AN ABSTRACT OF THE THESIS OF

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The amylose component of cereal starch contains lysophospholipid, predominantly lysophosphatidylcholine (LPC) as inclusion complexes. As starch undergoes degradation by amylases during barley germination, lysophospholipase (LPL) an enzyme which degrades lysophospholipids appears in the starchy endosperms and becomes associated with the starch fraction. The enzyme has been reported to have a pH optimum at 8 with an inflection near 5.

A study with two varieties of Glacier barley, one with a high amylose content, was conducted to assess the influence of starch composition on its mobilization and on other factors during germination. It was found that there is essentially no difference between the two varieties in the rate at which either total starch or amylose is degraded during germination. Included lipid also has no influence on the rate of amylose degradation.

Both LPL activities (pH 8 and 5) increase markedly at day 5 of germination. The proportion of the alkaline activity associated with the starch is greater than for the acidic activity. However, there is no correlation between amylose content or amylose included lipid and

starch-bound LPL activity.

LPL activity has been purified and characterized from a 4 day, unkilned brewing barley variety Advance. It displays the properties of a basic, lipophilic glycoprotein with a pH optimum at 8. The most active glycoprotein (LPL I) has Km 30 μM, Vmax 200 μmol/min/mg protein against LPC-1-palmitoyl as substrate. It has molecular weight 41,000; pI 8.8 and contains 12% carbohydrate on the basis of molecular weight reduction after chemical deglycosylation. Two other unresolved glycoproteins (LPL II) have Km 30 μM, Vmax 50 μmol/min/mg protein and molecular weights 41,000 and 40,000. They also have pI 8.8 and the same molecular weight polypeptide after deglycosylation as deglycosylated LPL I. No phospholipase A, lipase or transacylase activity is shown by the LPLs. Their substrate specificity coincides with the most abundant barley starch lipids.

Mouse antibodies raised against the LPLs have been used to measure LPL in whole endosperms. Although enzyme activity does not increase markedly until day 4 of germination, immunologically active enzyme increases at day 3. The presence of an enzymatically inactive polypeptide in the aleurone, presumably a pre-LPL which is undergoing processing e.g. by glycosylation, is consistent with the observation.

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LYSOPHOSPHOLIPASE OF GERMINATING BARLEY SEEDS

CHAPTER I. INTRODUCTION

Barley Starch Degradation

The mobilization of the barley starch reserves during germination has been the subject of intensive study dating back to 1890 by Brown and Morris (1). The starchy endosperm undergoes degradation by the carbohydrases α- and β-amylase. α-Amylase hydrolyzes the α-1,4 glycosidic links between glucose residues at random points throughout chains of amylose, a linear glucan, and amylopectin, a branched glucan, the two polysaccharides of starch. It degrades amylose into maltose and glucose but because of its inability to hydrolyse the α-1,6 branch points of amylopectin, highly branched areas of glucose units are produced from the branched glucan. β-amylase cleaves successive maltose units from amylose and amylopectin starting at the non-reducing end of the polymer. β-Amylase action on amylopectin yields a branched core of undegraded glucose units, a limit dextrin. The limit dextrins are further hydrolysed by debranching enzyme, R-enzyme and limit dextrinase, to yield small linear oligosaccharides which are then subject to amylolysis (2). The degradation products from the starchy endosperm are absorbed by the scutellum which provides the nutrients to the growing embryo.

Shortly after hydration of barley seeds at the beginning of germination, the aleurone, a layer of cells surrounding the starchy endosperm, produces and releases several hydrolytic enzymes into the

starchy endosperm of the seed. The synthesis and/or release of hydrolytic enzymes from the aleurone layer occurs in response to gibberellic acid (GA). Among these enzymes are α-amylase (3-7) and lysophospholipase (8,9). The significance of the presence of the latter activity lies in a unique feature found in the cereal starches. These starches contain monoacyl lipids which are present as inclusion complexes with the amylose component of the starch (10,11).

Amylose-lysophospholipid inclusion complex

The helical structure of amylose first proposed by Freudenberg et al (12) provides the basis for a starch-lipid interaction. The interior of the amylose helix is hydrophobic (13) and may be occupied by lipophilic substances with suitable dimensions (10,13). X-ray diffraction patterns (14) of amylose-fatty acid adducts (15) were compatible with the formation of an amylose-lipid inclusion complex. In addition, Acker and Becker (10,16) found that low amylose, waxy maize starch has very little lipid whereas high amylose amylomaize starch has more lipid than normal maize starch. A correlation between amylose and lipid contents of starch is clearly suggested. Finally, absorption bands characteristic of a mixture of lysophosphatidylcholine (LPC) and amylose disappear when the lipid is present as an inclusion complex with the amylose.

Lipids present as inclusion complexes with cereal starches (starch lipids) are only removed with extreme difficulty, unlike non-starch cereal lipids which may be extracted with the usual organic solvents (11).

Barley starch lipids are about 1% of the weight of the starch. In composition they are present as lysophosphatidylcholine (LPC), 62%; lysophosphatidylethanolamine, 6%; lysophosphatidylinositol, 3%; and free fatty acid, 4% (10,16). Palmitate and linoleate are the major free fatty acids in the starch lipid mixture and also the major fatty acids providing the acyl chains in LPC (10). The location of the acyl chain in LPC isolated from wheat starch has been shown to be at the C-1 position (17) by the resistance to enzymatic hydrolysis of the lysophospholipid to a phospholipase A_2 from *Crotalus atrox* venom. The resistance of the inclusion complexes to phospholipase B (10,16) and their susceptibility to phospholipase D (18) suggests that the acyl chains of the lipids are oriented inside helical segments of the amylose structure with the polar head groups exposed (Fig. I-1).

The lysophospholipid-amylose complex of cereal grains has been studied by many researchers and several roles for the function of the lipid in starch synthesis and degradation have been proposed. Downton and Hawker (19) suggested that the presence of LPC within amylose molecules might inhibit the enzymatic transformation of amylose to amylopectin by inhibiting the action of the branching enzyme. In support of this they cite the absence of LPC in low amylose, waxy starches. Acker and Becker (16) showed that in developing barley, increasing amylose content paralleled accumulation of lipid phosphorus and choline. Also, Baisted (20) demonstrated a requirement for sucrose, and presumably starch synthesis, for incorporation of acetate-^{14}C and choline-^{14}C into starch lipid in developing barley. Organization of helical segments of amylose by the presence of the included lipids was suggested as contributing to the starch granule

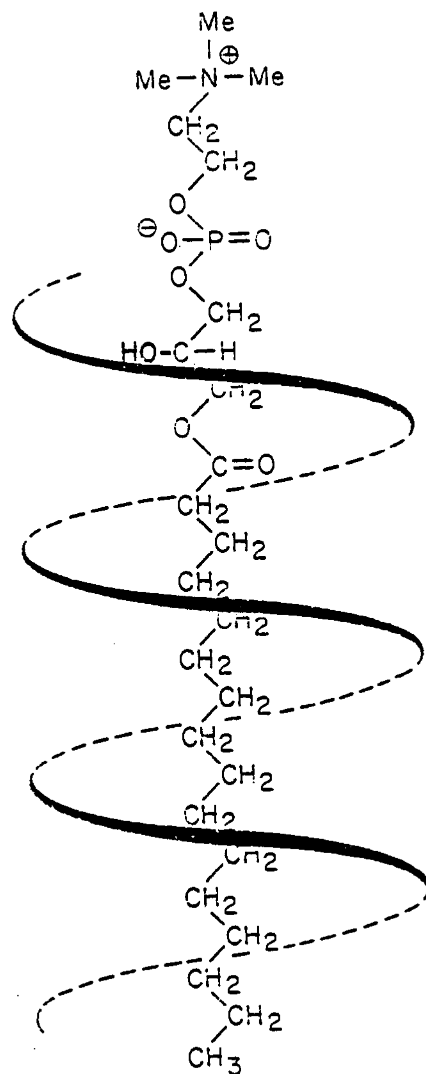

Fig. I-1 Representation of an inclusion complex of lysophosphatidylcholine in an amylose helix (26).

structure. There is no direct evidence however for the role of an LPC-amylose complex in starch synthesis.

Starch degradation occurs mainly by α- and β-amylases. Kugimiya and Yamamoto reported that β-amylase activity was inhibited by adding LPC when amylose was the substrate but that no influence by LPC was found when amylopectin was the substrate (21). On the other hand, Holm et al reported that the LPC-amylose complex displayed a substantially reduced susceptibility to α-amylase in vitro, but by adding a large excess of the enzyme the complex was completely hydrolysed (22). For barley it is known that β-amylase is present in seeds as a latent form and is activated by protease or reducing agents which cleave disulfide bonds (23). This activation of β-amylase appears at an early stage of germination. In contrast, α-amylase is an enzyme synthesized *de novo* and its activity appears after 3 to 4 days of germination (24). These facts might suggest that the LPC-amylose complex may regulate the rate of digestion of the starchy endosperm to supply the appropriate amount of nutrients to the growing embryo at the right time. However it is evident that during germination of barley the rate of digestion of the starchy endosperm is not affected by LPC-amylose complex (Chapter II). Clearly, the timing of the production and release of the amylases themselves is the primary control.

The role of LPC after digestion of the starchy endosperm still remains a puzzle. Weltzier described the influence of LPC on membrane properties such as membrane fusion, cell-surface morphology and the activity of membrane-bound enzymes (25). During germination the digestion of the endosperm tissue results in cell membrane-disruption. The released LPC may accelerate the transport of nutrients to the

embryo and it may also stimulate the release of membrane-associated enzymes. Whatever role is played by LPC in starch synthesis and its degradation, the steady state level of free LPC in the seed is maintained relatively low by an enzyme, lysophospholipase (26).

Lysophospholipase

Lipolytic acyl hydrolases are degradative enzymes which catalyze the transfer of acyl chains from neutral or polar substrates to water. In the case of a neutral substrate such as an acylglycerol the enzyme is called a lipase and may be readily assayed using the artificial substrate, p-nitrophenyl palmitate. In the case of polar substrates such as a phospholipid (A) (Fig. I-2) or lysophospholipid (B) the enzymes are called phospholipase (I) and lysophospholipase (LPL) (II) respectively. An enzyme activity closely related to lysophospholipase is a transacylase (III) in which an acyl chain is transferred from one lysophospholipid molecule to another thereby generating a phospholipid.

LPL activity has been mainly studied in animals in relation to phosphatidylcholine synthesis. In 1965 the activity was first described in rat liver along with transacylase and acyl-CoA dependent acyltransferase (27). A single enzyme which has both LPL and transacylase activities was purified from rat (28)(29) and rabbit (30) lung, and rabbit heart (31) and a reaction mechanism was proposed (31). An enzyme which has exclusively LPL activity was purified from beef pancreas (32) and liver (33), and rabbit heart (34). LPL activity has also been identified in rat stomach mucosa (35).

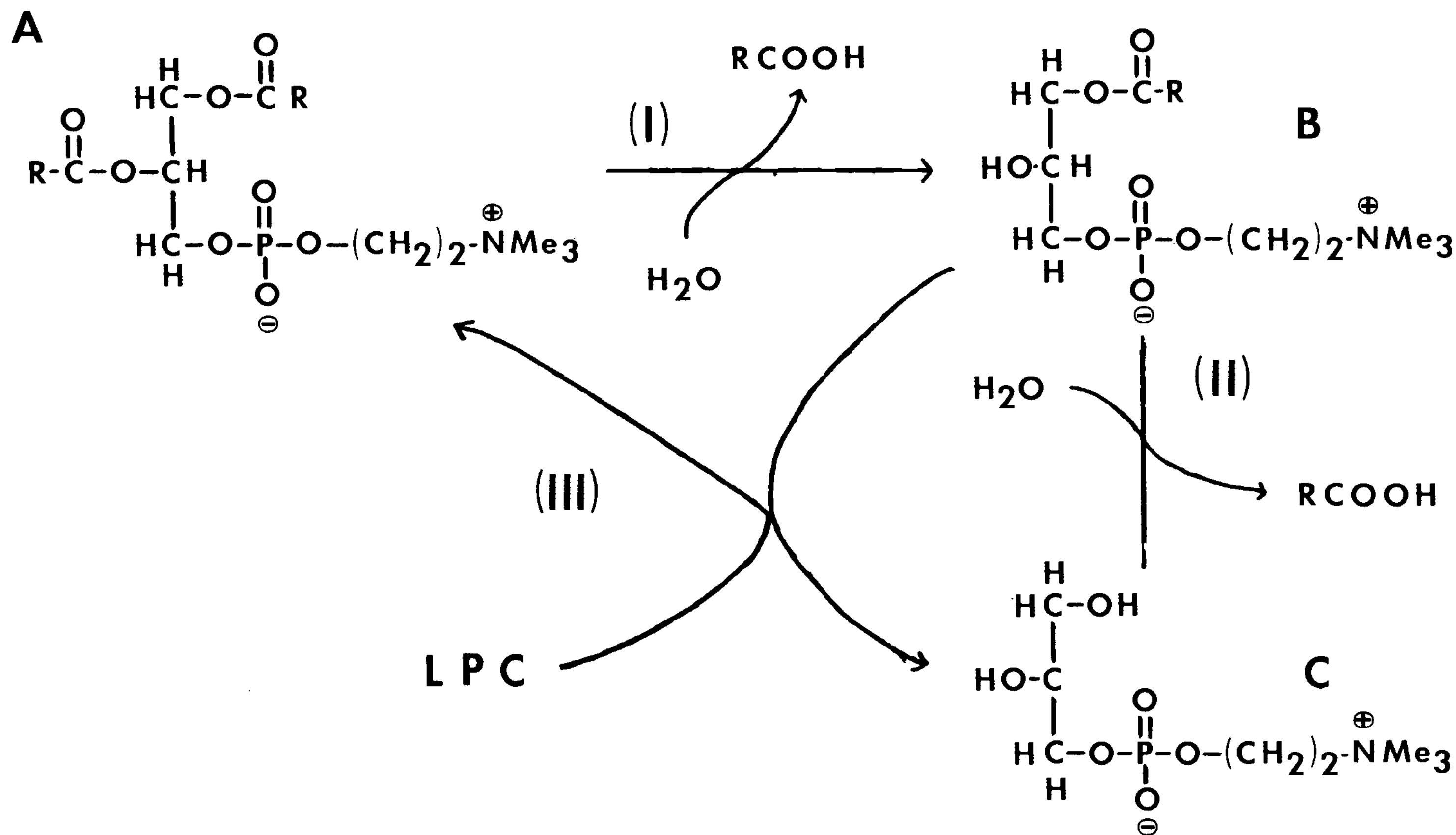

Fig. I-2 Enzyme reactions of lysophosphatidylcholine metabolism A, phosphatidylcholine; B, lysophosphatidylcholine; C, glycerylphosphorylcholine; RCOOH, free fatty acid; I, phospholipase; II, lysophospholipase; III, LPC:LPC transacylase.

