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AUTOLYSIS OF PHOSPHOLIPIDS IN HOMOGENATES OF VARIOUS PLANT TISSUES

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Abstract—The rates of autolysis of endogenous phospholipids were measured in homogenates of 17 cultivated plant tissues. For seven out of the 17 samples, the rates of autolysis of phosphatidylcholine (PC) were greater than 10% hydrolysis per hr (at pH 7.5 and 4°). The addition of dibucaine (2 mM) inhibited the autolytic rates in homogenates of 12 out of 17 samples. The only plant homogenates where dibucaine stimulated the autolytic rates were those of potato tubers. Although dibucaine inhibited the rate of autolysis of PC in potato leaf homogenates there was no advantage to adding it during homogenization and preparation of differential centrifugation fractions from potato leaves. Homogenization with different types and concentrations of osmotica affected the rates of autolysis of PC. Buffering the homogenates at pH 8 drastically inhibited the rates of autolysis in potato leaf homogenates but had little effect on bean leaf homogenates. Various strategies for controlling the rate of membrane lipid breakdown in different plant extracts are discussed.

INTRODUCTION

Many plant tissues contain high levels of either lipolytic acyl hydrolase activity (which exhibits phospholipase B and galactolipase activity) or phospholipase D activity [1, 2]. In 1970 Galliard reported that 26% of the endogenous phospholipids in potato tuber homogenates were degraded in 10 min (pH 5.5 and 0°) [3]; we have recently confirmed these experiments [4]. Homogenates of potato leaves have also been shown to undergo rapid degradation of membrane lipids [5–7].

The present study was undertaken to compare the high rates of autolysis of phosphatidyl choline (PC) which had previously been observed with potato tubers [4] and potato leaves [6, 7] with those rates obtained with 15 other plant tissues under comparable conditions. Experiments were also conducted to compare the properties of some of the lipolytic enzymes from different plant tissues, that is the effect of inhibitors and the influence of the pH of homogenization buffers.

RESULTS

We have previously reported that the rate of hydrolysis of endogenous PC in potato tuber homogenates was 15.0% hydrolysed per 4 hr at 4° (based on the original amount of PC) [4]. With potato leaf homogenates the rates were 18.3% hydrolysed per 4 hr at 4°, and 14.6% hydrolysed per 30 min at 25° [7]. In the first experiment (Table 1) the rates of PC hydrolysis in homogenates of potato leaves and tubers were compared with those of 15 other common plant tissues. The data were reported by listing the rates of hydrolysis at 4° from highest to lowest. Although potato tubers and leaves are often noted for containing high levels of lipolytic enzymes [1], more than half of the other plant tissues had even higher rates of autolysis (Table 1). The highest rates of hydrolysis were observed with bean leaf homogenates. At 4° PC hydrolysis was detectable in all of the plant homogenates except those of pea and spinach leaves. The rates of hydrolysis were higher at 25°, but there was no apparent correlation between the rates of hydrolysis at 4° and 25°. Because most biochemical studies of plant tissue are conducted at 0–4°, the remainder of the studies were conducted at 4°. Although the initial levels of PC in the 17 different plant homogenates varied from 92 to 1226 nmol/g fr. wt there was no correlation between initial PC content and the rate of PC hydrolysis at either temperature.

Table 1. Comparative study of the rate of phosphatidylcholine (PC) hydrolysis which occurs in homogenates of various plant tissues

	% PC hydrolysed during 1 hr (pH 7.5)		Initial ($T = 0$) nmol PC per g fr. wt
	4°	25°	
Bean leaf	56.4 ± 3.1	80.3 ± 2.3	605 ± 23
Eggplant leaf	21.7 ± 2.2	49.5 ± 0.7	635 ± 4
Eggplant fruit	20.3 ± 1.8	28.5 ± 2.8	92 ± 6
Pumpkin leaf	15.7 ± 0.8	66.8 ± 1.1	817 ± 34
Pepper leaf	12.2 ± 1.2	35.5 ± 1.0	918 ± 4
Radish leaf	11.1 ± 0.7	25.1 ± 1.5	607 ± 5
Barley leaf	10.1 ± 2.0	21.3 ± 1.8	595 ± 12
Carrot leaf	7.0 ± 2.1	32.3 ± 0.4	790 ± 34
Potato leaf	6.1 ± 0.5	47.3 ± 2.6	1009 ± 11
Tomato leaf	4.6 ± 0.9	17.4 ± 1.1	1226 ± 31
Soybean leaf	3.9 ± 1.5	60.1 ± 0.5	645 ± 3
Potato tuber	3.8 ± 0.4	20.0 ± 1.3	110 ± 6
Tobacco leaf	3.6 ± 0.8	11.3 ± 1.2	319 ± 9
Barley root	2.9 ± 1.3	11.0 ± 1.7	584 ± 17
Beet leaf	2.6 ± 1.1	6.3 ± 1.1	404 ± 12
Pea leaf	0.0	12.0 ± 0.5	670 ± 15
Spinach leaf	0.0	4.7 ± 0.6	532 ± 11

