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ENHANCEMENT BY GIBBERELLIC ACID AND ASYMMETRIC DISTRIBUTION OF LYSOPHOSPHOLIPASE IN GERMINATING BARLEY

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Key Word Index—*Hordeum distichum*; Gramineae; barley; half-seeds; germination; lysophospholipase; starchy endosperm; scutellum; aleurone; gibberellic acid.

Abstract—Germination of whole barley seeds for 4 and 6 days followed by measurement of lysophospholipase (lysolecithin acyl hydrolase, LAH) in the embryo-containing and embryo-free halves revealed a gradient of activity between the two halves of the seed. Most of the activity appeared in the embryo-containing half. This gradient decreased slightly in the aleurone and dramatically in the starchy endosperm during the 2 day germination interval. Embryo-containing and embryo-free half seeds of surface sterilized barley were placed separately on sterile agar plates. After 4 and 6 days LAH was observed in both the aleurone and starchy endosperm of the embryo-containing halves. In the embryo-free halves, LAH appeared at low levels in the aleurone and was virtually absent in the starchy endosperm. The scutellum of germinating seeds contains LAH activity. Exposure of embryo-free half seeds to GA_3 for 24 hr showed enhancement of acidic and alkaline LAH activities in the aleurone fraction and in the GA_3-medium in which the half seeds were treated. The LAH activity of the starchy endosperm of these half seeds was little changed by GA_3 treatment. Exposure of isolated aleurones to GA_3 for 24 hr resulted in substantial enhancement of acidic and alkaline LAH activities in the bathing medium and in fractions prepared from the aleurone. The physiological significance of the influence of GA_3 on LAH activity during barley germination is discussed.

INTRODUCTION

Recently, we reported the disappearance of starch-bound lysophosphatidylcholine (LPC) during germination of barley [1]. The occurrence of lysophospholipase (LAH) in barley was first reported by Contardi and Ercoli[2]. We have described [3] the occurrence of soluble and particulate LAH in the aleurone and starchy endosperm of germinating barley. Measurements of these activities over an 8-day period showed particulate activity from the starchy endosperm to undergo a 5-fold increase between days 4 and 5 of germination coincident with the production of maximum amylase activity and the most rapid disappearance of starch-bound LPC. The aleurone LAH activities increased relatively steadily and represented a 20–30-fold increase during the 8-day germination period.

The enhancement of hydrolytic enzymes of barley aleurone layers by GA_3 is well documented ([4–6] and references cited therein). In this communication we report on the changes occurring in LAH activity in the embryo-free and embryo-containing halves of a barley seed at two stages of germination; on the occurrence of the activity in the scutellum of germinating seeds; and of enhancement of the activity in embryo-free barley half seeds and aleurone layers by GA_3.

RESULTS AND DISCUSSION

Aleurone and starchy endosperm fractions of barley reveal marked increases in the total and specific activity of LAH during germination [3]. The activity shows pH optima under acidic and alkaline conditions, with the alkaline LAH being the more active. Preliminary observations on the stability of the two activities and of their purification indicate they are different enzymes (unpublished results). In the previous study [3] we measured the acidic activity because the α-amylase, which we believe is the likely candidate responsible for the release of the starch-bound LPC, has an acidic pH optimum [7, 8].

The changing distribution of LAH activity in the germinating seed is illustrated by the data in Table 1 for days 4 and 6 of germination. It is during this period of germination that the most rapid decline in starch-bound LPC and the most dramatic rise in the starchy endosperm particulate LAH occurs [3]. Consequently, it was anticipated that marked changes in the distribution of LAH in the seed would be observed. In all fractions the LAH activity is greater in the embryo-containing half of the seed than in the distal half. This gradient is shown by the column (+/−) in Table 1. For each tissue fraction the amount of LAH activity present increases but the gradient between the two halves declines as germination progresses from days 4 to 6. The most striking change in this gradient of activity is found in the starchy endosperm particulate fraction in which it changes from 5.6 to 1.8. This occurs at the same time that the embryo-containing half shows a 3.5-fold increase in LAH

Table 1. LAH (pH 5) activities of fractions from the embryo-containing (+) and embryo-free (−) halves of germinated barley seeds

Germination period (days)	Amount of palmitate released (nmol/min/total fraction)											
	Aleurone						Starchy endosperm					
	Soluble			Particulate			Soluble			Particulate		
	+	−	+/−	+	−	+/−	+	−	+/−	+	−	+/−
4	15.1	6.0	2.5	5.0	3.4	1.5	6.8	1.8	3.8	4.5	0.8	5.6
6	18.8	12.4	1.5	15.4	11.7	1.3	7.5	3.3	2.3	15.5	8.4	1.8

