Plant Physiol. (1984) 76, 92–95
0032-0889/84/76/0092/04/$01.00/0

Characteristics of a β-Galactosidase Associated with the Stroma of Chloroplasts Prepared from Mesophyll Protoplasts of the Primary Leaf of Wheat[1]

Received for publication March 7, 1984 and in revised form May 7, 1984

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ABSTRACT

Chloroplasts prepared from mesophyll protoplasts of the primary leaf of wheat (*Triticum aestivum* L. cv Egret) contain about 50% of the cellular β-galactosidase (EC 3.2.1.23) activity. More than 80% of this activity is associated with the stroma and most of the remainder, although tightly bound to the thylakoids, can be washed free with sodium pyrophosphate. The vacuole contained about 20% and the remaining enzyme was presumed to be cytoplasmic or associated with one of the other organelles. Both the vacuolar and chloroplast enzymes were capable of releasing galactose from the galactolipid monogalactosyldiacylglycerol. Apart from their distinct locations within the cells, we conclude that the enzymes are different because they differed with respect to assay pH-optimum, comparative activity against the synthetic substrates phenyl-β-D-galactoside, 4-methylumbelliferyl-β-D-galactoside, 6-bromo-2-naphthyl-β-D-galactoside, the disaccharide lactose, and the inhibitors D-galactose and D-galactono-1,4-lactone.

Senescence of mesophyll cells is a highly ordered and programmed phenomenon. At the ultrastructural level, the first detectable sign of cellular deterioration can be detected in the chloroplast where the internal features, especially the thylakoids, are degraded or disrupted (2–4, 9, 14, 15). Coincident with the disruption of the thylakoids is the appearance of osmiophillic granules. Apart from these changes in the appearance of the thylakoids, senescence is usually associated with degradation of Chl and a decline in photosynthetic electron transport (10, 13).

The most abundant lipids of the thylakoid membranes are MGDG[2] and DGDG (6). Three enzymes are required for complete hydrolysis of these compounds and their role is summarized in Figure 1. This scheme can be attributed to Sastry and Kates (16) who observed that glycerol, galactose and free fatty acids were the ultimate products of galactolipid degradation by crude extract from runner bean leaves. The intermediates monogalactosylglycerol and digalactosylglycerol were not detected. The presence of galactolipase in chloroplasts was subsequently established by Anderson *et al.* (1). In this paper we wish to report that chloroplasts contain a distinctive form of β-galactosidase.

[1] Supported by a grant to M. J. D. from the Wheat Industry Research Council of Australia. During the course of this study P. L. B. held a Melbourne University Research Fellowship.

[2] Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.

MATERIALS AND METHODS

Plant Material. Wheat seeds (*Triticum aestivum* L. cv Egret) were planted in a composted soil mixture and grown in a naturally-illuminated phytotron at 20°C. The plants were watered daily but received no additional nutrients.

Isolation of Protoplasts. Protoplasts were isolated from the primary leaves of 8- to 10-d old seedlings by the method of Waters *et al.* (20).

Isolation of Chloroplasts. Chloroplasts were released after rupture of the protoplasts by passage through a 10 cm long × 0.1 mm i.d. needle (12). The chloroplasts were purified by twice centrifuging (1500*g*, 5 min) through a layer consisting of 3% (w/v) Ficoll (Pharmacia, Uppsala, Sweden) in 0.5 M sucrose, 25 mM Tris-HCl, 1 mM EDTA pH 7.8. The pellet containing intact chloroplasts was resuspended finally in a large volume of 0.5 M sucrose, 25 mM Tris-HCl, 1 mM EDTA pH 7.8 and pelleted to remove the Ficoll. Chloroplasts prepared in this manner were estimated to be at least 90% intact based on the latency of the Hill-reaction in the presence of ferricyanide. In addition, they are essentially free of contamination by enzymes indicative of cytoplasm, vacuole, mitochondria, and peroxisomes (20).

Isolation of Vacuoles. Protoplasts were ruptured by passage through a 10 cm long × 0.1 mm i.d. needle and the vacuoles harvested by flotation in a sucrose:sorbitol discontinuous gradient as described by Waters *et al.* (20).

