ± 13	37±8 (52)	34±7 (48)
20 mM EDTA (2)	290 ± 90	215 ± 60	17±5 (7)	198±61 (93)

electrophoretic gel of gel filtration fractions used for production of the anti-serum is shown in Fig. III-3. An anti-serum against this protein preparation was obtained from a mouse and an IgG fraction separated. The procedures used were those employed by Fujikura in this laboratory (5). LPL activities to be tested were isolated by homogenization of aleurone layers, and by removal of the incubation media of half-seeds and isolated barley aleurone layers which had been stimulated by GA_3. The three LPL activities have previously been shown to have distributions of the acidic and basic forms that are approximately equal, predominantly basic and predominantly acidic, respectively. Inactivation of the LPL activity in each sample by increasing concentrations of the IgG fraction is shown in Fig. III-4. Also shown is the inactivation of a homogeneous LPL I isolated by Fujikura (5). It is evident that regardless of the proportion of the acidic activity in each of the three samples, the inactivation profiles are the same. Interestingly, 15-20% of the activity is resistant to the IgG and probably represents a non-specific lipolytic acyl hydrolase present in each of the samples. As expected, the LPL I activity was completely inactivated. It is evident from this experiment that the acidic and basic LPL activities are antigenically similar.

Molecular Size of the Acidic LPL

The incubation medium of GA_3-stimulated aleurone layers containing mainly acidic LPL was subjected to gel filtration on BioGel P100. The elution profile is shown in Fig. III-5A. The activity emerges mainly

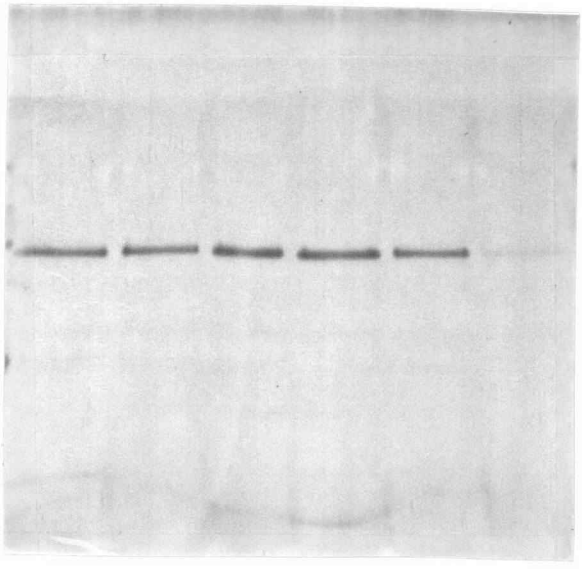

Fig. III-3 SDS-polyacrylamide gel electrophoresis of gel filtration fractions from a purified LPL activity according to Fujikura (5). The fractions shown were combined and used for antiserum production.