After 4 and 6 days of germination the roots and shoots were removed from batches of 10 barley seedlings. The remaining tissue was cut transversely to give halves with (+) and without (−) the embryos. LAH activity was measured in soluble and particulate fractions from the aleurone and starchy endosperm from (+) and (−) halves as described in the Experimental.

activity in this fraction. A duplicate experiment gave data similar to that shown in Table 1. Although LAH activity isolated from a tissue fraction will vary among experiments [3] the changes in the gradients were greatest in the starchy endosperm particulate fraction.

An asymmetric modification of endosperm tissue in barley germination was first noted by Brown and Morris [9] and the subject has been extensively reviewed more recently by Palmer [10]. Okamoto and Akazawa [11] have shown, in an elegant study of germinating rice seeds, the initiation of amylase activity in the epithelium followed later by induction of additional amylase molecules in the aleurone cells. More recently, Akazawa *et al.* [12] have shown that the scutellar epithelium in barley is the site of initial formation of proteinase, RNAase and α-amylase. Briggs had earlier shown [13] that 6.5% of the endospermic α-amylase of malt was of embryonic origin. Clearly, the pattern of starch digestion is initiated at the embryonic tissues. We have shown alkaline and acidic LAH activities to be present in the scutellum of germinating barley (Table 2) but we do not know that these are the source of the LAH in the starchy endosperm.

It is known that during the first 2 days of germination of barley, gibberellic acid is produced in the scutellum and later the embryo axis [14]. From the embryo axis the liberated gibberellins diffuse across the endosperm to the aleurone cells. The induction of α-amylase, along with several other hydrolytic enzymes by these cells, occurs in response to GA [4–6]. As the disappearance of the starch-bound lysophospholipids occurs simultaneously with the production of maximum amylase activity and the latter is induced by GA, it might be speculated that LAH activity might also respond to GA.

The synthesis of gibberellin is in the embryo [15] so that if LAH activity is under its control then embryo-free half seeds, after imbibition of water, should be devoid of LAH, but the embryo-containing half should possess the activity.

A comparison of the acidic LAH in aleurone and starchy endosperm fractions of the two barley half seeds is shown in Table 3. The data clearly reveal that the LAH activities are found in the aleurone and starchy endosperm fractions of the embryo-containing half seed. The activity present in the embryo-free half is much less, and almost exclusively resides in the aleurone fractions. It is clear from these experiments that although LAH may be enhanced by GA produced in the embryo, some LAH appears independent of GA as evidenced by the activity in the embryo-free tissues. Conceivably, the LAH activities have different roles. The function of the GA-stimulated activity may be directed towards controlling the level of lysophospholipids liberated by the action of GA-induced α-amylase on the amylose–inclusion lipid complexes. The GA-independent LAH may be a hydrolase working in concert with phospholipases for the general breakdown of endosperm membrane lipid. It is noteworthy that in spite of LAH being present in the aleurone tissue of embryo-free half seeds it is only in the embryo-containing halves that significant amounts of LAH appear in the starchy endosperm. If the origin of the starchy endosperm LAH is an aleurone activity then it is possible that GA may play an additional role in contributing to its release into the starchy endosperm. A comparison of the aleurone LAH activities in the embryo-containing halves shown in Tables 1 and 3 reveal substantially higher

Table 2. Lysophospholipase in the scutellum of germinating barley

Germination (days)	Amount of palmitate released (nmol/min/10 scutella)	
	pH 5	pH 8
2	14.0	42.0
3	19.5	70.0
4	26.0	106.5
5	23.5	74.0
6	15.5	67.5

Scutella from 10 seeds at each germination stage were homogenized in 0.01 M phosphate buffer, pH 7. The enzyme was isolated and assayed as described in the Experimental.

Table 3. LAH (pH 5) activities of fractions from embryo-containing (+) and embryo-free (−) barley half-seeds

Time of imbibition (days)	Amount of palmitate released (nmol/min/total fraction)							
	Aleurone				Starchy endosperm			
	Soluble		Particulate		Soluble		Particulate	
	+	−	+	−	+	−	+	−
4	49.5	6.2	22.2	1.8	11.1	<0.2	4.5	<0.2
6	42.2	9.2	27.1	3.1	23.8	2.3	11.7	0.4

Two batches each of 10 barley seeds were cut transversely to give half-seeds with (+) and without (−) the embryos. Each group was surface sterilized and placed on 1% agar plates. At intervals of 4 and 6 days LAH activity was measured in soluble and particulate fractions from the aleurone and starchy endosperm from (+) and (−) half seeds as described in the Experimental.

levels of LAH in the isolated half-seeds described in Table 3. This probably reflects the influence of the elevated concentration of GA that may be present in such half seeds as compared with the whole seed tissue.

