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TURNOVER OF STARCH-BOUND LYSOPHOSPHATIDYLCHOLINE IN GERMINATING BARLEY*

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Key Word Index—*Hordeum distichum*; Gramineae; barley germination; amylase; starch-lipid; lysophosphatidylcholine; acyl composition; amylose; inclusion complex.

Abstract—Free and starch-bound lysophosphatidylcholine (LPC) in germinating barley was isolated and quantified during the first 8 days of germination. During the first 4 days the starch-bound LPC remained at a relatively constant level (*ca* 0.4 μmol/seed) and then declined during the next 2 days to *ca* 0.1 μmol/seed. There appeared to be no further loss of this starch-bound lipid on further germination. The decrease in the content of starch-bound LPC is not due to the action of phospholipase C and/or D on the starch lipid because there was no corresponding accumulation of starch-bound lysophosphatidic acid or monoacylglycerol. The free LPC remained relatively constant at 0.02 to 0.04 μmol/seed during the entire germination period indicating that the LPC released from the starch during days 5 and 6 is further metabolized. Amylase activity was also measured in the germinating seed and increased 20-fold between days 2 and 4 which just precedes the rapid decline in starch-bound LPC. The starch content of the seed however declined to *ca* one third of the original value by day 5. LPC represents 65–70% of the starch-bound lipid phosphorus in the dry seed. Through days 5 and 6 when the loss of LPC is most rapid there is no marked change in this percentage. After 8 days, however, the LPC is only *ca* 30% of the starch-bound lipid phosphorus. Of the two major populations of starch-bound LPC, the one bearing a linoleyl group appears to decline more rapidly during days 4–6 than does that carrying a palmitoyl group. The role of starch-bound LPC in barley development and germination is discussed.

INTRODUCTION

It has been shown that the mobilization of starch in cereal grains is accompanied by a rise in the amylose/amylopectin ratio [1]. Furthermore, during this breakdown the MW of amylose does not undergo much change [2]. The presence of lysophospholipids as inclusion complexes with the amylose component of cereal starches [3] might account for the apparent resistance of amylose to digestion during germination. If this were the case then it might be anticipated that the starch-bound lysophospholipid content of the endosperm tissue of cereal grains would remain constant throughout germination. In this paper the loss of starch-bound lysophosphatidylcholine in germinating barley seeds correlated with the increase in amylase activity is reported. In addition, of the two major populations of starch-bound lysophosphatidylcholine present, the one bearing a linoleyl group disappears slightly more rapidly than that bearing palmitoyl. Possible roles for the starch-bound lysophospholipid in cereal grain development and germination is discussed.

RESULTS AND DISCUSSION

The amylose component of cereal starches contain *ca* 1% lipid of which LPC is the predominant component [3]. It is believed that the lysophospholipid occurs as an inclusion complex with the amylose helix as shown in Fig. 1. In such a form it might be anticipated that an enzymatic

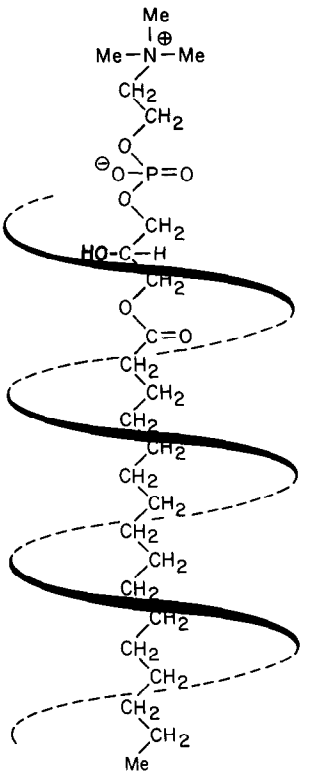

Fig. 1. Representation of an inclusion complex of lysophosphatidylcholine in an amylose helix. The amylose is shown as a left-handed helix with a pitch of 0.8 nm [15].