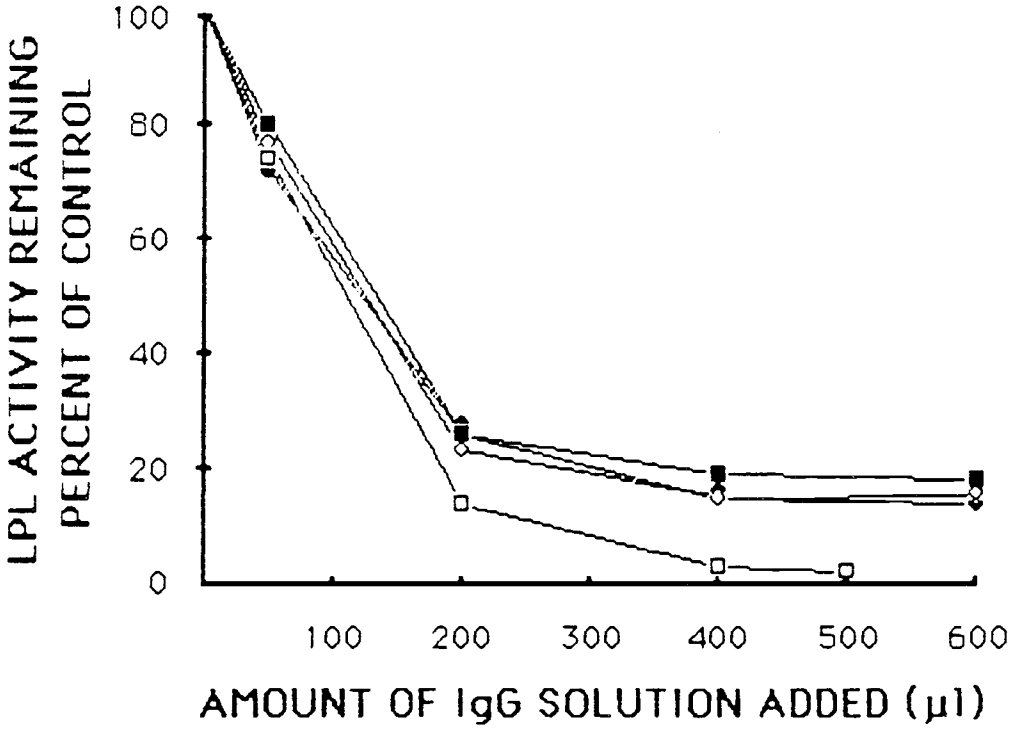

Fig. III-4 Inactivation of LPL activity by immunoabsorption with IgG. An equivalent amount (40 nmol/min) of LPL activity was incubated overnight at 21°C with varying amounts of anti-LPL IgG. Details are described in Materials and Methods. LPL activity from, ●: aleurone layer GA_3 incubation medium; O: half-seed GA_3 incubation medium; ■: aleurone layer homogenate; □: purified LPL.