A lysophospholipase (LPL) isolated from E. coli (36) acts on lysophosphatidylethanolamine (LPE) but displays no phospholipase activity against phosphatidylethanolamine; neither does it show lipase activity against p-nitrophenyl palmitate.

Plant acyl hydrolases are commonly non-specific, e.g. those isolated from runner bean leaves hydrolyse galactolipids but have higher activities with lysophospholipids and monoacylglycerol (37). The non-specific acyl hydrolases of potato tubers have received much attention (38) and similar activities have been found in several plant species (39). Reports of *Phaseolus multiflorus* (runner bean) acylhydrolase activities describe procedures for the separation, purification and characterization of two such enzymes (40). The relative activities of these enzymes towards phosphatidylcholine and glycosylglycerides have been studied. One activity revealed a preference for phosphatidylcholine rather than glycosylacyl glycerol and the other had the opposite specificity. The common features of the plant lipolytic acyl hydrolases then are their broad specificity and the difficulty with which they are purified.

In 1981 Baisted reported that the loss of starch-bound LPC in germinating barley seeds correlated with an increase in amylase activity. The released LPC did not accumulate. In fact, free LPC was maintained at a very low level during the entire germination period (26). Noting that the loss of LPC from the starch did not occur by the action of phospholipase C or D on the starch-bound lipid, Baisted looked for the presence of a lysophospholipase (LPL). Baisted and Stroud found LPL activity in both the aleurone and endosperm of barley seeds (41). In addition, the activity in embryo-free half seeds is enhanced by

gibberellic acid (8), in a manner similar to that for α-amylase (42). They reported that the soluble activity of LPL in the starchy endosperm increased during the first 3 days then diminished and remained low. In contrast, the particulate activity of LPL in the starchy endosperm increased sharply between day 4 and 5, and remained moderately high. The increase in particulate LPL activity of the starchy endosperm at day 5 interestingly follows the sharp increase in amylase activity at day 4.

The interactions of enzymes with starch was first noted by Akazawa and Murata (43). They found that a soluble starch synthetase could be isolated from glutinous rice in which the starch is amylopectin. A granular complex of the synthetase is formed on addition of amylose or of the high amylose starch granules of non-glutinous rice. Evidently it is the amylose component of cereal starch that is important in the binding. Also Sargeant and Walker (44) and Sargeant (45) have shown that during germination of wheat, the four α-amylase isoenzymes (group I) were readily adsorbed onto undamaged starch granules and subsequently degraded them. An ionic interaction may be a result of binding to protein present on the surface of the starch granules or to the presence of the exposed polar head groups of the included lipids in the amylose component of the starch.

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Chapter II. CHANGES IN STARCH-BOUND LYSOPHOSPHOLIPIDS AND LYSOPHOSPHOLIPASE IN GERMINATING GLACIER AND HIGH AMYLOSE GLACIER BARLEY VARIETIES

ABSTRACT

Total starch, amylose content and amylose-included lipid phosphorus and lysophosphatidylcholine (LPC) were measured in normal Glacier (G) and Hi Amylose Glacier (HA) barley varieties during germination. From days 3 to 6, alkaline and acidic lysophospholipase (LPL) activity in the starchy endosperm was measured and the distribution of these activities between a soluble and starch-bound form determined. During germination the amylose content of the starches increase as the total starch levels decline. The starch-bound LPC and lipid phosphorus disappear at the same rate between days 3 and 6 in both barley varieties, indicating no discrimination among the different lipid-included amylose population for degradation. However, both lipid phosphorus and LPC disappear more rapidly in the G than in the HA variety. This is presumably due to the slightly larger content of LPC per mg amylose of the G than of the HA variety, equivalent to 134 and 150 anhydroglucose residues/lipid molecule in G and HA, respectively. There is no increase in starch-bound lipid phosphorus or LPC expressed as nmoles of P or LPC per mg amylose as amylose content declines, indicating no selective resistance of lipid-included amylose to degradation. The alkaline and acidic LPC activities in each variety increase 2-4 fold between days 4 and 5. In both varieties _ca_. 30% of the acidic LPL and _ca_. 50-60% of the alkaline LPL is particulate from days 3 to 6. No correlation can be made between the content of amy-

lose or amylose-included lipid and particulate LPL activity. However, the possibility that particulate LPL activity is associated with specific populations of residual amylose-included lipid molecules cannot be excluded.

INTRODUCTION

Starch-bound lysophosphatidylcholine (LPC) present as an inclusion complex in the amylose component of barley starch, disappears as germination progresses (1). The lipid does not accumulate and is degraded by a lysophospholipase (LPL) which appears in the scutellum, aleurone and starchy endosperm (2,3). The enzyme activity which is enhanced by gibberellic acid in barley half seeds and isolated aleurone layers (3) appears in a soluble and a particulate form and has activity over a broad pH range with a peak activity near pH 8 and an inflection near pH 5. The particulate activity of the starchy endosperm appears to be associated with the starch fraction and can be solublized by salt extraction (unpublished observation). The transformation of a soluble starch synthetase to a particulate form in the presence of amylose, first observed by Akazawa and Murata (4), suggested to us that differences might be observed between the two varieties of barley, Glacier and Hi Amylose Glacier, with respect not only to the relative amounts of soluble and particulate LPL, but also to the level of starch lipids and to their metabolism. This communication presents the results of experiments examining such possible differences between these two varieties of barley.

MATERIALS AND METHODS

Materials

Seeds of Hordeum vulgare L. cultivars Glacier and Hi Amylose Glacier 1970 crops were kindly supplied by Dr. Robert F. Eslik, Montana State University. The average dry weight of the HA variety was 48 mg and that of the G variety, 52 mg. Hordeum distichum L. cultivar Georgie was supplied by RHM, Crops Department, High Wycombe, U.K. L-α-lysophosphatidylcholine 1-[1-14C] palmitoyl and L-α-phosphatidylcholine di-[1-14C] palmitoyl were obtained from Amersham, U.K. Unlabelled 1-palmitoyl lysophosphatidylcholine and wheat starch were from Sigma. Potato amylose was a product of the U.S. Biochemical Corporation, Cleveland, Ohio.

Seed germination

The seeds were germinated in the dark by total immersion in water for 24 hours and then transferred to moist paper towels in loosely covered dishes at 20-23 C for specified times. The seeds were rinsed with water each day.

Measurement of starch and amylose

Starch was measured according to the procedure of ref. (5). For each germination interval the starchy endosperms were separated from duplicate groups of 10 seeds. Each of the duplicate endosperm samples

was macerated in (2 ml of) 10 mM phosphate buffer (pH 7), with a pestle and mortar. The starch pellet produced by centrifugation of the resulting suspension at 800 g for 5 minutes was washed repeatedly with 80% EtOH. The washed starch was digested with 52% HClO4 and the starch content was measured by the anthrone-H_2SO_4 method as follows. The anthrone reagent was made by mixing 2 g of anthrone and 1 ℓ of 95% sulfuric acid. Five ml samples containing 25-100 ug of hexose were placed in 25 x 250 mm borosilicate glass tubes. To each sample was added 10 ml of cold anthrone reagent. The tubes were shaken vigorously to ensure complete mixing and were heated in boiling water bath for 7.5 minutes. Then the tubes were cooled in a water bath to 25 C and A620 was measured. Glucose solutions containing the same amount of perchloric acid as that in the sample were used as a standard. A standard curve was prepared daily. The amylose content of the starch was determined by the method described by McCready and Hassid (6). An aliquot of the $HClO_4$ solution containing 0.1 mg of each starch sample was diluted to 4.95 ml with water, 0.05 ml of I_2-KI solution was added and after 30 minutes A660 was measured.

The blank was starch free $HClO_4$ solution. The I_2-KI solution was made up of 250 mg of I_2 and 2.5 g of KI in 125 ml of water. A standard curve was made using a mixture of known composition of potato amylose and wheat amylopectin (7).

Isolation and measurement of starch-bound lipid phosphorus and LPC

The starchy endosperm of duplicate samples of 10 seeds at specific germination intervals were separated and ground with 3 ml of 10 mM

phosphate buffer. The homogenates were washed with distilled water 5 times and were dried in a vacuum oven overnight. Free lipid was removed from the pulverized endosperm by rapid extraction with cold water-saturated butanol (WSB). Starch-bound lysophospholipids were then isolated from the residual seed material by repeated extraction with boiling WSB (8). Non-lipid contaminants of the starch-bound lipid were removed by first evaporating the solvent from the extract and passing the residues in Folch lower phase, $CHCl_3$-MeOH-H_2O (86:14:1), through a column of Sephadex G-25 previously equilibrated with Folch upper phase (3:48:47) (9). After evaporation of the solvent, the lipid was dissolved in $CHCl_3$ and stored at -20 C. An aliquot of the $CHCl_3$ extract containing 0.1-1.0 μmole of phosphorus was dried completely. To the sample was added 0.4 ml of 70% $HClO_4$ and the mixture was heated on a sand bath until a clear solution was obtained. After cooling, 4 ml of distilled water was added. Then 0.6 ml of ammonium molybdate-H_2SO_4 solution (mixture of 50 ml of 5% ammonium molybdate and 100 ml of 5 N H_2SO_4) and 0.2 ml of Fiske-Subbarow reagent (mixture of 0.5 g of 1-amino,2-naphthol,4sulfonic acid in 200 ml of freshly prepared 15% sodium bisulfite and 1.0 g of anhydrous sodium sulfite) were added. The mixtures were vigorously agitated and after 15 minutes the absorbance at 660 nm was measured. Inorganic phosphate solution was used as a standard (10). LPC was separated by the thin layer chromatography using 0.1 ml of the $CHCl_3$ extracts. LPC-phosphorus was measured from scraped LPL zones. The lysophospholipids were separated on activated Si gel G plates using $CHCl_3$-MeOH-AcOH-H_2O (50:30:8:3) in solvent-saturated tanks. The lipids were located with I_2.

Isolation and assay of LPL of the starchy endosperm

Batches of barley were germinated for 1-6 days. Duplicate groups, each of nine seeds, were used for the measurements. The starchy endosperms were removed from the seeds and macerated with 6 ml ice-cold phosphate buffer (10 mM, pH 7) with a pestle and mortar. The suspension was spun at 4000 g for 5 minutes. The pellet was resuspended in the phosphate buffer to give a total volume of 3 ml. Both the supernatant and the pellet suspension were assayed as follows. LPC (12,000 cpm, 200 nmol) was evaporated in a 15 ml centrifuge tube. The substrate was dissolved in 10 mM Tris-HCl (pH 8, 0.8 ml) for the alkaline LPL assay, and in 10 mM sodium acetate (pH 5, 0.6 ml) for the acidic assay. Appropriate amounts of either supernatant or pellet suspension were added to give final volumes of 1.0 ml. The assays were run at 25 C and were stopped with 6 ml heptane-iso-PrOH-0.5 M H_2SO_4 (60:40:1). One half of the heptane extract was counted in 10 ml of a scintillation fluor composed of 4 g 2,5-diphenyl oxazole in 1 ℓ toluene-Triton X-100 (95:5). Counting was on a Beckman LS200 liquid scintillation counter.

RESULTS AND DISCUSSION

The mobilization of starch and the decline in its amylose component during germination of the Hi Amylose Glacier (HA) and Glacier (G) barley varieties is shown in Figs. 1a and 1b. In each case, the convergence of the starch and amylose data points as germination progresses illustrates the increasing proportion of amylose in the residual starch (Fig. II-1) and confirms the previously observed increase in the amylose/amylopectin ratio which accompanies the mobilization of starch in cereal grains (11). The accessibility for attack by β-amylase of the large number of non-reducing ends of each amylopectin molecule compared with the single non-reducing end of an amylose molecule is clearly an important factor contributing to the increasing amylose-amylopectin ratio. We have also suggested (2) that the presence of inclusion lipids in the amylose component of the starch may hinder attack by this enzyme, thereby contributing to the increasing amylose composition of the starch.

The two Glacier barleys used in this study differ in the amylose content of their starches. As the starch-bound lipid phosphorus is in the form of an inclusion complex with the amylose component of the starch (12), different metabolic profiles of starch mobilization and starch lipid metabolism during germination might be anticipated. Figures 1a and 1b show that substantial metabolism of the starch reserves occurs between days 3 and 6 of germination; consequently, starch-bound lipid phosphorus and LPC were measured at one-day intervals in seeds which had imbibed water for one day and had germinated for 3 to 6 days. The data is shown in Table II-1.

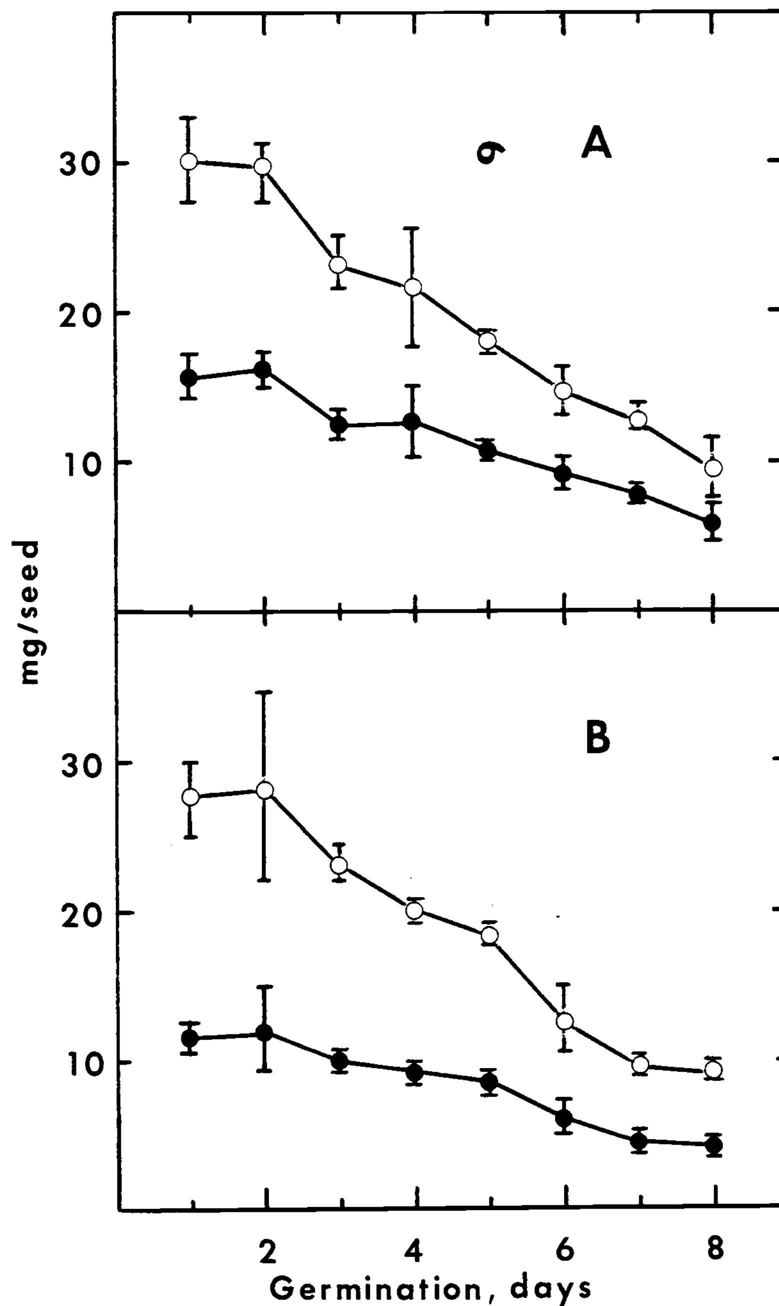

Fig. II-1 Starch (○) and amylose (●) contents of the starchy endosperms of germinating HA (A) and G (B) varieties of barley. The methods used for the measurement of the polysaccharides are described in the Materials and Methods. Each data point represents the average of at least duplicate measurements made on duplicate groups of 10 seeds each throughout the germination interval shown.