An enhancement of acidic and alkaline LAH activities in embryo-free barley half seeds in response to GA_3 is shown in Table 4. The half seeds were in contact with 1 μM GA_3 in an acetate buffer. After 24 hr the LAH activities were enhanced *ca* 4–7-fold in soluble and particulate fractions of isolated aleurones compared with those from control half seeds. The incubation medium of the control seeds was devoid of activity but the activities were clearly evident when GA_3 was present. This activity in the medium was most likely released from the aleurones as the activity in the separated starchy endosperm was unchanged from zero in the controls of the particulate fractions and only increased from zero to less than 2 units in the soluble fraction of the GA_3-treated samples (data not shown). One unit of activity is defined as 1 nmol of palmitate released per min under the assay conditions described for Table 4.

In Table 5 the response of isolated aleurones to GA_3 is shown by an experiment similar to that conducted with half seeds. Aleurones isolated from embryo-free half seeds which had imbibed water were incubated with 1 μM GA_3 in acetate buffer for 24 hr. The increases in the LAH activities were much greater in all fractions assayed than in the experiment with half seeds and probably reflected the much better penetration of GA_3 into the isolated aleurone.

These data prompt further study of the nature of the GA_3-induced enhancement of LAH activities, especially with respect to their timing relative to the GA_3-induced formation of α-amylase. A time lag

Table 4. Influence of GA_3 on acid and alkaline LAH activities in aleurone fractions of embryo-free barley half seeds

		Amount of palmitate released (nmol/min/total fraction)		
			Aleurone	
		Incubation medium	Soluble	Particulate
Control	Acidic	<0.2	2.8	1.3
	Alkaline	<0.2	7.0	4.4
1 μM GA_3	Acidic	2.8	16.0	5.6
	Alkaline	9.2	43.8	31.4

Half seeds were surface-sterilized with subsequent processing conducted under sterile conditions as described in the Experimental. The half seeds were placed on 1% agar plates for 3 days at 21° and then groups of 10 transferred to flasks containing either 1 mM acetate (pH 5) or 1 μM GA_3 in the same buffer. After 24 hr at 21° the half seeds were washed free of the incubation medium with the buffer and the aleurones separated. Acid and alkaline LAH were measured in the soluble and particulate fractions of this sample and in the incubation medium as described in the Experimental.

Table 5. Influence of GA_3 on acidic and alkaline LAH activities on isolated aleurones from embryo-free barley half seeds

		Amount of palmitate released (nmol/min/total fraction)		
			Aleurone	
		Incubation medium	Soluble	Particulate
Control	Acidic	<0.2	4.0	1.6
	Alkaline	7.0	8.3	7.9
1 μM GA_3	Acidic	6.3	53.8	14.0
	Alkaline	15.3	124.8	70.4

Aleurones were separated from half seeds which had imbibed water from 1% agar plates as described in the legend to Table 4. Groups of 10 aleurones were incubated in either 1 mM acetate (pH 5) or 1 μM GA_3 in the same buffer. After 24 hr at 21° the tissues were washed free of the incubation medium. The acid and alkaline LAH were measured in the aleurone soluble and particulate fractions and in the incubation medium as described in the Experimental.

between the appearance of α-amylase and that of LAH could be of potential advantage in the germination process. It is probable that the formation and release of α-amylase into the starchy endosperm leads initially to the breakdown of those starch granules adjacent to the aleurone layer. The starch lipids, principally lysophospholipids, would be freed from the amylose helices and could modify the adjacent aleurone cell membranes so as to promote the secretion of GA-induced hydrolases. Lysophosphatidylcholine is a noted membrane-fusing agent [16, 17] and secretion is a process involving membrane-fusion. Thus, a lag period between α-amylase and LAH appearance might be anticipated.