Potential techniques for evaluating rates of autolysis of PC in other plant tissues

The data reported in Table 1 revealed that the rate of autolysis of PC is very high in homogenates of many types of plant tissues. Unfortunately, the techniques which were used to measure the rates of autolysis are quite laborious. The next experiment (Table 2) was undertaken in order to evaluate whether either of two convenient fluorometric assays might be useful in predicting the rates of autolysis of PC in homogenates of plant tissues. 4-Methylumbelliferyl laurate (4-Mu-Laur) is a common esterase substrate and has been used as a substrate for plant acyl hydrolases [8] and lipases [9, 10]. Since we recently showed that the rate of autolysis of endogenous PC in potato leaf homogenates was nearly identical to the rate of hydrolysis of 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl] phosphatidyl choline (C_6-NBD-PC) [11] it seemed essential to investigate whether a correlation also existed with other plant tissues. The results of Table 2 reveal that neither the 4-Mu-Laur assay nor the C_6-NBD-PC assay appear to be useful techniques for predicting rates of autolysis of PC in plant homogenates.

Effect of dibucaine on the rates of autolysis of PC in plant homogenates and membrane fractions

We have recently demonstrated that the local anaesthetic, dibucaine (at 0.5–5 mM), inhibited the rate of autolysis of PC in potato leaf homogenates [7]. However, with homogenates of potato tuber we found that very low concentrations of dibucaine (25–100 μM) inhibited PC hydrolysis, but higher concentrations (1–2 mM) caused a 5- to 6-fold stimulation [4]. To investigate the inhibition of autolysis by dibucaine (2 mM), 15 other plant tissues were surveyed (Table 3). In 12 of the plant homogenates the addition of dibucaine to the homogenates immediately after homogenization and filtration inhibited PC hydrolysis as we previously reported for potato leaves [7]. In four of the homogenates dibucaine had no effect. The stimulation of PC hydrolysis by dibucaine as previously reported [4] was only observed with potato tuber homogenates, suggesting that this response may be unique.

Because 2 mM dibucaine significantly inhibited the rate of PC hydrolysis in 12 out of 17 types of plant tissues, the next experiment (Table 4) was designed to investigate whether including dibucaine during homogenization would improve the recovery of membrane fractions during differential centrifugation. When potato leaves were homogenized with dibucaine (1 or 2 mM) in the grinding buffer, the resulting 13 000 *g* and 100 000 *g* pellets actually contained less PC than the controls. Even when the fractions were stored for 24 hr at 4° there was still less PC in the dibucaine treatments than in the controls. Although dibucaine caused a 65.5% inhibition in the rate of PC hydrolysis in potato leaf homogenates (Table 3), it

Table 2. Investigation of the possible correlation between the rate of PC autolysis with that of two fluorometric substrates

	% PC hydrolysed during 1 hr at 4° (pH 7.5)	Fluorometric assays 4-Mu-Laur (μmol/min/g fr. wt)	C_6-NBD-PC (nmol/min/g fr. wt)
Bean leaf	56.40	0.015	1.25
Eggplant leaf	21.70	221.0	nm*
Eggplant fruit	20.30	47.0	nm
Pumpkin leaf	15.70	0.068	nm
Pepper leaf	12.20	1.87	nm
Radish leaf	11.10	0.125	nm
Barley leaf	10.10	2.2	0.017
Carrot leaf	7.00	0.003	nm
Potato leaf	6.10	8.7	8.09
Tomato leaf	4.60	0.05	1.05
Soybean leaf	3.90	0.009	2.5
Potato tuber	3.80	10.4	4090.0
Tobacco leaf	3.60	0.113	0.75
Barley root	2.90	12.2	0.895
Beet leaf	2.60	0.056	nm
Pea leaf	0.00	0.122	nm
Spinach leaf	0.00	0.105	0.021

*nm = Not measured.