Table II-1 Loss of Starch Lipid Phosphorus and LPC from HA and G Varieties

Germination (days)	Phosphorus (umol/seed) G	Phosphorus (umol/seed) HA	Phosphorus (nmol/mg amylose) G	Phosphorus (nmol/mg amylose) HA	LPC (umol/seed) G	LPC (umol/seed) HA	LPC (nmol/mg amylose) G	LPC (nmol/mg amylose) HA
1	0.50	0.50	43	32	0.38	0.38	33	25
3	0.50	0.53	50	41	0.36	0.34	36	28
4	0.44	0.50	48	34	0.28	0.33	31	26
5	0.32	0.44	38	40	0.23	0.27	27	26
6	0.26	0.36	50	43	0.18	0.23	30	26

The lipid phosphorus determinations were made as described in the Materials and Methods. Each value is the average of two measurements, each of which is reproducible to within 10% of the average. The values for mg amylose were the average used in Fig. I-1.

The starch-bound lipid phosphorus (μmoles/seed, Table II-1) appears to suffer a sharper decline during the 3-6 day germination period in the G than in the HA variety; ca. 50% loss in G and 32% in HA. These are the same percentage losses shown in the starch-bound LPC column for the respective barleys over this germination interval. This is consistent with the earlier observation (2) that there is no discrimination by degrading enzymes for amylose molecules included with LPC than for amylose molecules included with any other lysophospholipid.

Comparisons of Figs. II-1-A and II-1-B reveal that the overall rate of degradation of amylose in the two varieties between the third and sixth day of germination is essentially the same. The explanation for the increased rate of disappearance of lipid phosphorus in the G variety is found in Table II-1 in which the lipid phosphorus is expressed as per mg of amylose. Clearly, although the G variety has a lower amylose content, there is more included lipid per amylose molecule and therefore a greater loss of included lipid upon degradation of the polysaccharide. From Table II-1, over the 4 days of germination, the average bound lipid phosphorus is 46 and 41 nmoles/mg amylose for the G and HA varieties, respectively. Assuming the total lipid phosphorus to be present in a form similar to LPC, these values represent 134 and 150 anhydroglucose residues for each included lipid for the G and HA barleys, respectively. These values fall within the range of 100 to 200 calculated by Acker for the included lipids of the cereal starches (6).

Evidently the presence of included lipid in the amylose does not affect its rate of degradation. Were the lipid to exert an inhibitory effect, the lipid phosphorus per mg of amylose would increase during

germination. These data in Table II-1 suggest that molecules of amylose included with the lysophospholipids are no more resistant or susceptible to degradation than amylose molecules not included with lipid. If the capping of the non-reducing ends of included amylose by the polar head groups of lysophospholipids were to inhibit attack by β-amylase, then it is likely that the degradation of included amylose occurs from attack by α-amylase.

Alkaline and acidic LPL activities dramatically increase in the starchy endosperm of both the HA and G barley varieties between the 4th and 5th day of germination as shown in Figure II-2. The proportion of each activity which is associated with the particulate fraction is shown in Table II-2. It is clear that the alkaline activity is associated more with the particulate fraction than is the acidic activity. It is also evident that no direct correlation can be made between bound enzyme activity and either amylose or starch lipid content; the amount of LPL activity associated with the particulate fraction is increasing during this 3 to 6 day germination period and at the same time both the amylose and included lipid contents of the starches are decreasing (Fig. II-1 and Table II-2). This does not preclude the possibility that the enzyme is binding to a specific population of amylose or lipid-included amylose molecules. However, as it is the polar head group of the included lipid which apparently distinguishes one amylose population from another, then it seems reasonable to assume that if the particulate enzyme is associated with starch it is the lipid-included amylose that may be the site of binding.

Table II-2

Percentage of Total LPL Activities Associated with The Starch Fraction in HA and G Varieties

Germination (days)		Total LPL associated with the starch fraction (%) 3	4	5	6
Alkaline	HA	43	54	57±4	47
	G	48	64	61±7	65
Acidic	HA	30	32	36±4	25
	G	32	32	31±1	32

The acidic and alkaline LPL activities were measured on the crude supernatant obtained from a 4000 x g centrifugation of a homogenate of the starchy endosperms of the two barley varieties. The pellet fraction was resuspended and the LPL activities assayed as described in the Materials and Methods. The values represent the percentage of the total LPL activity in the starchy endosperm that is associated with the 4000 x g pellet. With the exception of day five which was run on duplicate batches of germinated seed, the remaining values are single determinations.

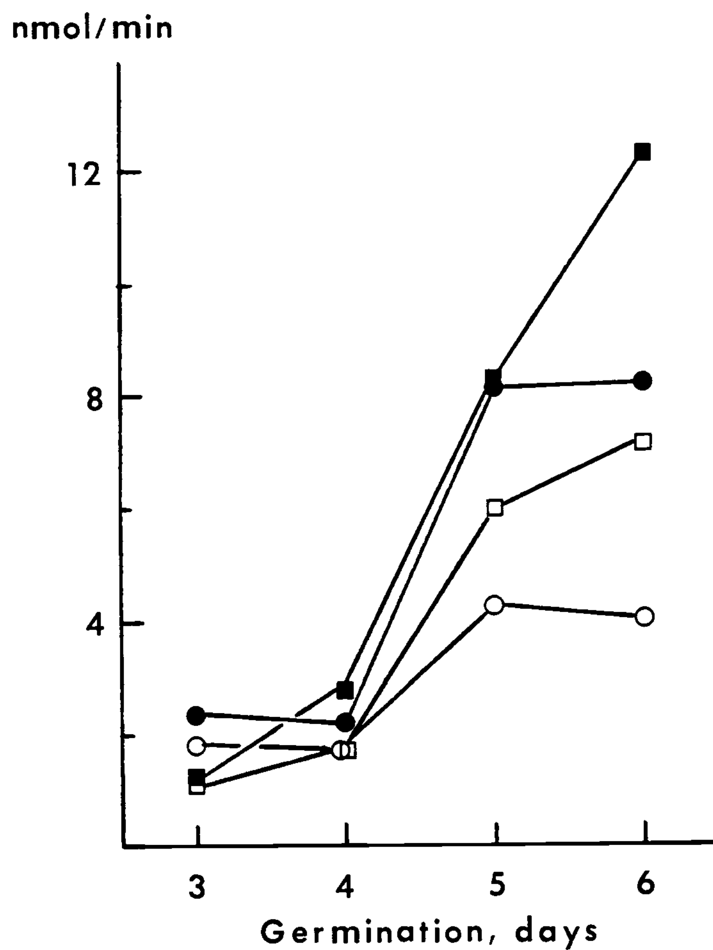

Fig. II-2 Lysophospholipase activities per seed of the starchy endosperms of barley.

G: alkaline (■) and acidic (□) activities; HA: alkaline (●) and acidic (○). Isolation and measurements of the lysophospholipase activities are described in the Materials and Methods.

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Chapter III. PURIFICATION AND CHARACTERIZATION OF BASIC BARLEY LYSOPHOSPHOLIPASE

ABSTRACT

Lysophospholipase from germinating barley seeds has been isolated using methods which take advantage of the fact that the activity is basic, lipophilic and contains carbohydrate. There appears to be at least three enzymic forms of the activity, two with molecular weights at 40,000 and one at 41,000. They co-migrate with a pI of 8.8 on isoelectric focussing and they all undergo deglycosylation to give a 36,000 dalton polypeptide indicating 10 to 12% carbohydrate in the original glycoproteins.

The enzyme is inactivated by sulfhydryl reagents and has a tendency to aggregate. The latter property may be attenuated with mercaptoethanol with which the activity is stable for more than three months at 4°C. The barley enzyme has a specific activity 20 times greater than that isolated from animal sources. It has no phospholipase, lipase or transacylase activity. It is most active on lysophosphatidylcholine with a saturated 16 carbon or unsaturated 18 carbon chain; these are the predominant molecular species of lysophospholipid present as inclusion complexes in barley starch. The role of the barley lysophospholipases in barley germination is discussed.

Mouse antibodies raised against the LPLs have been used to measure LPL in whole endosperms. Although enzyme activity does not increase markedly until day 4 of germination, immunologically active enzyme

increases at day 3. The presence of an enzymatically inactive polypeptide in the aleurone, presumably a pre-LPL which is undergoing processing e.g. by glycosylation, is consistent with the observation.

INTRODUCTION

Lysophosphatidylcholine (LPC) is the major starch lipid of barley. It exists as an inclusion complex with the amylose component of the starch (1). Although α- and β-amylases are major hydrolases for the digestion of the starchy endosperm, it has been reported that the amylose-LPC complex is resistant to β-amylase (2) and its susceptibility to α-amylase is reduced (3). This suggests that one of the roles of LPC may be to regulate the rate of starch breakdown during germination. An alternative role has been suggested in which the membrane-perturbing properties of lysophospholipids may promote an increase in the fluidity of the aleurone plasma membrane and thereby enhance the secretion of the cereal hydrolases during germination (4). Similarly, uptake by the scutellum plasma membrane might be enhanced by free lysophospholipids in the starchy endosperm thereby promoting the uptake of metabolites. To avoid cell lysis, the level of free lysophospholipids must be controlled. Lysophospholipases which actively degrade LPC have been identified in the starchy endosperm of germinating barley (5). Isolated aleurones have also been shown to secrete lysophospholipase (LPL) in response to gibberellic acid (6). In the whole seed, LPL activity occurs in the barley aleurone, scutellum and starchy endosperm from early germination onwards (7). LPL activity reaches a maximum in the starchy endosperm after 5 days, shortly after α-amylase activity has peaked, suggesting that its role is to dispose of lysophospholipids released from the degraded starch. Most of the LPL activity in the starchy endosperm is particulate. Although soluble starch synthetase can be

converted to a particulate form by amylose (8) the association of LPL with starch is unrelated to the starch amylose content (9).

Lysophospholipases have been purified and characterized from animals and bacteria (10-13). In this communication we report the purification, properties and role of a basic LPL in germinating barley.

MATERIALS AND METHODS

Materials

Hordeum vulgare var. Advance germinated for 4 days was obtained frozen in dry ice from the Great Western Malting Company, Vancouver, Washington. Lysophosphatidylcholine, [1-^{14}C]-palmitoyl (56 mCi/mmol, ^{14}C-LPC) and phosphatidylcholine, di-[1-^{14}C]palmitoyl were obtained from Amersham Corporation Ill., USA. Unlabelled LPC and protein molecular weight markers for SDS gel electrophoresis and anti-mouse goat IgG alkaline phosphatase conjugate were from Sigma, St. Louis, Mo. Protein molecular weight markers were from Schwartz/Mann Standards and proteins for isoelectric focussing (Iso Gel pI Markers) were from FMC Corporation. Ampholytes for isoelectric focussing were from BioRad and were available as a 40% solution called Biolyte with a pH range 3.5-9.5.

Methods. Purification of lysophospholipase

All purification procedures were carried out at 4°. About 200 g of frozen seeds were homogenized in a Waring blender with 200 ml of buffer containing 30 mM phosphate, pH 7, and 0.2 M NaCl. The whole homogenates were centrifuged at 7000 x g for 10 min. The centrifuged residues were reextracted with an additional 100 ml of the same buffer and the combined supernatants were dialyzed for several hours, first against 4 ℓ of 30 mM phosphate (pH 7, containing 30 mM β-mercaptoethanol) and then overnight against 4 ℓ of buffer of the same

composition to which glycerol was added to give a 10% concentration (v/v) (Buffer A). The dialyzed extract was centrifuged at 10000 x g for 10 min to remove insoluble protein. The supernatant was applied to a column (3x50 cm) of carboxymethylcellulose (CMC) previously equilibrated with buffer A. Flow-through protein was eluted with buffer A until fractions showed less than 0.100 O.D. at 280 nm. Basic LPL was fractionated by elution with a 0 - 0.5 M NaCl gradient. The active fractions eluting in the range 0.1 -0.18 M NaCl were combined and SDS added to give a concentration of 0.01% (w/v). The enzyme solution was applied to a column (1.6x4.0 cm) of octyl agarose previously equilibrated with buffer A containing 0.01% SDS (Buffer B). Inactive protein was eluted from this column first with buffer B (50 ml) and then with buffer B, 2 M in NaCl (50 ml). LPL was removed from the column with a 50% ethanol buffer containing 0.5 M NaCl and 10 mM $NaHCO_3$. Active fractions were collected and dialyzed overnight against buffer B. The dialyzed sample was applied to a column (1.6x3.5 cm) of Con A-sepharose previously equilibrated with buffer B containing 0.1 M NaCl. After washing the column with the same buffer, a gradient of α-methyl mannoside in buffer B was applied. However, only trace amounts of LPL activity were removed by elution of the column with 1.0 M α-methyl mannoside. Subsequently, LPL was eluted in 1.0 M NaCl in buffer B. A second hydrophobic chromatography was carried out on the combined active fractions from the lectin columns using the less hydrophobic phenyl agarose in a column (1.6x4.0 cm) with buffer B. A high specific activity LPL, designated LPL I, was eluted in one column volume of buffer. Further washing with the buffer did not remove the remaining activity. The 50%

ethanol-NaCl-HCO_3 used for removal of LPL from octyl agarose was used to elute a second LPL activity, designated LPL II. Both activities were concentrated against polyethylene glycol (MW 20,000) and then dialyzed against a buffer composed of 50 mM phosphate, pH 7, 30 mM β-mercaptoethanol, 150 mM NaCl and .01% SDS (Buffer C). Each LPL was subjected to gel filtration on BioGel P100 in a column (2x50 cm) using buffer C. The flow rate was 10 ml/hr and 1 ml fractions were collected. Fractions containing LPL, free of low molecular weight contaminants, were combined. The LPLs were stored at 4° for several months with no loss of activity.