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Table 1. Starch content of barley seeds during germination

Length of germination (days)	Starch content per endosperm (mg)
1	35.5 ± 1.6
2	27.9 ± 1.8
3	22.3 ± 0.9
4	15.5 ± 0.5
5	12.6 ± 1.4
6	9.7 ± 1.4
7	7.9
8	6.5

Starch was isolated from the endosperm of 5 seeds for each stage of germination as described in the Experimental. It was assayed by the anthrone method [29]. With the exception of days 7 and 8, which were single assays, the measurements were made in duplicate or triplicate. The values are the averages of these replicates with the deviations from each average shown.

attack on such an amylose molecule by β-amylase or phosphorylase would be hindered as compared with an attack by α-amylase. The increase in the ratio of amylose to amylopectin during germination [1] is presumably a result of the larger number of non-reducing ends of the amylopectin component. However, the presence of an amylose inclusion complex may be a contributing factor to the slower hydrolysis of amylose.

During the first 8 days of germination, the starch content of the barley seed declines as shown in Table 1. Both the decline in starch content and increase in amylase activity precede the loss of starch-bound LPC (Fig. 2). Indeed, more than 50% of the starch has been degraded by day 4 when the starch-bound LPC level has suffered no noticeable decline. From these data there appears to be a resistance to the attack of the hydrolytic enzymes on the polysaccharide carrying the lysophospholipid.

The procedures used in this study measure the LPC retained by the starch. Earlier studies have demonstrated

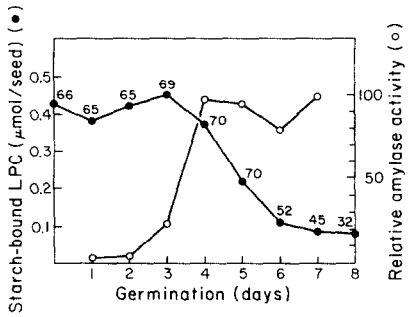

Fig. 2. Increase of amylase activity and loss of starch-bound LPC in germinating barley. The assay for amylase activity and the isolation and quantitation of the starch-bound LPC are as described in the Experimental. All data points are averages of replicate experiments. The range about the averages are < 0.07 μmol for LPC and < 10% for amylase activity. The numerals on the LPC curve are the LPC values expressed as a percent of total starch-bound lipid phosphorus.

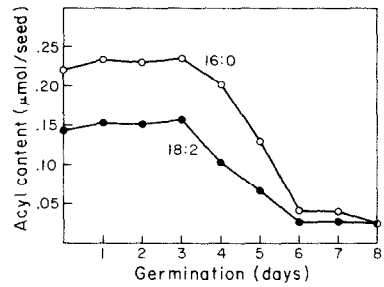

Fig. 3. Palmitoyl and linoleyl contents of starch-bound LPC in germinating barley. The acyl contents were determined from the area measurements under the two peaks related to that of the internal standard (see Experimental). Each measurement is the average obtained from duplicate runs. The deviation from each point is < 10%. The range about the average value for the ratio of 16:0 to 18:2 of the LPC acyl groups for each stage of germination varies < 15%.

the orientation of the LPC in the amylose helix by showing the susceptibility of the polar head group to attack by phospholipase D [3] and the resistance of the acyl ester group to phospholipase B [4, 5]. Attack by phospholipase C and/or D on the starch-bound LPC during germination would also result in a decline of the starch-bound LPC content of the barley, but a concomitant increase of starch-bound monoacylglycerol and/or lysophosphatidic acid would also be expected. The extracts of the starch-bound lipids at all stages of germination showed only trace amounts of these compounds to be present (< 0.005 μmol/seed) as measured by acyl analysis of the separated TLC zones.

Although between days 4 and 6 the starch-bound LPC content falls from *ca* 0.4 to 0.1 μmol/seed the free LPC level in the seed remains unchanged at 0.02 to 0.04 μmol/seed. Clearly, this membrane-disrupting lysophospholipid is rapidly metabolized as germination progresses. Nevertheless, even though it may have a transient existence, it is conceivable that one of its functions during germination is to disrupt membranes in the endosperm and thereby promote the mobilization of hydrolysates for the embryo. The distribution and nature of the enzymatic activity which turns over the free LPC is currently under investigation.