Table 3. Effect of the addition of dibucaine (2 mM) to the homogenates (immediately after homogenization and filtration) on the rate of PC hydrolysis in the homogenates of various plant tissues

	% PC hydrolysed during 1 hr at 4°		
	Control	Dibucaine	% Inhibition
Bean leaf	56.4 ± 3.1	21.3 ± 0.4	62.2
Eggplant leaf	21.7 ± 2.2	10.0 ± 0.5	53.9
Eggplant fruit	20.3 ± 1.8	13.9 ± 1.9	31.5
Pumpkin leaf	15.7 ± 0.8	0.0	100.0
Pepper leaf	12.2 ± 1.2	9.5 ± 0.7	22.1
Radish leaf	11.1 ± 0.7	7.1 ± 1.6	36.0
Barley leaf	10.1 ± 2.0	11.8 ± 1.2	0
Carrot leaf	7.0 ± 2.1	1.3 ± 0.9	81.4
Potato leaf	6.1 ± 0.5	2.1 ± 0.2	65.5
Tomato leaf	4.6 ± 0.9	2.8 ± 0.2	39.1
Soybean leaf	3.9 ± 1.5	0.0	100.0
Potato tuber	3.8 ± 0.4	22.3 ± 0.8	0*
Tobacco leaf	3.6 ± 0.8	2.3 ± 0.6	36.1
Barley root	2.9 ± 1.3	1.2 ± 0.4	58.6
Beet leaf	2.6 ± 1.1	2.6 ± 1.3	0
Pea leaf	0.0	0.0	0
Spinach leaf	0.0	0.0	0

*587% stimulation.

Table 4. Effect of dibucaine in the grinding buffer during the fractionation of potato leaves

Composition of grinding buffer	nmol PC/1 ml Fraction			
	13 000 *g* pellet		100 000 *g* pellet	
	0 hr	24 hr	0 hr	24 hr
Control	761 ± 34	688 ± 47	458 ± 38	405 ± 20
+ 1 mM Dibucaine	640 ± 31	546 ± 7	437 ± 12	404 ± 3
+ 2 mM Dibucaine	614 ± 41	537 ± 37	327 ± 19	299 ± 28

actually had deleterious effects upon the recovery of PC during differential centrifugation (Table 4). We previously reported a similar observation with potato tuber homogenates [4]; low concentrations of dibucaine (100 μM) inhibited the rate of PC hydrolysis by more than 50% but when the same concentration was added during differential centrifugation the pelleted fractions contained less PC than the controls.

Effect of pH and osmoticum on the rates of autolysis of PC

One of the recommended strategies for controlling the breakdown of membrane lipids during cell fractionation studies is to buffer the fractions at pH 7.5–8 [12]. We previously documented that this technique is quite effective with potato tuber homogenates [4]. When potato leaf homogenates were prepared (Fig. 1A) the rates of PC hydrolysis were found to be 65, 18 and 6% hydrolysed per hr at 4° when buffered at pH 6, 7 and 8, respectively. When bean leaf homogenates were similarly prepared (Fig. 1B) the rates of PC hydrolysis were extremely high at pH 6 or pH 8. This experiment shows that buffering membrane preparations at pH 7.5–8 does not effectively control the rate of PC hydrolysis in all plant extracts.