Assay of LPL activity

The activity was measured according to the procedure described by Baisted and Stroud (5). To LPC-[1-14C]-palmitoyl (200 nmoles, 10^4 cpm) dissolved in 0.9 ml 0.1 M Tris pH 8 in a 15 ml centrifuge tube is added 0.1 ml of enzyme solution. The reaction is run at 21° with slow shaking for a period of time to ensure linear kinetics. The reaction is stopped by the addition of 6 ml of a mixture of heptane-isoPrOH-1NH_2SO_4 (60-40-1) followed by vigorous agitation on a vortex mixer. After separation of the mixture into two layers by centrifugation on a clinical centrifuge, one-half of the organic phase is taken for quantitation of ^{14}C in the liberated palmitic acid. Radioactivity was measured by scintillation counting on a Beckman LS200 instrument using a fluor composed of 4g PPO in 1 ℓ of toluene-Triton X100-H_2O (6:3:1).

The pH optimum for the activity was run by dissolving the substrate in 0.1 M buffers: acetate (pH 4-6); phosphate (6.5-7.5) and

tris (7.5-9) before the addition of enzyme.

For the influence of potential activators and inhibitors on the LPL activities LPL I and II were first dialyzed overnight against 30 mM phosphate, pH 7. Additions were made to the substrate just before addition of enzyme for all the chemicals except phenylglyoxal. For the latter, the enzyme was preincubated at 25° for 50 min before conducting the assay.

Coomassie blue methods for protein quantitation

The reagent solution was prepared according to the method of Bradford (14). 5 ml of Coomassie blue solution (100 g of Coomassie blue in 50 ml of 95% of EtOH) and 10 ml of 85% phosphoric acid were mixed well and made up to 100 ml with water, and filtered. To 0.1 ml of the sample containing 2-20 ug of protein was added 2 ml of the reagent solution and the mixture quickly agitated. After 5-10 minutes the absorbance at 595 nm was measured. Bovine serum albumin was used as the standard (15).

SDS-gel electrophoresis

The procedure is based on Laemmli's method (16). The separation gel (10%) was made by mixing 2 ml of pH 8.9 buffer (9.6 ml of 1 N HCl, 7.26 g of tris-(hydroxymethyl)aminoethane(Tris) and 46 µl of tetramethylethylenediamine (TEMED) in a total volume of 20 ml with H_2O), 4 ml of 20% acrylamide containing 0.4% N,N-bis-methylene acrylamide and 2 ml of 0.14% ammonium persulfate containing 0.4% SDS. The stacking

gel (5%) was made by mixing 0.3 ml of pH 6.8 buffer (9.6 ml of 1 N HCl, 1.2 g of Tris, and 92 ul of TEMED in a total volume of 20 ml with H_2O), 0.6 ml of 20% acrylamide containing 0.4% N,N-bis-methylen acrylamide, 0.3 ml of 0.004% riboflabin, 0.9 ml of 40% sucrose (W/V), and 0.3 ml of 0.8% of SDS. First the separation gel was polymerized in a slab (9x6x1) cm between two glass plates. The stacking gel solution was then poured. A Comb with 7 mm wide wells was inserted assuring about 7 mm in depth for the stacking gel. The stacker was polymerized under UV light. The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Protein samples (50 μl) were mixed with 25 μl of 0.02% bromophenol blue containing 40% glycerol and a small amount of SDS and mercaptoethanol. The mixtures were immersed in boiling water for 5 min, then cooled and applied to the gels. Electrophoresis was carried out first at 50 V until the stacking dye reached the top of the separating gel then at 110 V until the dye reached the bottom.

Silver nitrate staining

After electrophoresis the gel was soaked in 50% MeOH with gentle agitation overnight. The staining solution was prepared by adding dropwise 4 ml of silver nitrate (20%) into an alkaline solution (21 ml of 0.36% NaOH and 1.4 ml of 58% ammonium hydroxide) with constant stirring. The volume was then increased to 100 ml with H_2O. The gel was stained in the solution for 15 min with constant agitation. Destaining of the gel was accomplished by first washing with deionized water for 20 min. This was repeated two more times with gentle agita-

tion. The gel was then transferred to the developer (10 mg of citric acid and 10 µl of 37% formaldehyde in 200 ml of H_2O). The protein bands appeared in 10-15 min. Staining was stopped by washing the gel with 50% MeOH (17).

Periodic acid silver nitrate staining for glycoproteins (18)

After electrophoresis the gel was fixed in 25% iso-PrOH 10% AcOH overnight with gentle agitation and then soaked in 7.5% AcOH for 30 min. The gel was transferred into 0.2% periodic acid and stored at 4 C for 1 hour. The gel was washed for 3 hours with gentle agitation in several changes of H_2O. The washed gel was subjected to the silver nitrate staining procedure (17).

Deglycosylation with TFMS

Deglycosylation of the barley LPLs was achieved using the procedure described by Edge et al. (19). After gel filtration, LPL I and II were separately dialyzed against H_2O and 40 µg of each freeze-dried. To each sample was added in a Teflon-lined capped tube 0.5 ml of a mixture of anisole/TFMS (1:2,v/v) and the reaction run at 4° for 3 hours with stirring. Cold ether at -40° (1.0 ml) and ice-cold pyridine (50%, 1.5 ml) were added in order and the mixture thoroughly agitated. The ether layer was discarded and the mixture extracted three more times with ether. The aqueous samples were subjected to SDS-PAGE after dialysis against 2 mM pyridine acetate buffer pH 5.5. The reagents used in this procedure are hazardous and caution is required

in their handling.

Substrate specificity of LPL activity.

Chain length specificity was determined by conducting the assay using a mixture of 200 nmoles each of non-radioactive LPCs with acyl chain lengths ranging from C12:0 to C18:0 and C18:1 as the substrate. Fatty acid composition of the product resulting from enzyme-catalyzed hydrolysis was by GLC of the derived methyl esters. Polar head specificity was investigated by comparing the amount of palmitate released from 200 nmoles of LPC with that from 200 nmoles of lysophosphatidylethanolamine, each carrying C16:0, in two separate assays. When the reactions were terminated, one ml of each extract and 25 or 50 μg of a C17:0 fatty acid in $CHCl_3$ solution as internal standard were transferred to a Teflon-lined capped tube. The mixtures were evaporated under N_2 and the residues esterified with 1 ml of BF_3-MeOH at 100° for 2 min. One ml of saturated NaCl was added to the cooled reaction mixtures and the esterified esters extracted with 0.5 ml heptane. Analyses of the fatty acid methyl esters (FAME) was by GLC on a 6 ft x 1/8 in stainless steel column packed with 15% OV 275 on Chromosorb W, acid-washed, 80-100 mesh. The column was run at 160° with a N_2 flow rate of 30 ml/min on a Varian Model 3700 gas chromatograph equipped with a flame ionization detector. The output was recorded and automatically integrated on a Varian 4270 integrator. One μl samples of each extract were analyzed and all analyses were in triplicate. The area response for each FAME relative to that for the internal standard was used to compute the amount of each fatty acid released in the

assay.

Phospholipase A assay

This was similar to the procedure described by Robertson and Lands (20). Phosphatidylcholine-[1-^{14}C]-1,2-dipalmitoyl (25,000 cpm, 200 nmole) in toluene-ethanol was evaporated in a Teflon-lined, screw-capped tube and then dissolved in 2 ml of ether. LPL I (0.1 ml) and 0.1 ml of 0.5 M Tris pH 7.5, 8 mM in $CaCl_2$ were added to the substrate. A control tube had the enzyme omitted. The capped tubes were vigorously shaken for 75 min at 21°. The mixtures were washed into conical flasks with methanol (5 ml) and chloroform (10 ml) and the solutions dried over anhydrous Na_2SO_4. The extracts were evaporated to small volumns on a rotary evaporator and the products along with standards for phosphatidylcholine and palmitic applied to silica gel G TLC plates. The plates were developed in hexane: Et_2O: formic acid (60:40:2 by vol.). Lipids were located by placing the plate in an I_2 tank. Radioactive zones were located by scanning the plates with a Packard TLC scanner and the radioactivity measured by scraping the zones into vials for counting.

Non-specific lipase assay

9.5 ml of p-nitrophenyl palmitate was dissolved in 2.4 ml of 10% Triton-X 100/$CHCl_3$. The solution was evaporated with nitrogen gas. To the residue was slowly added 30 ml of 0.1 M acetate (pH 5) with vigorous shaking using a vortex mixer. 0.3 ml of the enzyme solution

was added to 2.9 ml of the substrate to start the assay. After 30 minutes at 21 C 0.8 ml of 0.2 M Tris was added to stop the reaction and the absorbance at 400 nm was measured. A p-nitrophenol solution was used as the standard. Duplicate measurements were conducted for each assay.

Transacylase assay

The procedure was based on that of Gross et al. (21). LPC (50,000 cpm, 1 μmole) was evaporated in a 15 ml test tube and dissolved in 0.9 ml of 0.1 M phosphate (pH 7). 0.1 ml each of the purified LPL I and II were added to the substrate in separate tubes. The reaction was run for 40 min at 21° C. Reaction products were extracted into 500 μl of butanol by vortexing and the layers were separated by centrifugation at 1000 x g for 5 minutes. 100 μg of the organic (upper) layer was applied to a Si gel G plate using developing solvent of $CHCl_3$-MeOH-AcOH-H_2O (65:50:1:4) (22) in a solvent-saturated tank. The lipids were visualized with I_2 and identified by running standards adjacent to the unknown on the same plates. The distribution of radioactivity on the plates was measured with a thin layer chromatogram scanner, Packard Model 201. Radioactivity was measured by scraping off the radioactive zones into vials and counting.

Production of anti-LPL antibody

The mixture of LPL I and II from Con-A sepharose chromatography was subjected to a Bio-Gel P-100 gel filtration. The purity of the

enzymes was checked by the silver nitrate staining of SDS-gel electrophoresis. The active fractions were pooled and concentrated to 20 μg/ml in dialysis tubing in contact with polyethlene glycol. The concentrated solutions were dialysed against phosphate buffered saline (PBS: NaCl 8 g, KCl 0.2 g, KH_2PO_4 0.3 g, and $Na_2HPO_4.7H_2O$ 2.17 g in H_2O 1 ℓ) overnight with two changes. A mouse (Balb/c female) was injected with 0.4 ml of a vigorously stirred mixture of the antigen solution and Freund's complete adjuvant (1:1). The mixture of PBS and the adjuvant was injected into a second mouse to obtain control serum. Two weeks later the injections were repeated except the incomplete adjuvant was used instead of the complete. One week later a third injection was made. 10 days later 0.2 ml of SP-2 myeloma cells (ascites tumor cells at 10^6 cells/ml) was injected to boost the antibody production. Then two weeks later the sera were collected from the mice. The tappings were repeated weekly for the next several weeks. The pooled sera were centrifuged at 1200 x g for 15 minutes. Sodium azide was added to the supernatants to give a concentration of 0.1% and the sera were kept at 4 C (23).

Purification of immunoglobulin fraction

5 ml of the antiserum was adjusted to pH 8.6 with 0.2 N NaOH. 1.46 g of ammonium sulphate (50% saturation) was added to the serum and the mixture centrifuged at 10,000 x g for 10 minutes. The pellet was washed at 4 C with 3 ml of 1.75 M ammonium sulphate to remove albumin, transferrin, and a-protein including haptglobin and haemoglobin. The final white pellet was dissolved in 10 mM phosphate pH 7

and dialysed overnight against the buffer. The precipitated lipoproteins were removed by centrifugation at 10,000 x g for 10 minutes. The supernatant was used as the immunoglobulin solution (24).

Enzyme linked immuno-sorbent assay (ELISA)

The sample antigen solution was dialysed against PBS overnight. To each well of a micro titer plate (Falcon 3070) was added 200 µl of the sample solution (.03 to 1 µg/ml). The plate was incubated at 37 C for 30 minutes and at 4 C overnight. The plate was washed four times with PBS/Tween 20 which contains 0.5 g of Tween-20 in 1 ℓ of PBS. 200 µl of various concentrations of antiserum and control serum diluted with PBS/Tween-20 were placed in the designated wells. After 3 hours of incubation at room temperature the plate was washed four times with PBS/Tween-20. To each well was added 200 µl of alkaline phosphatase-conjugated anti-mouse goat IgG. The plate was incubated at room temperature for 3 hours and then subjected to the same washing procedure five times. 200 µl of p-nitrophenyl phosphate (1.0 mg/ml in 0.1 M glycine pH 10.4) was added to each well. After 30-60 minutes the enzyme reaction was stopped by the addition of 50 µl of 2 N NaOH and the absorbance at 405 nm was measured by EIA READER (BIO-TEK Instrument Inc.) (25).

RESULTS AND DISCUSSION

Purification of the Basic Lysophospholipase.

LPL has been found in the starchy endosperm, aleurone and scutellum of germinating barley (7). A time-course of appearance of the activity shows that after 4 days germination the barley LPL becomes associated with the starch fraction (5). The activity is readily released from homogenates of germinating seeds by extraction with NaCl. LPL is strongly inhibited by sulfhydryl reagents and the presence of a SH protector, e.g. mercaptoethanol, was found to be necessary to minimize losses of activity. Both mercaptoethanol and glycerol maintained in the buffers improved the recovery of the activity. However, it is evident that the basic barley LPL poorly tolerates high ionic strength solutions.

Most of the LPL activity is a basic protein as is evident from the elution pattern from the carboxymethyl cellulose column (Fig. III-1). Most of the barley protein however, is acidic and is eluted with low ionic strength buffer. In this fraction a small amount of an acidic LPL appears.