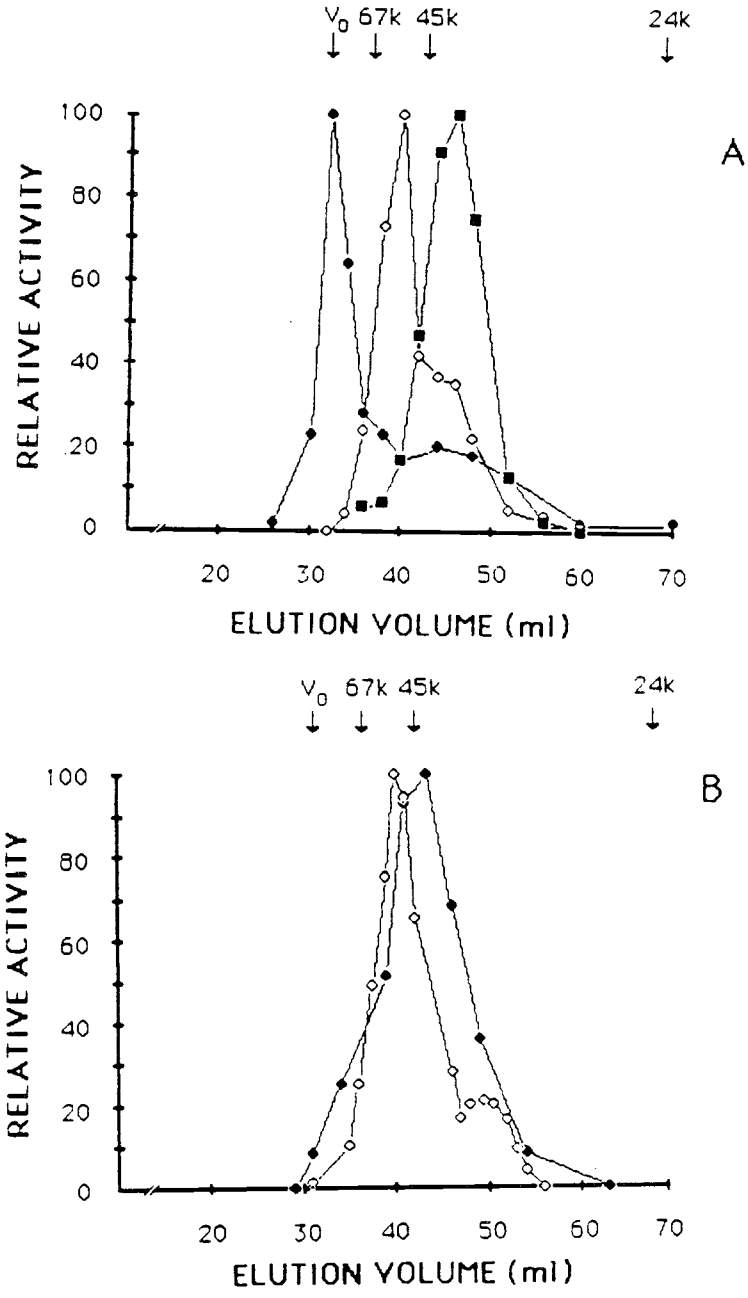

Fig. III-5 Gel filtration (BioGel P100) elution profile of acidic and basic LPL. The 36 hour incubation medium of twenty aleurone layers incubated with the GA_3 buffer containing 20 mM $CaCl_2$ was prepared and applied to the column as described in Materials and Methods section. Enzyme activities are relative to the maximum activity for each hydrolase assayed. Arrows refer to elution positions of molecular weight markers in a separate run.

A. Incubation medium of aleurone layers, ●: LPL activity; 0: acid phosphatase; ■: amylase.

B. Incubation medium as in A above treated with 20 mM EDTA, ●: LPL activity; 0: acid phosphatase.

in the exclusion volume of the column suggesting the nature of the activity to be a large molecular weight complex. A smaller proportion of the activity appears in a diffuse lower molecular weight range with a peak located at an elution volume equivalent to that for the basic LPL. In contrast, two other hydrolases secreted with LPL, acid phosphatase and α-amylase, appear in elution volumes consistent with their known molecular weights and clearly distinguishes LPL from these and possibly other hydrolases secreted by barley aleurone layers. Addition of EDTA to a second sample of the same incubation medium and application to the same column shows a shift of the LPL activity from a high molecular weight complex to an elution volume more consistent with the molecular weight of the characterized basic LPL (Fig. III-5B).

Fractions eluted off a BioGel P300 column (Fig. III-6) and applied to an electrophoretic gel (Fig. III-7) show the association with other proteins. Interestingly, the protein pattern is relatively uniform across the peak and suggests a specific association of proteins with the LPL to give it the acidic character. The basic LPL is a very hydrophobic protein and its association with specific proteins during its transfer from the intracellular to the extracellular environment may be the means by which tight and irreversible binding to membrane lipids is avoided. On entering the starchy endosperm, the association with these proteins is broken. The possibility that the disaggregation would lead to a more efficient basic enzyme with a lower K_m prompted measurement of a K_m for the aggregated enzyme. This is shown in Fig. III-8. The value of 42 μM is comparable with that of 30 μM for the purified basic LPL (5) and