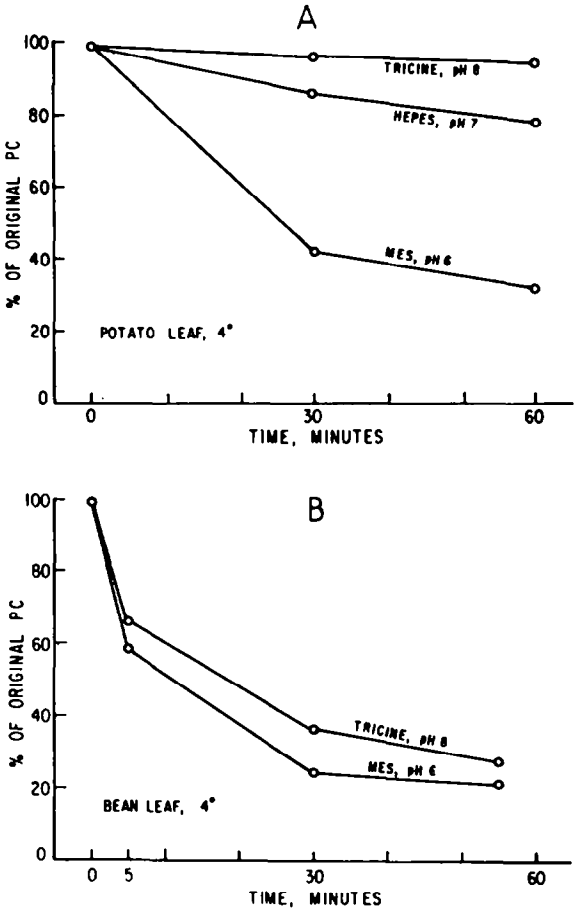

Fig. 1. Effect of pH of the grinding buffer on the rate of hydrolysis of phosphatidylcholine in homogenates of potato leaves (A) and bean leaves (B). All buffers were 0.1 M and contained NaOH as the counterion.

In the final experiment (Table 5) the rates of PC hydrolysis were compared in potato leaf homogenates prepared with various osmotica commonly used in plant organelle isolation. In the previous sections of this paper and in the previous reports from this laboratory [4, 6, 7] all plant homogenates were prepared with homogenization buffer which contained 0.3 M sucrose. When homogenates were prepared without sucrose or with 0.6 M sucrose the rates of PC hydrolysis were higher than with 0.3 M sucrose (Table 5). When homogenates were prepared with 0.6 M mannitol the rates of PC hydrolysis were more than 40% lower than those with the other three treatments. This experiment demonstrated that varying the osmoticum can significantly influence the rates of PC hydrolysis. However, further work is required in order to determine the ideal chemical species and concentration of osmoticum to minimize membrane breakdown in various plant homogenates.

DISCUSSION

This study has demonstrated that high rates of membrane lipid autolysis are not unique to homogenates of potato leaves and tubers. Indeed, nine of the 17 homogenates of plant tissues which were tested had higher rates of autolysis (at 4°) than either potato leaves or tubers.

The autolytic rates measured in this study were based on rates of disappearance of PC in the various plant homogenates. In higher plants PC is commonly hydrolysed by two enzymes, lipolytic acyl hydrolase or phospholipase D [1, 2]. For this study no attempt was made to try to determine which type of enzyme activity was present in each tissue. It has previously been reported that lipolytic acyl hydrolase activity is high in bean leaves and potato tissues [1] and phospholipase D activity is highest in rapidly growing plant tissues (storage tissues and seeds) [2]. However, the lipolytic properties of most of the tissues tested in this study have not previously been reported.

Among the several techniques which were evaluated for their effectiveness at controlling the rates of membrane lipid autolysis several conclusions can be drawn. Although dibucaine inhibited the autolytic rates in 12 out of the 17 plant homogenates it did not improve the yield of membrane fractions prepared from potato leaves (Table 4). We have previously reported similar effects of

Table 5. Effect of various osmoticum on the rate of phosphatidyl choline (PC) hydrolysis in potato leaf homogenates*

Treatment	% Original PC hydrolysed during 4 hr at 4°
No osmoticum	20.5 ± 1.8
0.3 M Sucrose	14.5 ± 0.9
0.6 M Sucrose	16.3 ± 1.9
0.6 M Mannitol	8.1 ± 1.4

*All media were buffered with 0.1 M HEPES, pH 7.5, and contained 2 mM EDTA and 5 mM each of dithiothreitol and β-mercaptoethanol to control browning.

dibucaine on differential centrifugation fractions from potato tuber [4]. Because dibucaine and many of the other calmodulin antagonists are detergent-like [4], it is possible that they may solubilize the PC and cause it to not be precipitated at 100 000 *g* and thus lower the yield of membranes in the pelleted fractions. Buffering the homogenates at pH 7.5–8 as suggested by Galliard [12] was effective in controlling autolysis in homogenates of potato leaves, but not in those of bean leaves. Controlling the temperature at 4° provided some degree of inhibition of autolytic rates in all plant homogenates (Table 1) as also suggested by Galliard [12]. Preliminary experiments also indicate that the use of mannitol (instead of sucrose) as an osmoticum may also help control membrane lipid breakdown (Table 5). A possible reason for the lower autolytic rate with mannitol may be because chloroplasts are less likely to rupture (and release lipolytic enzymes) in mannitol than in sucrose [13].