In exploratory studies of the distribution of LPL we have found the activity to be enriched in the fatty layers of supernatant fractions of crude homogenates. This indicates the LPLs are lipophilic and may contain a high proportion of non-polar amino acids which occur in clefts or domains accessible to added lipids. The lipid nature of the substrate clearly indicates that the active site must possess non-polar residues involved in its binding. Based on these observations,

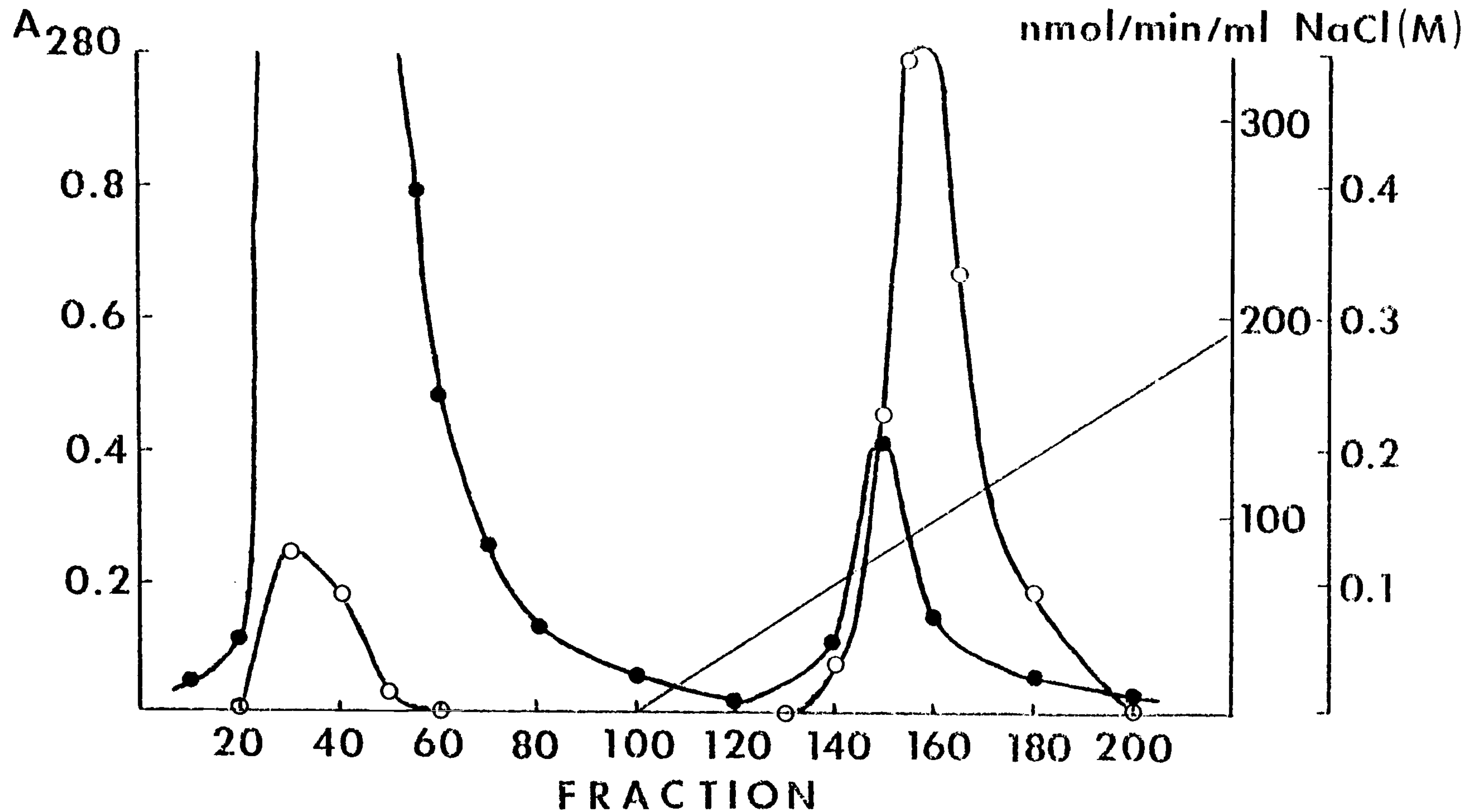


Fig. III-1 Purification of barley lysophospholipase by CMC column chromatography 8 ml fractions, (●), UV absorbance at 280 nm; (○), enzyme activity; (--), NaCl gradient.

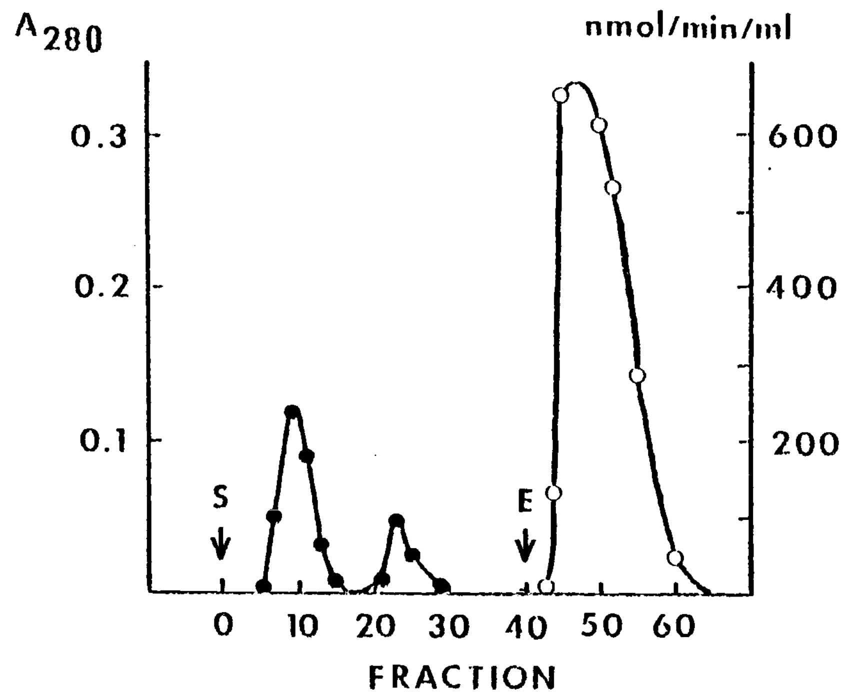


Fig. III-2 Purification of barley lysophospholipase by Octyl Agarose column chromatography. After the enzyme binding, the column was washed with buffer B and phosphate buffer, buffer B containing 2 M NaCl (S) and ethanol buffer (E) were applied. 1.5 ml fractions; (●), UV absorbance at 280 nm; (○), enzyme activity.

Table III-1 Purification of Lysophospholipase from Barley Malt

Step	Protein (mg)	Total Act. (μmol/min)	Sp. Act. (μmol/min/mg)	Yield (%)	Purification (-fold)
NaCl extraction	4200	201	.048	100	---
CM-Cellulose	260	101	.39	50	8
Octyl-Agarose	1.4	64	45	32	940
Con. A-Sepharose	.21	28	133	14	2770
Phenyl-Agarose	nd	nd	---	---	---
Bio-Gel P-100 (I)[a]	.009	1.8	200	0.9	4170
Bio-Gel P-100 (II)[b]	.087	4.4	51	2.2	1050

a Enzyme activity which does not bind to Phenyl-Agarose
b Enzyme activity which binds to Phenyl-Agarose
nd Not determined

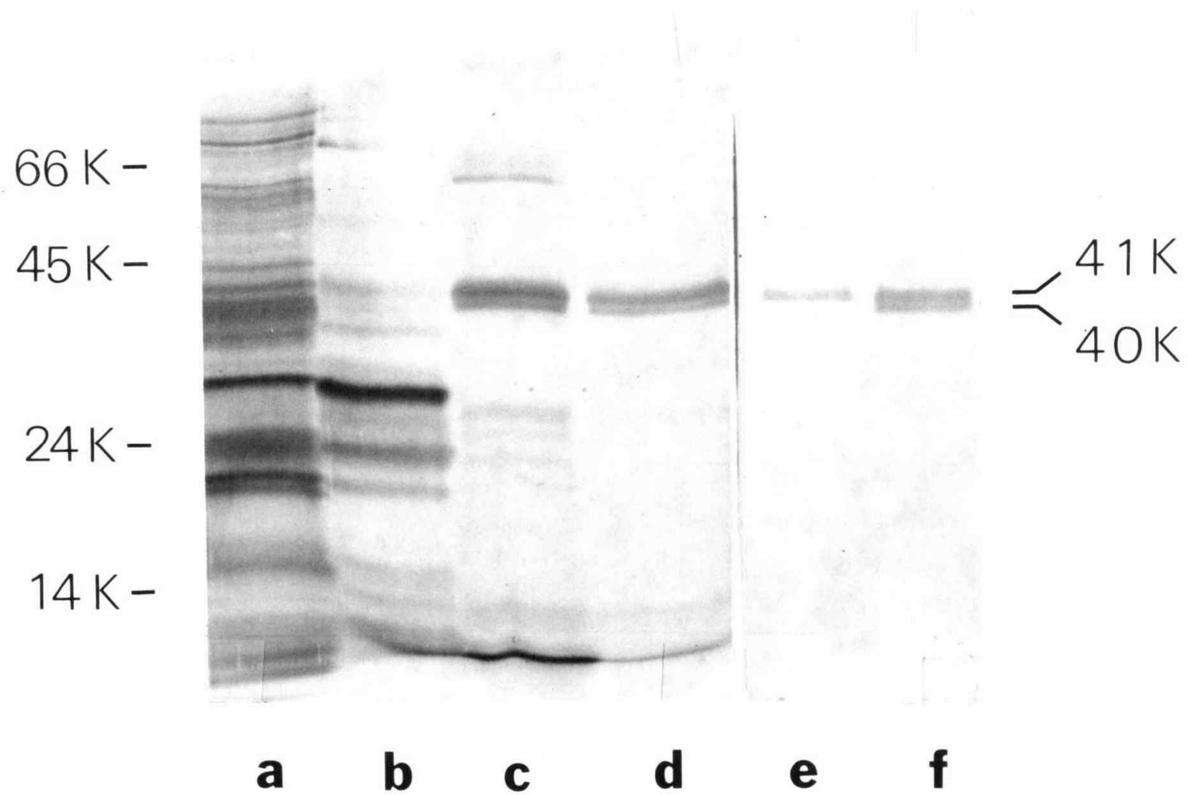
it was not surprising then that the fractions with basic LPL activity from the carboxymethyl cellulose column would have high affinity for a hydrophobic column of octyl agarose; the activity is only removed with a 50% ethanol buffer (Figure III-2). There is a substantial purification of the activity after the hydrophobic column treatment (Table III-I). The distribution of protein on SDS-gel electrophoresis (Figure III-3, column 'c') illustrates this very well and shows that silver staining of material in the 41000 molecular weight region of the gel is particularly heavy. These proteins also stain positively for glycoprotein with periodic acid-Schiff base and periodic acid-silver nitrate. Other hydrolases including esterase and acid phosphatase from barley malt have been shown to be glycoproteins (26). Carbohydrate in very small amounts has also been shown to be present in barley α-amylase (27).

These observations prompted us to use a lectin column for the next stage of the purification. LPL activity is extremely tightly bound to Con A sepharose. A gradient of α-methyl mannoside (0-0.5 M) failed to release the activity and only a trace was removed with α-methyl glucoside. The activity was removed with 1.0 M NaCl buffer. The binding of LPL to the lectin column evidently involves not only interactions with the sugar moieties but also strong ionic interactions. At this stage the activity from the lectin column shows only two major bands at 40,000 and 41,000 on SDS-gel electrophoresis (Figure III-3 'd'). Isoelectric focussing of the sample shows a single band at pI 8.8 (Figure III-4) which suggests that the difference between the two bands on SDS-gel electrophoresis lies in the microheterogeneity of their carbohydrate chains. Microheterogeneity within the carbohydrate

Fig. III-3 SDS-Polyacrylamide gel electrophoresis of barley lysophospholipase.

Aliquots from the purification steps were prepared for SDS gel electrophoresis and were applied to the slab gel. Electrophoresis was run at 50 V for 30 minutes and 110 V until the tracking dye reached the bottom. (a) 10 μg from NaCl extract; (b) 2 μg from CMC chromatography; (c) 400 ng from Octyl Agarose chromatography; (d) 70 ng from Con-A Sepharose chromatography; (e) 20 ng of purified LPL I; (f) 80 ng of purified LPL II.

Fig. III-3



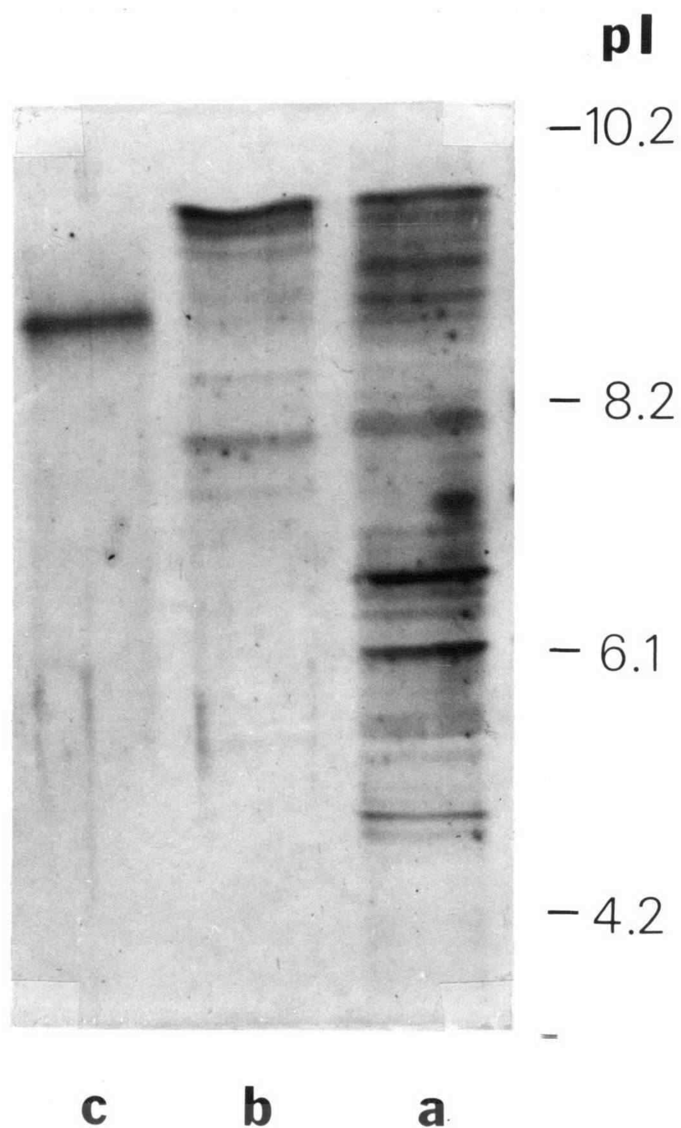


Fig. III-4 Urea-IEF of barley lysophospholipase. Aliquots from the purification steps were applied to the gel. (a), 10 ug from NaCl extract; (b), 1 ug from CMC chromatography; (c), 100 ng from Octyl Agarose chromatography.

moiety of the barley malt esterase has been indicated (26). In general, microheterogeneity of carbohydrate side-chains of glycoproteins is common (28,29) and may result from differences with respect to the point of attachment of the carbohydrates to the polypeptide chain or from different carbohydrate structures at the same point of attachment. A consequence of lipophilic glycoproteins having different degrees of glycosylation might be that they would exhibit small differences in lipophilicity. Conceivably, the protein with the higher molecular weight would be less lipophilic because of the potentially greater concealment of lipophilic domains by the carbohydrate moieties. To test this, a column less hydrophobic than octyl agarose was chosen to attempt to resolve the product obtained from the lectin column. Chromatography on phenyl agarose gave two active fractions; one did not bind to the column, (LPL I) and the second could only be eluted with a 50% ethanol buffer, (LPL II). Traces of low molecular weight contaminants in LPLs I and II were removed by gel filtration on BioGel P100. We observed that in the absence of mercaptoethanol the activity appeared in the void volume indicating that aggregation involving sulfhydryl groups of the glycoproteins had occurred. In the presence of mercaptoethanol the activity eluted in a volume consistent with a molecular weight of 38,000 (Fig. III-5). Gel electrophesis of the purified LPLs I and II are shown in Figure III-3, 'e' and 'f' respectively. Their molecular weights based on their mobility are shown in Fig. III-6.