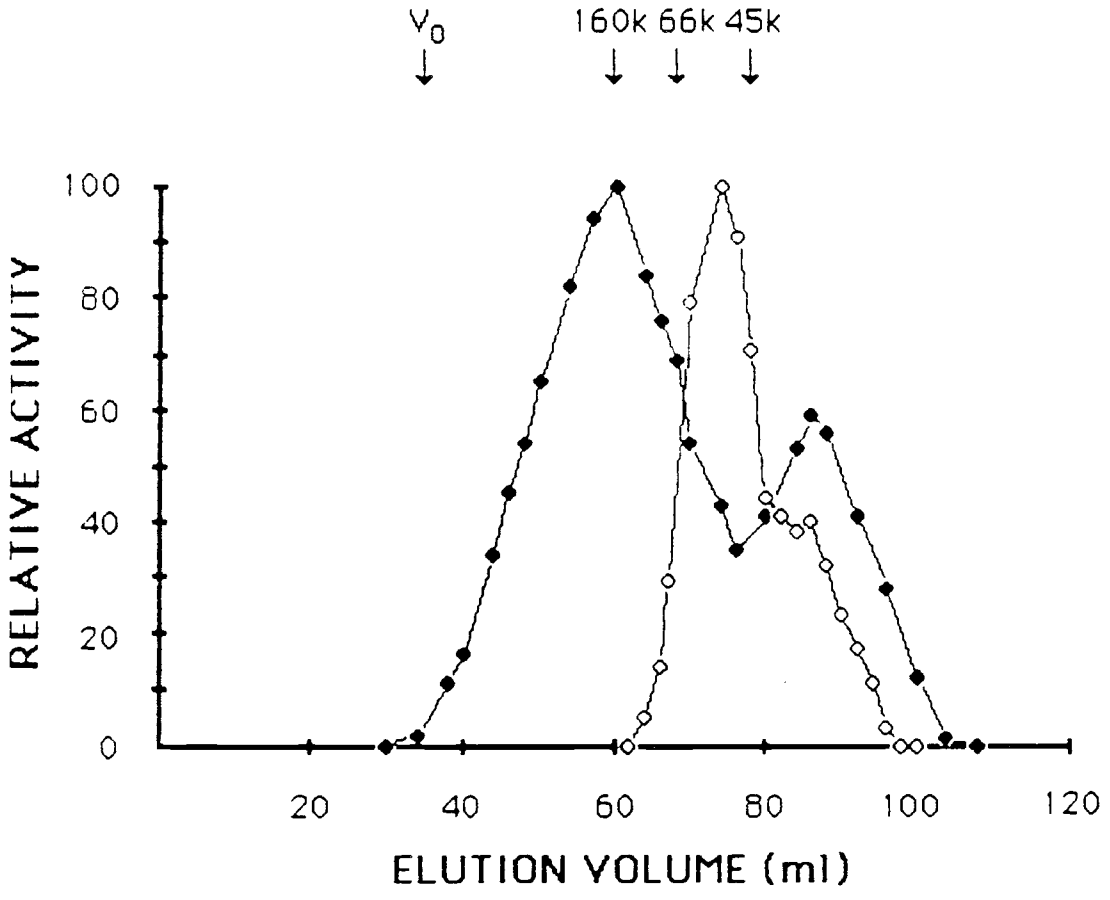

Fig. III-6 Gel filtration (BioGel P300) elution profile of aleurone layer incubation medium. The 28 hour incubation medium of twenty aleurone layers in GA_3 buffer containing 20 mM $CaCl_2$ was applied to the column as described in the Materials and Methods section. Arrows refer to the elution volumes of molecular weight standards. Enzyme activities are relative to the highest activity for each enzyme. ●: LPL; O: acid phosphatase.

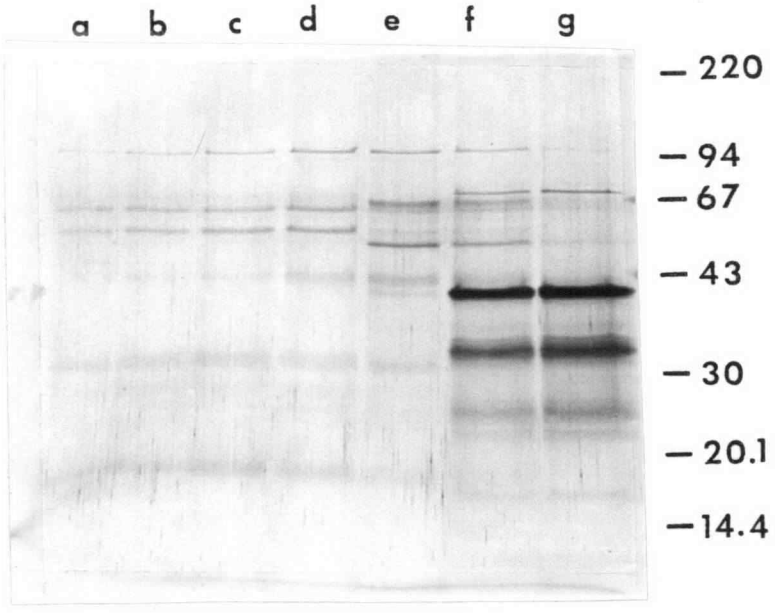

Fig. III-7 SDS-polyacrylamide gel electrophoresis of fractions eluted from BioGel P300. Eluted fractions from the gel filtration column (Fig. III-6) were concentrated 10-fold. Thirty µl was applied to each well as described in Materials and Methods. Numbers refer to the molecular weight ($M_r \times 10^{-3}$) positions of marker proteins. Protein samples applied to the gel have the following elution volumes. a: 41 ml; b: 46 ml; c: 50 ml; d: 60 ml; e: 70 ml; f: 78 ml; g: 84 ml.