EXPERIMENTAL

Materials. Leaves of barley, soybean and tobacco were obtained from plants growing in clay pots with commercial potting soil in a greenhouse for 1, 3 and 12 weeks, respectively. Seed potato tubers and spinach leaves were purchased locally. Barley seeds were germinated in the dark at 25° on filter paper moistened with 0.1 mM $CaCl_2$ for 4–5 days. All other plant tissue was obtained from mature plants grown in a local garden. The following cvs were used, bean (Kentucky wonder), egg plant (Burpee hybrid), pumpkin (Jack-O'-Lantern), pepper (California wonder), radish (sparkler), barley (unknown variety), carrot (Nantes), potato (Kennebec), tomato (Better boy), soybean (Harosoy), tobacco (LAFC 53), beet (Burpee's red ball), pea (Blue Bantam), spinach (unknown variety). 4-Mu-Laur was obtained from United States Biochemical Co. and C_6-NBD-PC from Avanti Polar Lipids, Birmingham, AL. Dibucaine was obtained from Sigma Chemical Co. All other reagents were the best grades commercially available.

Lipid analysis of plant homogenates. Homogenates were prepared essentially as previously described [4, 7]. Plant tissue (5 g) samples were homogenized in 20 ml of a soln containing 0.3 M sucrose, 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer (pH 7.5), 2 mM EDTA, 5 mM dithiothreitol and 5 mM β-mercaptoethanol in a chilled mortar and pestle. The homogenate was filtered through two layers of cheese-cloth and divided into 5 ml aliquots. For some studies the various potential inhibitors were added to some of the aliquots. All aliquots were vortexed and incubated in 4° or 25° shaking water baths. Triplicate 1 ml samples were removed immediately and after other designated times. The reactions were stopped by adding 50 μl of HOAc. Lipids were extracted with 7 ml of 6.7% (w/v) Na_2SO_4, spotted on 250 μm thick silica gel G TLC plates, developed in 85:15:10:3.5 $CHCl_3$–MeOH–HOAc–H_2O (17:3:2:0.7) and visualized with I_2. The spots which co-chromatographed with PC standards were scraped from the TLC plates and subjected to analysis for total P [7]. The rate of hydrolysis was expressed as the percentage of the total PC which was hydrolysed in 1 hr at 4° or 25°.

Differential centrifugation. Potato leaves were homogenized in grinding buffer, or grinding buffer containing 1 or 2 mM dibucaine. The filtered homogenate was centrifuged at 13 000 *g* for 15 min. The 13 000 *g* supernatant was centrifuged at 100 000 *g* for 50 min to obtain the microsomal fraction. The 13 000 *g* and 100 000 *g* pellets were each resuspended by adding 2 ml of original grinding buffer (which included the same concn of dibucaine as the originals). The resuspended pellets were incubated in a 4° water bath. Samples were removed at 0 hr and 24 hr and lipids were extracted, separated and analysed as described above.

Enzyme assays. Both assays were conducted with a fluorometer. The hydrolysis of 4-Mu-Laur was measured in a 2 ml reaction mixture containing 50 mM KPi buffer (pH 8), 40 μl of stock 20 mM 4-Mu-Laur in ethylene glycol monomethyl ether and 10–100 μl enzyme. The fluorometer was equipped with an excitation filter (360 ± 5 nm) and an emission filter (≥ 415 nm). The hydrolysis of C_6-NBD-PC was measured with slight modifications of a recently published procedure [14]. A 2 ml reaction mixture contained 5 μM C_6-NBD-PC, 50 mM Na-HEPES buffer (pH 7.1) and 10–100 μl enzyme. The fluorometer was equipped with an excitation filter (460 ± 5 nm) and an emission filter (≥ 535 nm).

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