In the dry seed LPC represents 65–70% of the starch-bound lipid phosphorus (Fig. 2). This value remains little changed during that period when the bulk of the starch is degraded (Table 1). Clearly, there is no selection by the hydrolytic enzymes for starch molecules bound to LPC anymore than there is for starch bound to any other lysophospholipid. Only in the starch remaining after the seedling is well established (day 8), do we find that this percentage value declines, suggesting a selection for degradation of starch molecules bound to LPC. This may reflect the different biogeneses of starch granules during development if the granules last to be formed are the first to be degraded. In this context the barley grain deposits starch granules of two distinct sizes and types [6]. Large granules having a diameter greater than 10 μm (the majority 15–35 μm) appear during the initial stages of starch deposition. Small granules having a diameter less than 10 μm (average being *ca* 5 μm) appear at a later stage of development. There are conflicting results concerning

the amylose and amylopectin composition of the two populations of granules [7–9]. A scanning electron microscope study of malting barley showed the small granules to be degraded first [10]. This might be anticipated by virtue of the much larger surface area available to hydrolytic attack as compared with the large granules.

An examination of the acyl component of the LPC residing in the starch as germination progresses is shown in Fig. 3. In the dry seed palmitoyl and linoleyl groups combined represent *ca* 90% of the total acyl content of the starch bound LPC [11]. This is also true for the barley variety in the present study. The ratio of the $C_{16:0}$ to $C_{18:2}$ is *ca* 3:2 through the third day of germination which corresponds to no apparent turnover of starch bound to LPC (Fig. 2). Days 4 and 5 in which the starch-bound LPC shows a substantial decline corresponds to a ratio of 2:1 for the $C_{16:0}$ to $C_{18:2}$ of the acyl groups in the residual LPC. Evidently there is some selection for hydrolysis of those starch molecules carrying the population of LPC molecules with the more unsaturated acyl group. Becker and Acker [12] have shown that during starch granule synthesis in developing barley there is an increasing proportion of linoleic acid incorporated into LPC whereas the palmitic acid content remains essentially constant. This could explain the data we find in the germinating seed if, again, the last-formed starch molecules are the first degraded. It is also interesting to speculate that the presence of two double bonds in the acyl chain of the included LPC may promote a sufficient strain in the amylose helix serving to accelerate the breakdown of such starch molecules.

Among the functions that have been suggested for the starch-bound LPC is that it may govern the amylopectin/amylose ratio in cereal starches [13]. It is suggested that the presence of lysolecithin might inhibit the branching enzyme. In support of this is the absence of lysolecithin in the low amylose waxy starches. It should be noted however that Shiefer *et al.* [14] have evidence suggested that the presence of lysolecithin might inhibit the synthesis of amylose and amylopectin in isogenic lines of maize varieties.

The generation of a helix by amylose in the presence of an inclusion compound and the ordered stacking of such helices within the granule would clearly be a useful function for LPC. The unit cell dimensions for such an ordering of helices in wet butanol-precipitated amylose has been made by Rundle and Edwards [15]. The LPC produced during development of the barley seed [16] would then serve at least two functions: As an inclusion compound it promotes helix formation of the newly synthesized amylose during development. In addition the polar head groups exposed at the ends of helical segments might promote the ordering of the helices through electrostatic interactions. Such ordered structures may provide the skeleton about which the amylopectin is deposited in the starch granule. It should be pointed out that Banks and Greenwood [17] argue on the basis of X-ray diffraction data that little ordering of amylose exists in the cereal starches until they have been gelatinized. These same authors also concede that the form of amylose in the starch granule is currently a matter of speculation.

A second function for LPC emerges during germination in that its membrane-disrupting property may aid in the liquefaction of the endosperm and the resulting transport of nutrients to the growing embryo.

In the context of biological functions of polysaccharide–lipid interactions, it should be noted that two polysaccharides, one containing 3-*O*-methylmannose and the other 6-*O*-methylglucose, form stoichiometric adducts with long chain (C_{16}–C_{24}) acyl CoA [18, 19]. Helical polysaccharide–acyl CoA inclusion complexes, postulated to be the products of the interaction [20], govern the rate of fatty acid synthesis in *Mycobacterium smegmatis* [21].

EXPERIMENTAL

Materials. Barley, *Hordeum distichum* L. cv Georgie, was grown from seeds supplied by RHM, Crops Dept., High Wycombe, U.K.

Germinating barley. Several batches of seeds were germinated by total immersion in H_2O for 24 hr and then transferred to moist paper towels in the dark for the next 7 days. The seeds were thoroughly washed with H_2O each day. After 24 hr, 50 seeds were taken for the isolation of starch-bound LPC. After each subsequent 24 hr interval, 50 seeds which had visibly germinated were removed for analysis. For each time interval the isolation and individual assays were at least in duplicate.