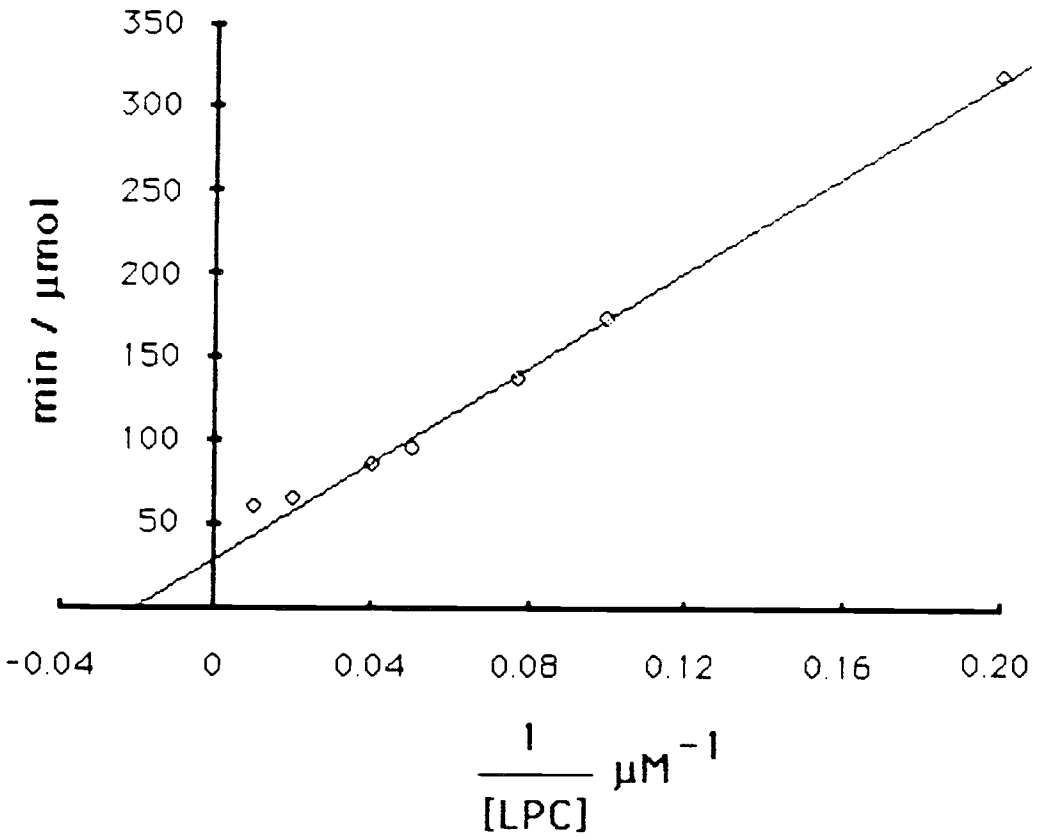

Fig. III-8 Lineweaver-Burk plot of acidic LPL. The 30 hour incubation medium of twenty aleurone layers in the GA_3 buffer containing 20 mM $CaCl_2$ was dialyzed and applied to an ion exchange column. Fifty µl of the (2.8 µg protein) acidic LPL activity was assayed for 15 minutes at 21°C with substrate concentrations from 5 to 100 µM. K_m = 42 µM.

therefore argues against this possibility.

The nature of the binding and its dissociation by EDTA or by a thermally-stable, proteolytic-insensitive barley starch hydrolysis product is unknown. Association of β-amylase with a hydrophobic 'Z' protein present in barley starch has been demonstrated (12). In addition, a protein α-amylase inhibitor in germinating barley has been shown to cause a small pI shift of an endogenous α-amylase isozyme (13). The basic LPL is a glycoprotein (5) and the possibility of lectin-like aggregation cannot be excluded. In this regard, EDTA-inhibition of aggregation of vegetative Dictyostelium discoideum has been observed (14). Inhibition of this EDTA-sensitive agglutination occurs with high concentrations of glucose (ca. 50 mM) and with N-acetylglucosamine (15).

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