LPL I is a single band at 41000 and has the higher specific activity (Table III-I). LPL II is composed of a pair of protein bands at 40,000 and 41,000. Since no further attempt has been made to

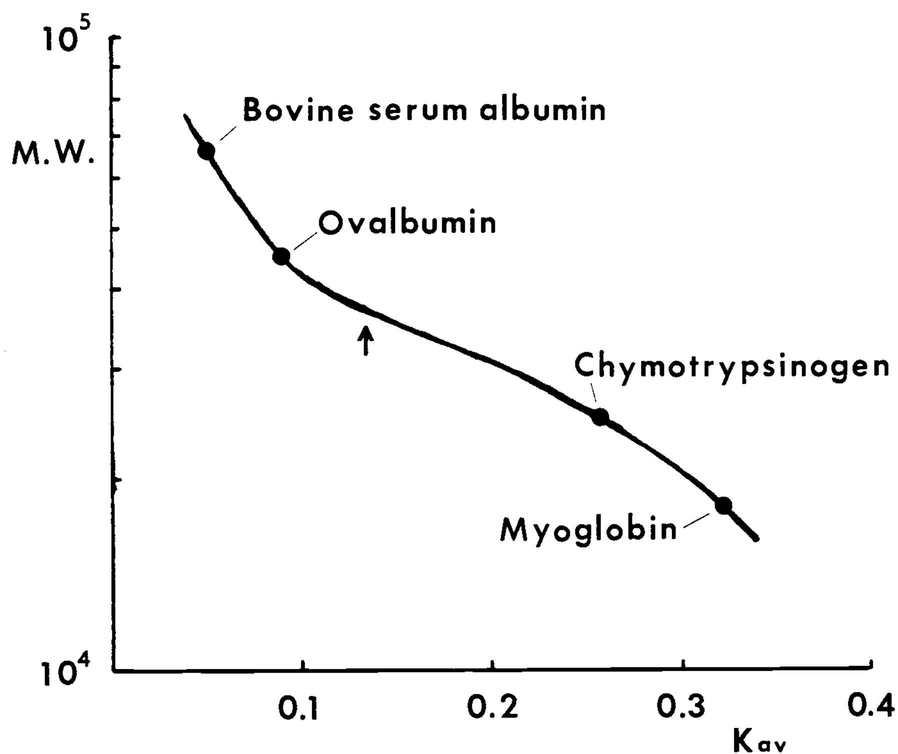


Figure III-5 Estimation of molecular weight of barley LPL by Bio-Gel P-100 gel filtration.

Mixture of standards (bovine serum albumin 67,000; ovalbumin 45,000; chymotrypsinogen 25,000; and myoglobin 18,000) and LPL after Octyl Agarose column was applied to Bio-Gel P-100 column (3 x 20 cm), and 1.4 ml fractions were collected.

The distribution coefficient (K_{av}) is defined as follows,

$$K_{av} = (V_e - V_o)/V_x$$

V_e, elution volume; V_o, void volume; and V_x, gel beads volume.

LPL activity (↑) came out around 38,000.

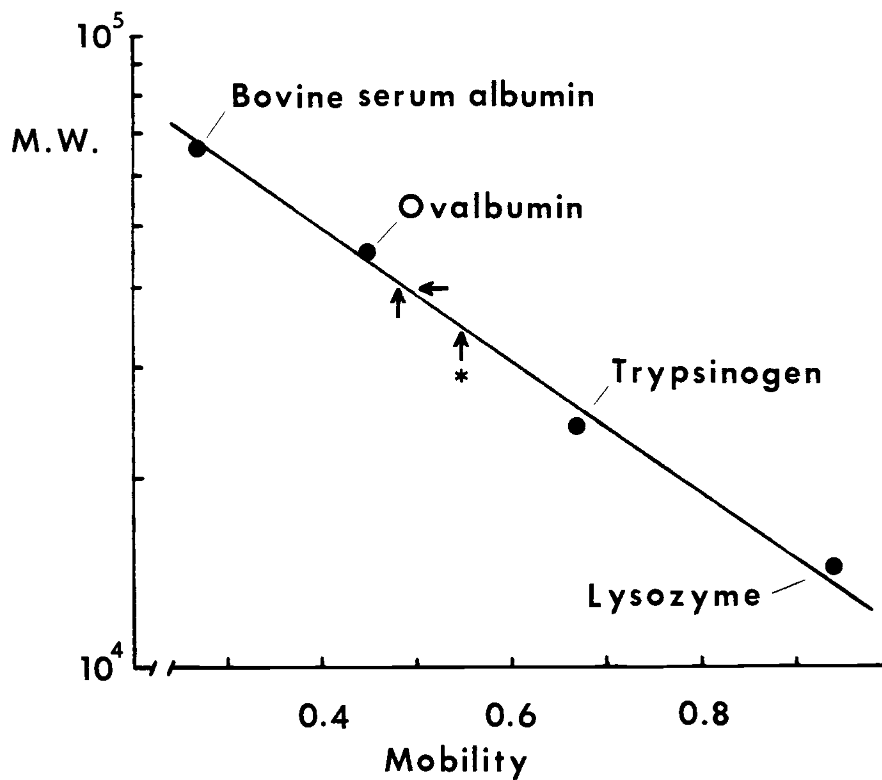


Fig. III-6 Estimation of molecular weight of barley LPL by SDS gel electrophoresis.

Molecular weight standards are bovine serum albumin, 66,000; ovalbumin, 45,000; trypsinogen, 24,000; and lysozyme, 14,300. From the figure, the molecular weights are LPL I (↑), 41,000; LPL II (←), 40,000; and deglycosylated LPL I and LPL II ($\underset{*}{\uparrow}$), 36,000.

resolve LPL II it is not known yet whether both proteins have LPL activity or only one. However, the result of deglycosylation of the LPLs suggests that there may be at least three basic, lipophilic glycoproteins with LPL activity.

pH Optimum and Kinetic Behavior of Basic Lysophospholipase.

LPL activity has a pH optimum of 8 in tris buffer (Figure III-7). This compares with the pH optima of basic LPLs from other sources which fall in the range 7.5 to 8.5 (12,13). The kinetic behavior of each purified LPL is shown in Figure III-8 and 9. They have essentially the same Km which is somewhat greater than that for LPL derived from animal sources (12,13). The Vmax is substantially larger for LPL I than for LPL II however and both are greater than Vmax reported for LPL from any other source. The critical micelle concentration of lysophosphatidylcholine is about 7 μM and it is clear from the kinetic data that the reaction must be taking place with the micellar form of the substrate.

Influence of Activators and Inhibitors on Lysophospholipase.

The influence of a number of potential activators and inhibitors, both metal ions and organic molecules on LPLs I and II, is shown in Table III-2-a and b). There is remarkably good agreement between the two throughout the entire Table. The Ca cation is a modest inhibitor of LPL activity. This contrasts with a closely related enzyme, phospholipase A2, which requires Ca for activity. The remaining metal

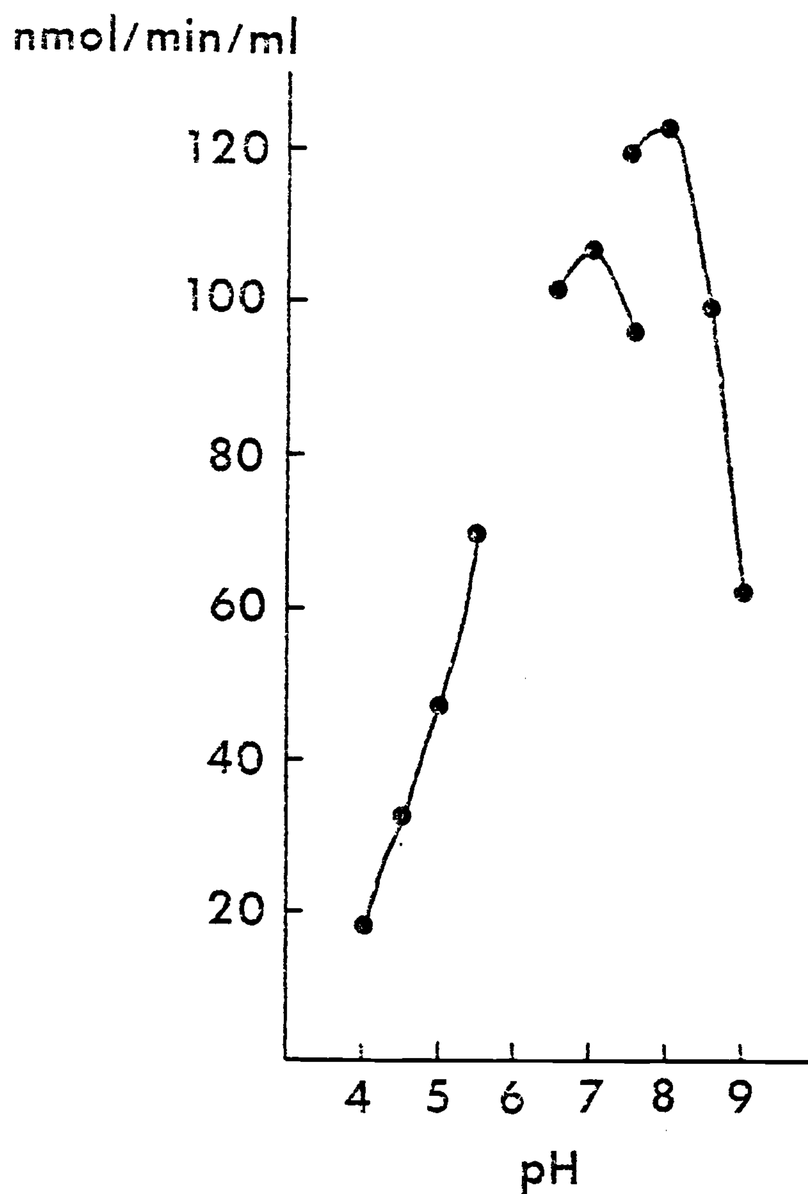


Fig. III-7 Effect of pH on barley lysophospholipase activity 0.1 M of sodium acetate (pH 4-6), potassium phosphate (pH 6.5-7.5) and tris-HCl (pH 7.5-9) were employed as the buffers.

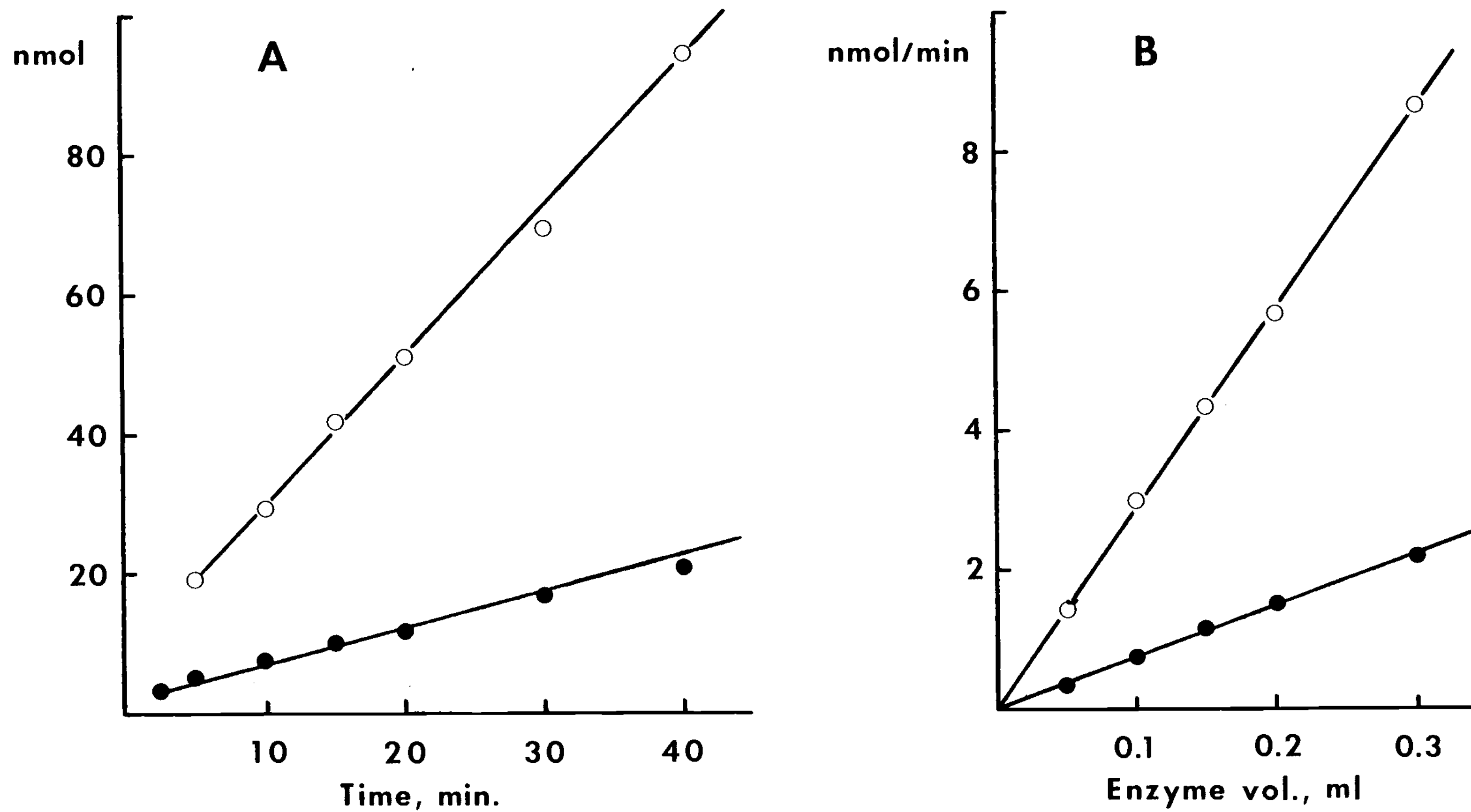


Fig. III-8 Enzyme activity studies. The diluted solutions (150 ng/ml) of purified LPL I (○) and II (●) were assayed at pH 8. (A), reaction time vs. amount of the product; and (B), enzyme volume vs. reaction rate.