Starch-bound LPC isolation. Germinated seeds were placed in boiling *iso*-PrOH for 1 min to inactivate phospholipases [22]. The tissue was removed and the *iso*-PrOH evaporated in a vacuum oven at 40° overnight. After removal of the root and shoot tissue the residual seed material was pulverized in a Moulinex coffee grinder. The starch-bound lysophospholipids were isolated essentially as previously described [16]. Free lipid was removed from the pulverized seed material by repeated extraction with cold H_2O-satd BuOH (WSB). This extract was combined with the *iso*-PrOH used for killing the seeds. Starch-bound lysophospholipids were then isolated from the residual seed material by repeated extraction with boiling WSB. Non-lipid contaminants were removed by first evaporating the solvent from the extract and passing the residue redissolved in Folch lower phase through a column of Sephadex G-25 in Folch upper phase [23]. The free lipids from the combined *iso*-PrOH and cold WSB treatments were similarly treated to remove non-lipid contaminants. The two lipid samples were each dissolved in a standard vol. of $CHCl_3$ (1–5 ml) and stored at −20°.

TLC of lipids. From aliquots (0.1 ml) of the $CHCl_3$ extract the lysophospholipids were separated on activated Si gel G plates using $CHCl_3$–MeOH–HOAc–H_2O (50:30:8:3) and (10:6:2:1) in solvent-satd tanks. The lipids were located with I_2 and the Dittmer–Lester phospholipid spray [24]. Monoglycerides and free fatty acids were separated on non-activated silica Anasil G in hexane–Et_2O–HOAc (60:40:1) in a solvent-satd tank. The lipids were visualized with I_2.

Quantitation of lipids. LPC was quantitated by a measurement of inorganic Pi by $HClO_4$ digestion of the TLC scrapings of the LPC zone according to the procedure of ref. [25]. The value was corrected for the background inorganic Pi of the Si gel. Typically, the correction was 0.02–0.04 μmol of Pi per assay.

Acyl composition of separated lipids. This was determined essentially according to the method of ref. [26]. Scrapings of the TLC zones of the LPC and monoacylglycerol were transferred to screw cap tubes fitted with Teflon-lined internal caps. To the scrapings was added a known amount (0.10–0.50 μmol) of heptadecanoic acid as internal standard. One ml of 0.5 M NaOH in MeOH was added to the scrapings and the capped vial heated for 5 min at 100°. To the cooled mixture was added 2 ml of 14% BF_3 in MeOH and the capped vial again heated for 5 min at 100°. Three ml of satd NaCl soln was added to the reaction mixture and the fatty acid Me esters were extracted with heptane (1 ml).

GC of fatty acid Me esters. The esters were separated on a 2 m × 4 mm glass column packed with 10% diethyleneglycol succinate on acid-washed Chromosorb W (80/100 mesh) at 190°.

Amylase activity. The enzyme was isolated according to a procedure of ref. [27] and assayed by the starch–I_2 method [28].

Five endosperms were used for each germination period. The tissue was ground with a pestle and mortar in 3 ml of ice-cold NaOAc (2 mM, pH 5.0), 2 mM in $CaCl_2$. The homogenates were centrifuged at 1000 *g* for 10 min and the supernatant used as the source of enzyme.

The starch soln for the assay was prepared by first boiling a mixture of 150 mg non-solubilized potato starch, 600 mg KH_2PO_4 and 151 mg $CaCl_2$ in 100 ml of H_2O for 1 min. The cooled soln was centrifuged at 10000 *g* for 15 min and the clear supernatant used for the assay. For each germination period a time-course was run by reacting 0.1 ml enzyme with 0.5 ml starch soln over 60 min at 25°. The reactions were stopped with 1.0 ml I_2 reagent. The reagent was prepared by adding 0.15 ml of an I_2 soln (6 g KI and 600 mg I_2 in 100 ml H_2O) to 50 ml HCl (0.05 M). After stopping the reaction, the mixture was diluted to 3 ml with H_2O and the *A* measured at 620 nm. Amylase activity was measured from the ΔA over the linear part of the time course for each sample.

Starch content of germinating barley. Five endosperms were removed from seeds which had germinated from 1–8 days. The endosperms were macerated in Me_2CO with a pestle and mortar and the dehydrated and finely powdered tissue further washed with 80% EtOH. The residue was transferred to either 100 or 250 ml volumetric flasks in H_2O. Aliquots of the suspended material were assayed for glucose by the anthrone method [29].

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