EXPERIMENTAL

Materials. Hordeum distichum L. cv Georgie was grown from seed originally supplied by RHM, Crops Department, High Wycombe, U.K. Gibberellic acid (GA_3), Grade III and unlabelled 1-palmitoyl LPC were obtained from Sigma. 1-[1-^{14}C]palmitoyl LPC (5 mCi/mmol) was a product of Amersham. For the LAH assays it was diluted with unlabelled LPC giving a sp. act. of 11000 cpm/200 nmol in 100 μl toluene–EtOH (1:1).

Germination and dissection of seeds. Batches of seeds were germinated by total immersion in H_2O for 24 hr. They were transferred to moist paper towels in loosely covered dishes in the dark at 20–23° for specified times. The seedlings were thoroughly rinsed with H_2O each day. After specific germination periods the seedlings were separated into starchy endosperm, scutellum and aleurone (plus testa and pericarp). The roots and shoots were carefully pulled free by first prying open the husk with forceps and a microspatula. The husk was easily removed with forceps. The seed coat (testa and pericarp) was broken near the scutellum and the starchy endosperm either slipped or was scraped free with a microspatula; its removal became easier as germination progressed. The scutellum, still attached to the seed coat was carefully pulled free, leaving the seed coat with the adhering aleurone layer.

Scutellar LAH activity. Scutella from groups of 10 seeds selected from each germination interval of 2–6 days were used. The tissue was ground in a pestle and mortar with ice-cold 0.01 M Pi buffer, pH 7 (4 ml). The crude homogenate was centrifuged at 800 *g* for 5 min and the supernatant was used as the source of enzyme.

Experiments with embryo-containing (+) and embryo-free (−) half seeds. The roots and shoots of seedlings germinated for 4 and 6 days were removed and the remaining tissue cut transversely with a razor blade. LAH was assayed in fractions isolated from the aleurone and starchy endosperm of the (+) and (−) halves from 10 seedlings at each time interval.

For experiments with the separated (+) and (−) halves, ungerminated dry seeds were cut transversely and the extreme tips of the (−) halves removed. The separated halves were surface-sterilized with 1% sodium hypochlorite soln for 20 min, then rinsed thoroughly with sterile H_2O. All subsequent transfers were made in a sterile laminar flow hood. Glassware and thermally stable solns were sterilized by autoclaving; other solns were filtered through Millipore filters with 0.45 μm pore size. Groups of (+) and (−) half seeds were transferred to Petri dishes containing 1% agar and stored in the dark at 21°. Batches of 10 half seeds from each group were sampled at 4 and 6 days for assay of LAH in the aleurone and starchy endosperm fractions.

Gibberellic acid (GA_3)-enhancement of LAH in (−) half seeds and in isolated aleurones. After storage for 3 days on 1% agar plates, groups of 10 half seeds were transferred to 25 ml control flasks. Control samples were incubated in 2 ml 1 mM NaOAc buffer (pH 5) and the exptal samples in 1 μM GA_3 in the same buffer. The flasks were stoppered with cotton plugs and shaken slowly at 21°. After 24 hr the incubation medium was removed and the half seeds washed twice with 2 ml of buffer. LAH was measured in the combined incubation medium and washings. The half seeds were separated into aleurone and starchy endosperm for measurement of LAH in soluble and particulate fractions.

GA_3 enhancement of LAH in isolated aleurones was conducted under conditions identical to those used for the half seeds. The aleurones were separated from the half seeds after the initial 3 day storage interval and then groups of 10 aleurones incubated with either the GA_3 in buffer or buffer alone. Measurement of LAH was made in the combined incubation medium and washings and in soluble and particulate fractions of the aleurone.

LAH isolation. The soluble and particulate enzymes were isolated as described in ref. [3].

LAH assays. [^{14}C]LPC was evaporated in a 15 ml centrifuge tube. For assay of the acidic LAH the substrate was dissolved in 0.6 ml NaOAc buffer (0.1 M, pH 5) and the reaction started by the addition of 0.4 ml enzyme. The mixture was gently shaken at 21° for 15–20 min. The alkaline LAH was measured by first dissolving the substrate in 0.8 ml Tris–HCl buffer (0.1 M, pH 8) and then starting the reaction by the addition of 0.2 ml enzyme. The mixture was shaken at 21° for 5–10 min. Each reaction was stopped with 6 ml heptane–*iso*-PrOH–1 N H_2SO_4 (60:40:1). The ^{14}C content of the released fatty acid in one-half of the heptane extract was measured.

Radioactivity counting. The ^{14}C content of the heptane extracts from the LAH assays was measured by scintillation counting using a fluor composed of 4 g PPO in 1 l. toluene–Triton X-100–H_2O (6:3:1).

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