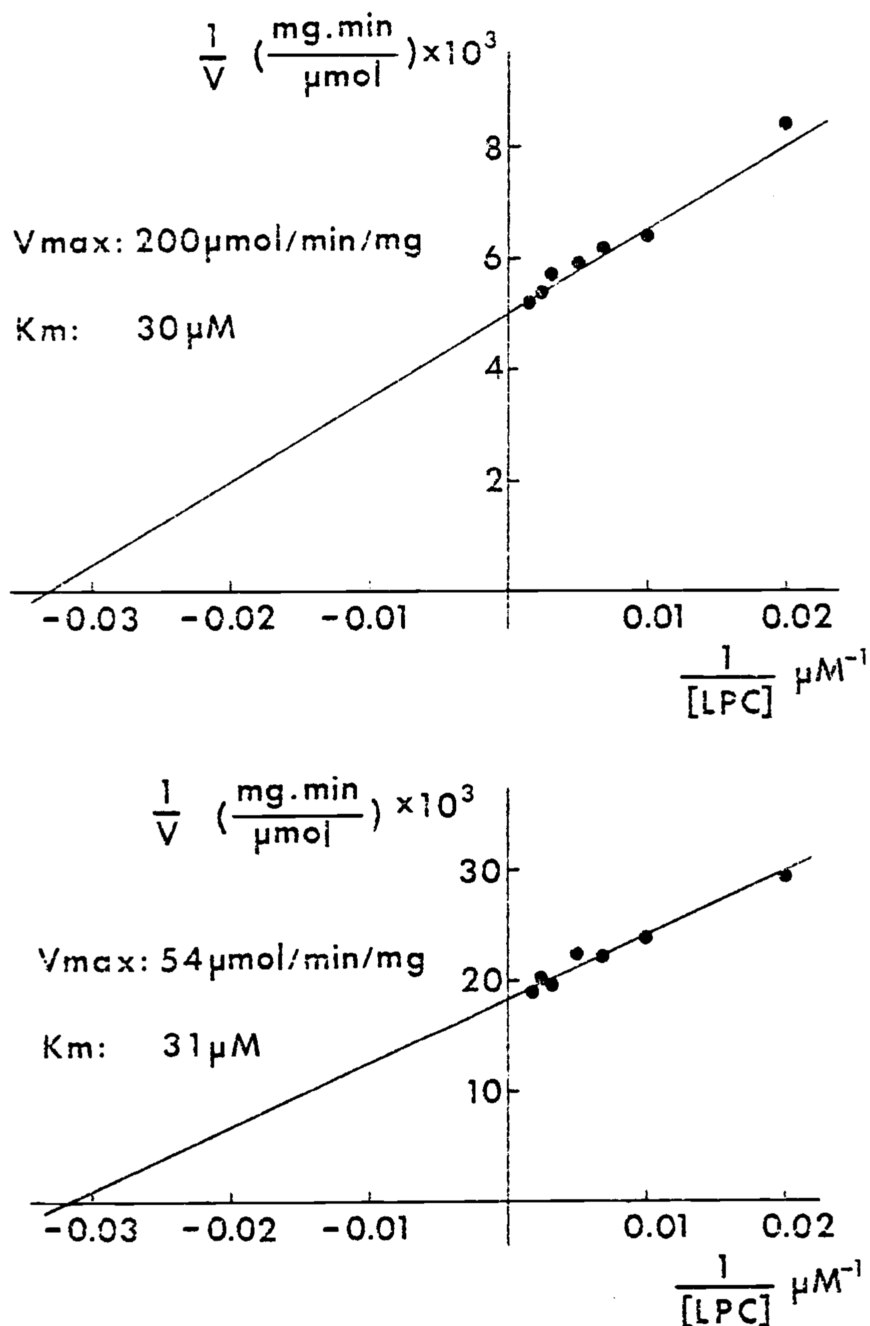


Fig. III-9 Lineweaver-Burk plot of barley lysophospholipase activity. Top, LPL I; bottom, LPL II. Suitably diluted samples of LPL I and LPL II were each used with substrate concentrations from 50-600 μM. The assays were run for 10 minutes at 21 C.

Table III-2-a Effect of Various Agents on Lysophospholipase Activity (I)

Addition	Concentration	(%) Recovery LPL I	LPL II
None	---	100	100
Ca^{++}	10 mM	65	65
Mg^{++}	10 mM	79	81
Hg^{++}	10 mM	114	116
Cu^{++}	10 mM	99	112
Zn^{++}	10 mM	91	92
EGTA	10 mM	77	76
EDTA	10 mM	72	87
BSA	0.1 %	79	79

Table III-2-b Effect of Various Agents on Lysophospholipase Activity (II)

Addition	Concentration	(%) Recovery LPL I	LPL II
None	---	100	100
p-Chloromercuibenzoate	10 mM	16	15
Iodoacetamide	20 mM	13	15
N-Ethylmaleimide	10 mM	87	90
Dithionitrobenzoic acid	1 mM	87	88
Phenylmethylsulfonylfluoride	1 mM	84	84
Phenylglyoxal	5 mM	68	77
Sodium deoxycholate	2.4 mM	47	52
Cetyltrimethylammonium	2.4 mM	8	9
Triton X-100	1.0 %	29	29
Palmitic acid	0.1 mM	106	102

ions and chelators have relatively small effects. Palmitic acid, a product of the assay does not inhibit the enzyme. This contrasts with the reported inhibition by palmitic acid of LPL from rabbit myocardial tissue (13).

There is strong inhibition by the sulfhydryl inhibitors, PCMB and iodoacetamide but little effect by more polar inhibitors of sulfhydryl groups, NEM and the Hg and Cu cations. This probably reflects the fact that a SH group important to the activity of the enzyme is located in a hydrophobic pocket. p-Methane sulfonyl fluoride, an inhibitor of serine proteases (30) has little effect indicating that enzyme catalysis does not involve transfer of the acyl chain of the substrate to an acidic serine OH. LPL is a basic activity and the amino acid analysis shows a high arginine content (Table III-3). Such residues have been shown to be involved in reactions involving phosphate-containing substrates where an ionic interaction between the positively charged guanidine side chain of arginine interacts with the phosphate anion of the substrate (31). Phenylglyoxal inhibits such reactions by blocking the specific arginine residue involved in the binding of substrate. The slight inhibition of LPL observed with the reagent may represent that population of molecules of heterogeneous glycoproteins in which the attachment of carbohydrate allows accessibility of the reagent to an active residue.

Although the substrate for the enzyme is a detergent, albeit a zwitterionic detergent, the enzyme is inhibited by anionic, neutral and cationic detergents, especially by the latter. We have found, however, that the enzyme will tolerate .01% SDS and that its recovery from hydrophobic columns is improved in the presence of this

Table III-3

Amino Acid Composition of
Deglycosylated Barley Lysophospholipase[a]

	LPL I	LPL II
Asx	30	26
Thr	16	16
Ser	23	23
Glx	16	18
Gly	32	37
Ala	30	31
Val	23	24
Met	4	4
Ile	11	12
Leu	36	35
Tyr	10	10
Phe	12	14
His	9	8
Lys	12	17
Arg	15	14

a Values are from a single analysis of Ortho-Phthalaldehyde method after HCl hydrolysis and are approximates of residues per molecule.

detergent.

Deglycoslyation of LPLs I and II.

LPLs I and II are composed of at least three glycoproteins with very similar properties. To test the possibility that they may be related through their having a common polypeptide chain but different carbohydrate moieties, they were deglycosylated and the derived polypeptides subjected to SDS-PAGE. Deglycosylation was effected with trifluoromethane sulfonic acid. A time-course for the reaction was established using BSA and the glycoprotein fetuin as controls. The conditions used, described by Edge et al. (19), hydrolyse all glycosidic bonds except the Asn-NAcGlu bond. Figure III-10 shows that both LPLs undergo a loss of molecular weight to give a single polypeptide at 36,000. The data indicate that the basic LPLs of barley are glycoproteins composed of a single polypeptide with 10 to 12% carbohydrate.

Amino Acid Analyses of the LPLs.

Amino acid analyses of the two deglycosylated LPL samples show marked similarities consistent with the similar properties the two activities display. They do not show any cysteine residues present (Table III-3). It may be a result of their destruction in the deglycosylation procedure as the inhibition of the activity by sulfhydryl inhibitors clearly indicates the presence of free cysteines. The high pI of LPL is a result of the large proportion of

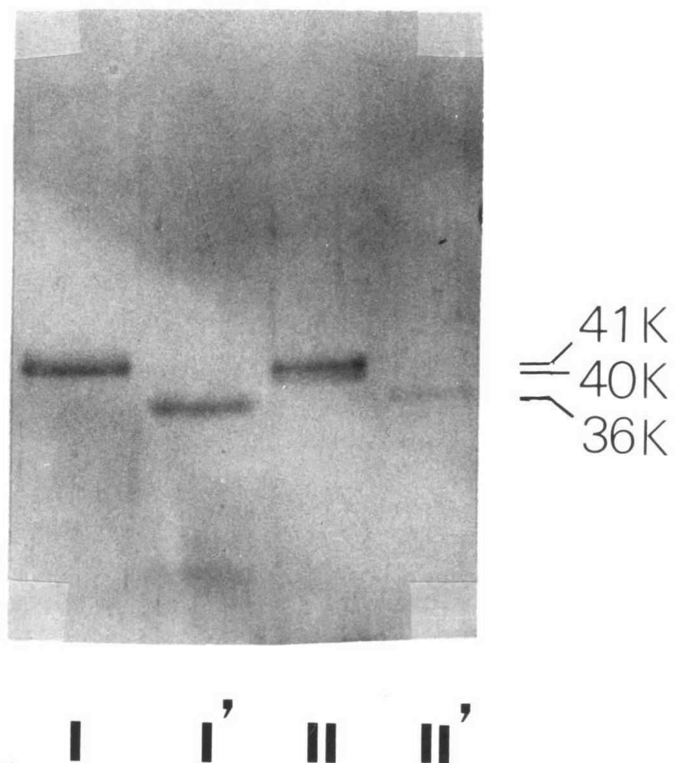


Fig. III-10 SDS-Polyacrylamide gel electrophoresis of barley lysophospholipase I and II before and after TFMS treatment. (I), LPL I; (I'), TFMS treated LPL I; (II), LPL II; (II'), TFMS treated LPL II.

arginine and lysine residues present; a large fraction of the acidic amino acids must have been originally present as the corresponding amide amino acids based on the large amount of ammonia produced in the protein hydrolysis. A high asparagine content would be consistent with a glycoprotein displaying microheterogeneity through different N-linkages for attachment of glycosidic groups to the polypeptide. The lipophilic character of LPL is evident from the large proportion of leucine, isoleucine and valine present.

Although cysteine, proline and tryptophan have not been quantitated the minimum molecular weight for the polypeptide of LPL I may be calculated from the amino acid analysis to be approximately 30000. Based on the molecular weight of the deglycosylated protein determined by SDS-gel electrophoresis, LPL I is 36,000. The glycoprotein LPL I has molecular weight 41,000 indicating it to be about 12% carbohydrate.

Specificity of LPL and Its Function during Germination

The combined action of LPLs I and II on an equimolar mixture of lysophosphatidylcholines carrying acyl chains located at C-1 and ranging in length from C12:0 to C18:0 and unsaturation as C18:1 is shown in Table III-4. It is clear that the preferred saturated chain length is C16:0 and that activity suffers a sharp drop on extending the chain by two carbons. If unsaturation is inserted into the C18 chain however there is a sharp increase in activity. The C16:0 and unsaturated C18 in the form of C18:2 are the most abundant acyl chains of the lysophospholipids occurring in barley starch (1,4). The

Table III-4

Relative Activity of Barley Lysophospholipase on
on Lysophosphatidylcholine ($C_{16:0}$) and Lysophosphatidylethanolamine ($C_{16:0}$)
and on LPCs carrying different acyl chains

Substrate		LPC		LPE	
Relative Activity		1.00		0.43	
Acyl Chain	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18.1}$
Relative Activity	0.37	0.80	1.00	0.17	0.53

influence of the lipid polar head group is shown by a comparison of the susceptibility to hydrolysis of lysophosphatidylcholine (LPC) with lysophosphatidylethanolamine (LPE), each carrying a C16:0 chain. The preferred substrate is LPC which is the most abundant lysophospholipid in barley starch.

The purified LPL shows no lipase activity against p-nitrophenyl palmitate; no phospholipase A activity against L-α-phosphatidylcholine di[1-^{14}C]palmitoyl assayed in the presence of Ca^{2+} and ether (20); nor does it display LPC-LPC transacylase activity measured according to the procedure of Gross et al. (21) (data not shown). The high degree of specificity by the purified LPL for the hydrolysis of lysophospholipids, especially for those carrying the phosphorylcholine polar head and the $C_{16:0}$ and C_{18} unsaturated acyl chains, strongly suggests that the function of this enzyme is directed to the disposal of the starch inclusion lipids during their release from the starch matrix during germination.

The activity of LPL under optimal conditions is very high compared with that of LPL isolated from any other source. Even at the pH of 5.0 to 5.2, the condition prevailing in the starchy endosperm during germination (32), the activity, which is approximately 30% of that at pH 8, is more than necessary to ensure that the lysophospholipids released during breakdown of the starch are rapidly hydrolysed.

