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SOLUBLE AND PARTICULATE LYSOPHOSPHOLIPASE IN THE ALEURONE AND ENDOSPERM OF GERMINATING BARLEY*

DEREK J. BAISTED and FRANCES STROUD

Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, U.S.A.

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Key Word Index—*Hordeum distichum*; Gramineae; barley germination; starch–lipid; lysophosphatidylcholine; lysophospholipase; aleurone; endosperm; amylase.

Abstract—Lysophospholipase was measured in extracts of germinating barley by determining the amount of free [^{14}C]palmitate released from [1-^{14}C]1-palmitoyl-lysophosphatidylcholine (LPC). Soluble and particulate lysophospholipase activity was measured at 1-day intervals in extracts from the aleurone and endosperm of barley seeds germinated for 8 days. The soluble and particulate activities of the aleurone increase approximately in parallel with one another and after 8 days of germination have 20–30 times more activity than at day 1. The activity profiles and the distribution of the activity between the soluble and particulate forms of lysophospholipase in the endosperm are markedly different. With the exception of the first 2 days when the aleurone activity is low, the endosperm activity is less than that associated with the aleurone. The soluble activity increases during the first 3 days and is more active than that of the aleurone. Thereafter it diminishes and remains low. The particulate enzyme, however, increases dramatically between days 4 and 5 and remains moderately high. The fourth and fifth day represent that stage of germination when starch-bound LPC is released in concert with the increase in amylase activity. It is proposed that it is this particulate form of the endosperm activity which may be responsible for maintaining the level of free LPC low in the endosperm of the germinating seed.

INTRODUCTION

It has recently been shown that the lysophosphatidylcholine (LPC), present in the amylose component of barley starch as a lipid inclusion complex, is released during germination [1]. No corresponding increase in free LPC in the seed was observed. In this communication we report the enhancement of lysophospholipase (LPL) activity, its distribution between the aleurone and endosperm, and its existence in both a soluble and a particulate form in germinating barley.

RESULTS AND DISCUSSION

The relationship of the decline in starch-bound LPC to the increase in amylase activity during barley germination is shown in Fig. 1. 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, however, remains unchanged at 0.02–0.04 μmol/seed [1].

At 1-day intervals, LPL activity was isolated from the aleurone and endosperm separated from barley seeds germinated from 1 to 8 days. Each tissue was ground in buffer and after the removal of cell debris by low-speed centrifugation a 105 000 *g* pellet fraction, designated the particulate enzyme, was obtained. After washing this fraction, activity was still retained by the pellet (data not shown) indicating the particulate nature of the activity. A soluble enzyme was found in the high-speed supernatant. The enzyme activity was assayed in each fraction by measuring the release of [1-^{14}C]palmitate from [1-^{14}C]1-palmitoyl LPC. The enzyme activity displayed two pH optima, one near 5 and the other near 8. Assay conditions were chosen so as to give a linear turnover of substrate during the assay conducted at pH 5. This pH optimum was chosen to coincide with that for α-amylase [2, 3], the enzyme most likely responsible for the release of the LPC.

The data shown in Fig. 2 were obtained using extracts of tissues from five seeds. It is clear that during germination the cumulative capacity of the endosperm and aleurone to turnover LPC increases markedly. This increase is most evident in the aleurone tissue (Fig. 2) where the activity is in both a soluble and a particulate form. Whether one of these forms is a precursor to the other or that the aleurone produces at least two isozymic forms of LPL is not established. In this regard, a soluble and a particulate form of LPL have been separated and purified in a mammalian system [4]. The activity profile for LPL in the endosperm is also soluble and particulate. However, unlike the aleurone tissue in which these activities increase for the most part in parallel, in the endosperm the activities appear to be unrelated. The soluble enzyme peaks after day 3 and then suffers a sharp decline. The fact that starch-bound LPC is not released until day 4 (Fig. 1) suggests that this soluble LPL activity may serve a function unrelated to the release of the starch-bound LPC. It may have a role, in association with phospholipase activity, in the early breakdown of phospholipids in the endosperm. It is likely that the