Comparison of the properties of barley LPL with those from animals and bacteria

The barley LPLs reported in this study are basic glycoproteins

with molecular weights about 40,000. They have a pH optimum at 8. They show high specificity for lysophospholipid substrates with a pre ference for LPL over LPE. They are inhibited by sulfhydryl reagents. No transacylase, phospholipase or lipase activity is shown by them. They have Kms of 30 μM and Vmax of 50 and 200 μmol/min/mg with LPL-LPC as substrate. All the other LPLs described to date are acidic proteins. They have pH optima between 7.5 and 8.5 except the LPL from rat lung which is 6.0 to 6.5 (33). There is no indication that any of them are glycoproteins. Their response to sulfhydryl reagents ranges from inhibition (10) to no effect (34). The highest specific activity is 7 μmol/min/mg reported for the rabbit heart LPL against LPC; the reported Kms range from 7 to 11 μM (12,13). The most common enzymatic activity accompanying LPL and associated with the same protein is a LPC:LPC transacylase in which an acyl chain is transformed one LPC to another thereby generating a phosphatidylcholine. All transacylases have LPL activity but not all LPLs have transacylase activity. A reaction mechanism of transacylase has been proposed (21). The molecular weights of LPLs associated with transacylase activity range from 50,000 to 60,000; those of LPL only from animal sources are 23,000 (13) and 25,000 (10). It is probably no coincidence that those enzymes catalyzing both transacylase and LPL activity have the larger molecular weights. The transacylase activities are found in lung, liver and heart where the requirement may be for both synthetic and degradative purposes. For the barley LPL the reaction takes place in germinating seeds especially in the starchy endosperm where reserve materials are digested and transported to the embryo. The reaction is clearly only degradative and no transacylase activity would be

expected. An LPL from E. coli has molecular weight 39,500 (11) and most closely corresponds to the barley enzyme. It is active against LPE, the major lysophospholipid of E. coli but displays no activity against phosphatidyl ethanolamine or p-nitrophenyl palmitate.

Enzyme-linked immunoabsorbent assay (ELISA) (23,25) for LPL

The anti-LPL serum from mice was obtained using a mixture of the purified LPL I and II as antigen. The assay was conducted using an anti-mouse goat IgG conjugated with alkaline phosphatase. Mouse antibody binds to antigen coated in the wells of the microtiter plate. Then the anti-mouse goat IgG bearing the alkaline phosphatase binds to the antibody. The phosphatase catalyzes the hydrolysis of phosphate from p-nitrophenol phosphate releasing p-nitrophenol which is measured for the enzyme assay. In Fig. III-11 it is clear that the antiserum from mice immunized with the LPL mixture show an increasing response with increasing serum concentration. Not surprisingly the response of the LPL I and II antigen mixture, and the separately purified LPL I and II parallel one another. The decline in response at high serum concentration may be a result of low concentration of the antibody in the serum. Serum from the control mouse shows a background response which may be due to specific or non-specific binding of IgG in the serum to the coated protein and probably to other protein-protein interactions as well (25). The two deglycosylated derivatives of LPL I and II also show minimal response to the serum. Alexander and Elder (35) have shown that for a number of viral glycoproteins, removal of the carbohydrate unit with endoglycosidase F gives products virtually

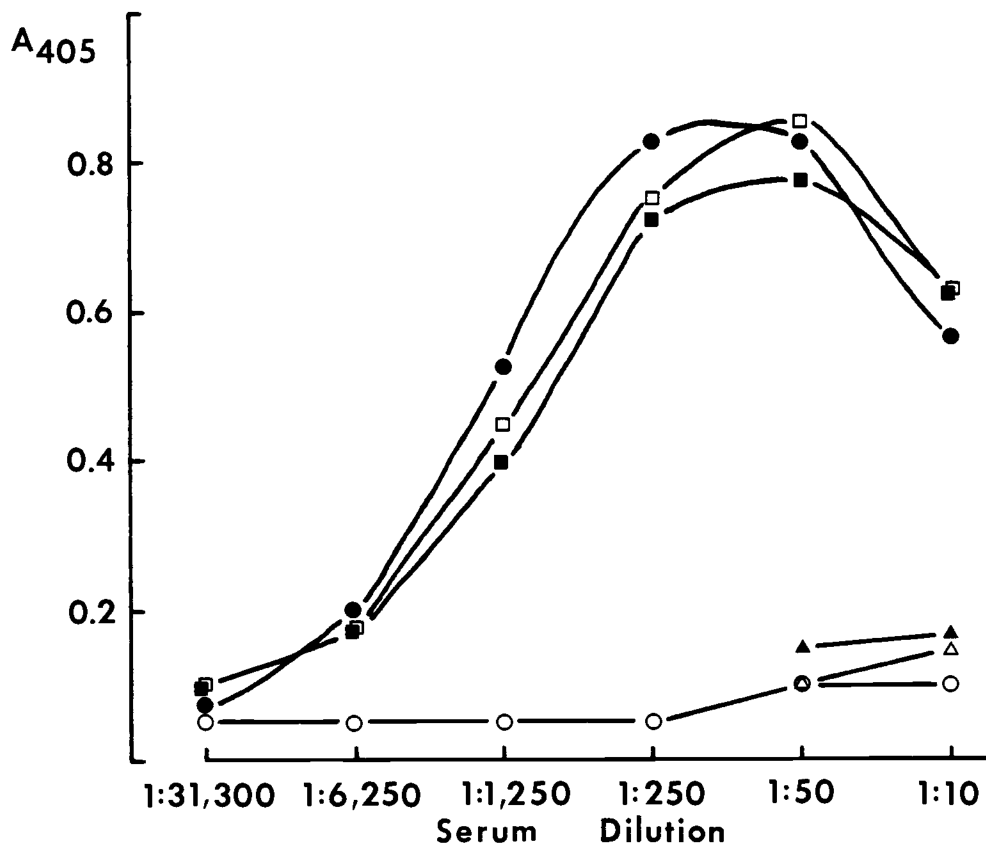


Fig. III-11 Titration with mouse anti-LPL and control serum wells were coated with the antigen mixture of LPL I and LPL II (●), LPL I alone (□), LPL II alone (■), deglycosylated LPL I (△), deglycosylated LPL II (▲). Response of control serum against the antigen mixture is shown (○).

unreactive to antibodies produced against the virus. In addition, they found that antibodies to synthetic peptide sequences of influenza virus hemagglutinin improved in reactivity against the hemagglutinin after carbohydrate removal. The concluded that, "immunogenicity is a direct measure of carbohydrate influence on the host's perception of foreign antigen."

Deglycosylation of LPL I and II employed TFMS and it is possible that some denaturation as well as deglycosylation occurred. However, Edge et al (19) have reported that the peptide backbone of a bovine nasal septum proteoglycan remains intact after TFMS deglycosylation and can act as an acceptor for UDP-D-xylose. The proteoglycan is, of course, non-enzymatic and may have limited tertiary structure. To the extent that exposed peptide sequences in the native glycoprotein of barley LPL participated in antibody formation, then a decrease in antigenicity may be, as a contributing factor, denaturation of the polypeptide.

Selection of antigen and serum concentration for quantitation of antigen is shown in Fig. III-12. In Fig. III-12-A, phosphatase activity has been plotted as a function of concentration of the coating antigen at different antibody concentration; Fig. III-12-B the enzyme activity is measured as a function of antiserum concentration at different concentration of coating antigen. From the former, a concentration of antigen >0.1 and <0.3 μg/ml is chosen and from the latter, a dilution of 1:125-1:25 for the immunoglobulin concentration.

A measurement of lysophospholipase enzyme in the endosperm of germinating barley variety Advance by the ELISA method is shown in Fig. III-13. Also shown is the total LPL activity in each sample. We know

this is predominantly the basic activity from the purification procedure. We have also demonstrated that there is almost no cross-reactivity of the antibody with the acidic enzyme. Most important is the observation that the increase in appearance of the basic enzyme in the endosperm precedes the appearance of enzyme activity. This probably reflects the synthesis and processing of the basic LPL in the aleurone in a yet inactive form, prior to its release in an active form into the starchy endosperm.

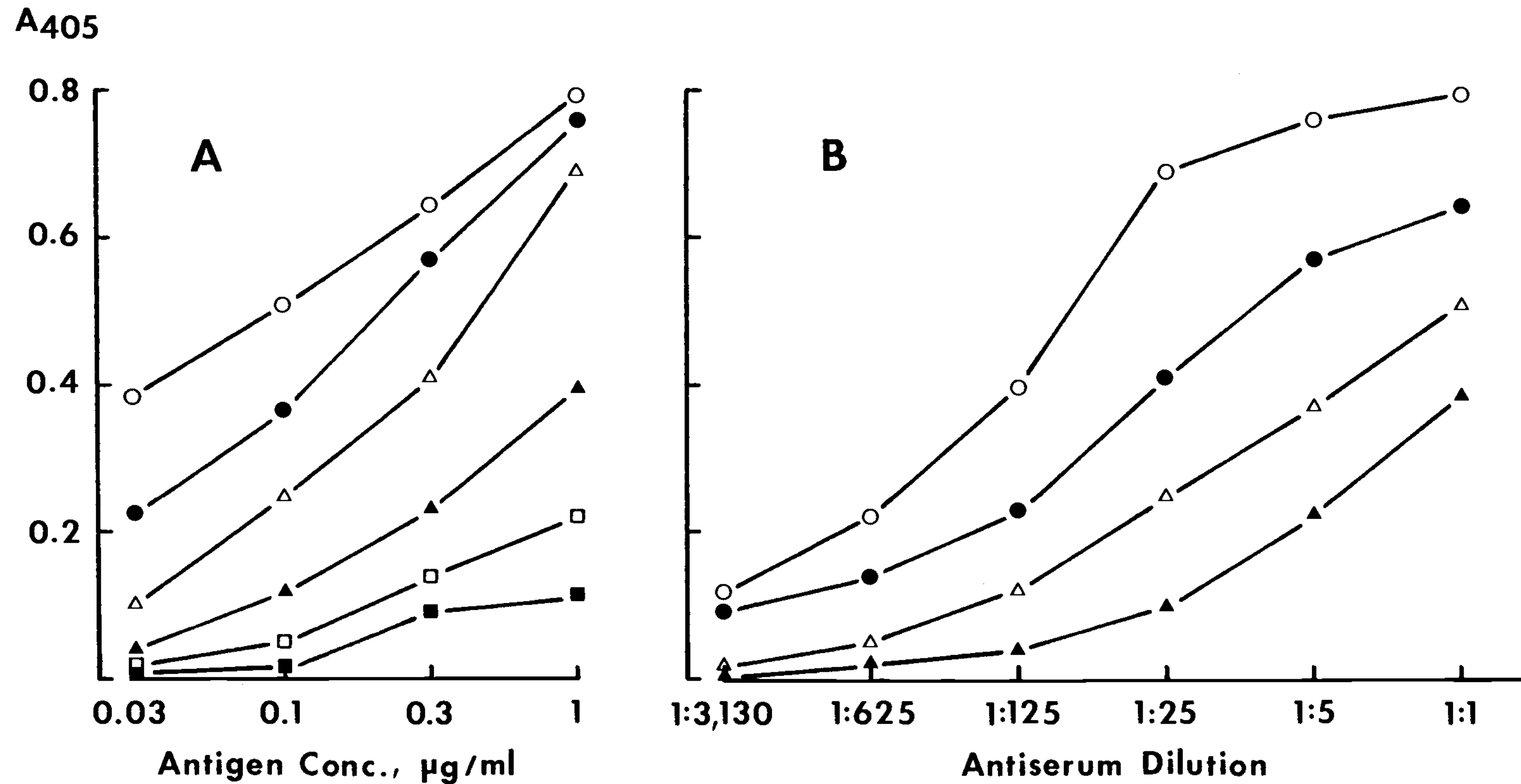


Fig. III-12 Checkerboard titration of the antigen concentration used for coating and of immunoglobulin concentration. (A) Enzyme activity as a function of antigen (LPL mixture) concentration used for coating and for immunoglobulin (200 μg/ml) diluted 1:1 (○), 1:5 (●), 1:25 (△), 1:125 (▲), 1:625 (□), and 1:3125 (■). (B) Enzyme activity as a function of immunoglobulin dilution in wells coated with LPL mixture 1 μg/ml (○), 300 ng/ml (●), 100 ng/ml (△), and 30 ng/ml (▲).

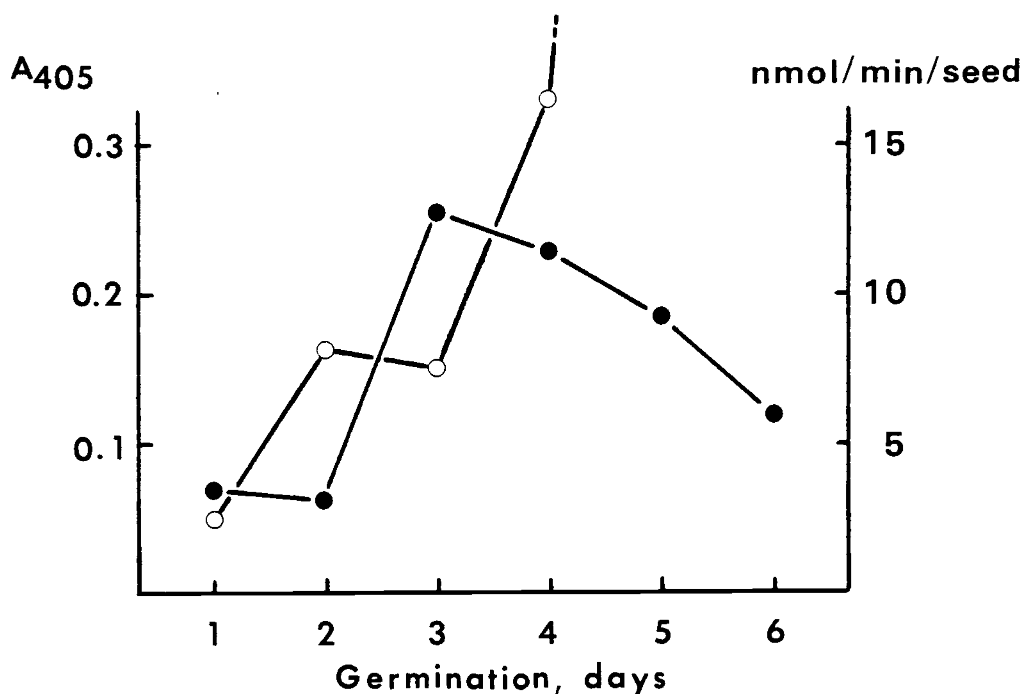


Fig. III-13 Antigenicity of germinated barley against anti-LPL immunoglobulin and LPL enzyme activity.

From 10 seeds of 1-6 day germinated barley (Advance), the starchy endosperm and aleurone combined were removed and ground with PBS. Each sample was made up to 2 ml with the buffer and centrifuged 1800 x g for 10 minutes. The supernatant were subject to further centrifugation 100,000 x g for 60 minutes in order to obtain clear samples. The procedure of ALISA is described in the Materials and Methods. Each data point is the average of two experiments (●). The control serum was employed as the reference. LPL activity is also shown for the same samples (○).

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