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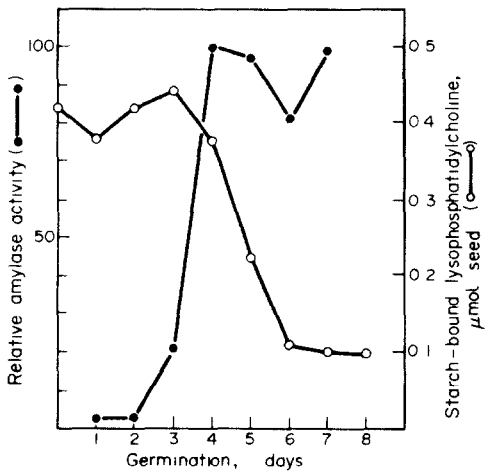

Fig. 1. Increase of amylase activity and loss of starch-bound LPC in germinating barley. The assay for amylase activity and the isolation and quantitation of the starch-bound LPC are as described in ref. [1]. All data points are averages of replicate expts. The range about the averages are <0.07 μmol for LPC and $<10\%$ for amylase activity.

particulate endosperm LPL is the activity responsible for metabolizing the released starch-bound LPC. The five-fold increase in its activity between days 4 and 5 coincides precisely with the disappearance of starch-bound LPC during germination. Furthermore, in the bound form it would be ideally situated to prevent excessive accumulation of lysophospholipid as it is released from the degraded amylose.

The change in sp. act. of the LPL from each of the four samples is shown in Table 1. The values for the activity of the particulate endosperm fraction from day 5 onwards were obtained from duplicate runs because the amount of residual endosperm material during this time period was so small. Comparison of these data for the aleurone enzymes with those of Fig. 2 shows that, although the total activities increase 20–30 fold during the 8 days, the sp. act. increase much less. This smaller increase reflects the active protein synthesis occurring in the aleurone during germination. Several different hydrolases, α-amylase [5], β-1,3-glucanase [6], ribonuclease [7] and protease [8] are known to be synthesized by the aleurone in response to gibberellic acid during barley germination. Although LPL may be another member of this family of hydrolases, its activation from a latent form is an alternate explanation for its appearance. The endosperm sp. act. contrast sharply with those of the aleurone and with each other. Both the total and the sp. act. of the soluble enzyme do not vary much during germination. The particulate enzyme, on the other hand, increases dramatically in both total and sp. act. between days 4 and 5 and the latter continues to increase as germination progresses. This continued increase in sp. act. reflects the disappearance of protein from the endosperm.

The biogenesis of the particulate form of the endosperm LPL activity may parallel that of the particulate form of starch synthetase during grain-filling in certain varieties of rice and maize. In such varieties which are devoid of amylose, the α-1,4 glucan synthase is present only in the soluble portion of the cell [9, 10]. An implication of such a finding is that the particulate form of the starch synthetase arises by its adsorption to the amylose component of the developing starch granule. Akazawa and Murata [11] have shown that in some cases amylose is capable of

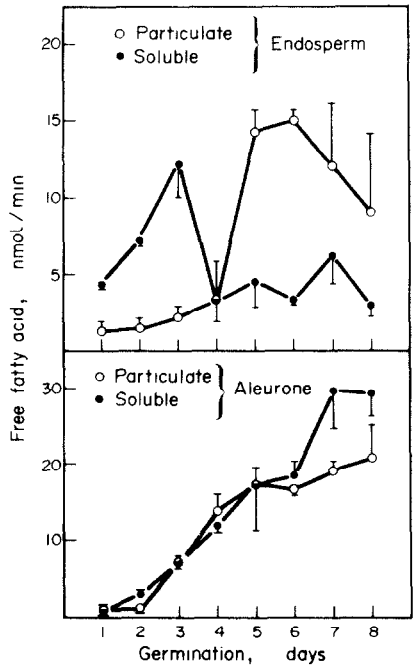

Fig. 2. Change in particulate and soluble lysophospholipase activities in the aleurone and endosperm of germinating barley. The isolation and assay of the enzyme are described in the Experimental. Each data point is the average from duplicate expts for enzyme isolation from five seeds. The vertical bars represent 0.5 the range of variation about the average for each pair of expts.

Table 1. Specific activities of lysophospholipase in germinating barley aleurone and endosperm tissue

Length of germination (days)	Sp. act. (nmol free fatty acid/min per mg)			
	Aleurone		Endosperm	
	Soluble	Particulate	Soluble	Particulate
1	2.5	2.0	1.0	2.0
2	5.0	5.0	2.0	1.0
3	4.5	9.5	3.0	2.0
4	7.0	11.5	6.0	5.0
5	8.5	15.0	n.d.	26.5 ± 0.5
6	7.0	14.0	3.0	35.0 ± 5.0
7	8.0	19.0	4.0	30.0 ± 5.5
8	10.0	20.5	4.0	46.0 ± 16.5

The fractions were isolated and assayed for lysophospholipase activity as described in Experimental. Protein determinations were by the Lowry method. The specific activities were determined on one series of germinations with the exception of the endosperm particulate fraction from days 5 to 8; these were for duplicate germination.

n.d., not determined.

adsorbing the synthase and transforming it into a particulate enzyme. Conceivably, the particulate form of the endosperm LPL in germinating barley may arise by adsorption of a soluble form of the enzyme to the amylose component of the endosperm.

Although we have described the barley enzyme as a LPL by virtue of its capacity to hydrolyse LPC, this enzyme will also readily hydrolyse *p*-nitrophenyl-palmitate. Clearly, we have in the barley cell fractions non-specific acyl hydrolases. There have been many reports of such activities in plants [12–15] and recent studies [15–19] have described the partial purification and characterization of two acyl hydrolases from *Phaseolus multiflorus* leaves. One activity revealed a preference for phosphatidylcholine rather than glycosyl acylglycerol and the other had the opposite specificity.

EXPERIMENTAL

Materials. Hordeum distichum L. cv Georgie was grown from seed supplied by RHM, Crops Department, High Wycombe, U.K. and germinated by total immersion in H_2O for 24 hr and then transferred to moist paper towels in loosely covered dishes in the dark for the next 7 days. The seeds were washed with H_2O each day.

Starch-bound LPC isolation and quantitation. Batches of 50 seeds were taken at 24-hr intervals for the isolation and quantitation of starch-bound LPC. The procedure used was that described in ref. [1].

Amylase activity. The enzyme was isolated according to the procedure of ref. [20] and assayed by the starch–I_2 method [21] as described in ref. [1].

Preparation of [1-^{14}C]1-*palmitoyl LPC.* This was prepared from [1-^{14}C]dipalmitoylphosphatidylcholine (114 mCi/mmol) obtained from Amersham Corporation. The radioactive material (2 μCi), diluted with 4 mg of dipalmitoylphosphatidylcholine obtained from Sigma, was hydrolysed with the phospholipase A_2 of *Crotalus adamanteus* from Worthington Corporation. The method used was adapted from that of ref. [22] described in ref. [23]. The dipalmitoylcholine, dissolved in 2 ml of dry Et_2O, was shaken for 1 hr at room temp. in a screw-capped tube with 20 units of the phospholipase in 0.1 ml of 0.1 M Tris, pH 7.5. To the mixture was added 30 ml $CHCl_3$–MeOH (2:1) and the soln dried over Na_2SO_4. The filtered soln was evapd to small vol. and then subjected to TLC on Si gel G in $CHCl_3$–MeOH–HOAc–H_2O (65:25:1:4). The labelled LPC was located using a radiochromatogram scanner and then eluted from the Si scrapings with $CHCl_3$–MeOH (1:4). ^{14}C content of the sample was measured by scintillation counting using a fluor composed of 4 g of PPO in 1 l. of toluene–Triton X100–H_2O (6:3:1). LPC was quantitated by measuring inorganic Pi of a sample according to the procedure of ref. [24].

Protein was measured by the method of ref. [25].

LPL isolation. The enzyme was isolated from either aleurone or endosperm by grinding the separated tissue from five germinated seeds in a pestle and mortar with 5 ml of ice-cold Pi (10 mM, pH 7). The homogenates were centrifuged first at 500 *g* for 5 min and the supernatant from this centrifugation then spun at 105 000 *g* for 90 min. The supernatant gave the soluble enzyme and the 105 000 *g* pellet, resuspended in 2 ml of the Pi buffer, gave the particulate enzyme. In some cases the pellets were washed with 2 ml of Pi buffer and recentrifuged at 105 000 *g* for 90 min and the pellet and supernatants assayed for activity.

LPL assay. LPC (11 300 cpm, 200 nmol) was evapd in a 15-ml centrifuge tube. The substrate was dissolved in 0.6 ml NaOAc buffer (0.1 M, pH 5.1) and the reaction started by the addition of 0.4 ml of enzyme. The mixture was gently shaken at room temp., 20°, for 20 min and stopped with 6 ml heptane–*iso*-PrOH–1N H_2SO_4 (60:40:1). One-half of the heptane extract containing the labelled free fatty acid was counted.

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