Estimation of β-Galactosidase Activity. *p-Nitrophenyl-β-D-Galactoside as Substrate.* A typical assay system contained 50 μl enzyme and 5 mM *p*-nitrophenyl β-D-galactoside dissolved in 150 μl 200 mM Na citrate-phosphate buffer pH 4.8. After incubation for 45 min at 37°C, each assay tube was centrifuged at 10,000*g* for 2 min, a 180-μl aliquot taken and added to 1 ml 0.3 M Na carbonate. After 30 min the liberated *p*-nitrophenol was measured at 410 nm.

Lactose and Substituted β-Galactosides as Substrates. Assay system contained 50 μl enzyme and 5 mM lactose and other substituted β-galactosides (*o*-nitrophenyl-β-galactoside, 4 methyl umbelliferyl-β-galactoside, phenyl-β-galactoside, 5-bromo-4-chloro-3-indoxyl-β-galactoside, and 6-bromo-2-naphthyl-β-galactoside) dissolved in 150 μl 200 mM Na citrate-phosphate buffer pH 4.8. After incubation for 150 min at 37°C, the reaction was terminated by boiling for 2 min. Galactose released was estimated as described below.

Galactolipid as Substrate. The assay was based on a procedure described by Gatt and Baker (7), 1 mg of MGDG was dissolved in chloroform:methanol (2:1, v/v) and added to 30 mg acid-washed Celite 535. After evaporating the solvent with dry nitrogen at 20°C, 150 μl 200 mM Na citrate-phosphate buffer 4.8 was added and the reaction started by adding 50 μl enzyme. Following

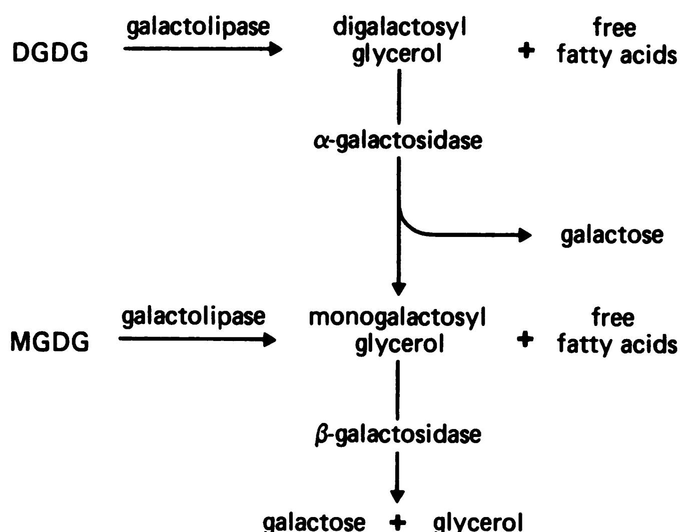

FIG. 1. Pathway for the degradation of MGDG and DGDG to galactose, glycerol, and free fatty acids through the concerted effort of galactolipase, β-galactosidase, and α-galactosidase (adapted from Sastry and Kates [16]).

incubation at 37°C for 270 min, the reaction was stopped by centrifuging at 10,000*g* for 5 min, thereby removing the substrate. The supernatant was collected and lyophylized. Activity against MGDG was detected by measuring the galactose released.

Estimation of Galactose. Galactose was estimated by the method of Kurz and Kurt (11). Lyophylized sample was dissolved in 600 μl 0.1 M Tris-HCl pH 8.6. Following the addition of 25 μl 16.5 mM NAD the A_{340} was measured; 5 μl galactose dehydrogenase (Sigma; *Pseudomonas fluorescens*; 8 units/ml) was added and after 60 min at 37°C the A_{340} measured. Galactose was estimated from the difference in A_{340} measured at time zero and after 60 min incubation.

RESULTS

Distribution of α- and β-Galactosidase between Chloroplast and Vacuole. Chloroplasts appear to represent the major location of β-galactosidase activity in mesophyll cells of the wheat primary leaf (Table I). Rupture of the chloroplasts and separation of the thylakoid and stromal components indicated that at least 80% of the chloroplast activity was associated with the stroma. The remainder was tightly associated with the thylakoids and although resisting being washed off with the lysis buffer, 25 mM Tris-HCl pH 7.8, the enzyme was readily removed by washing with 100 mM Na PPi. During the course of these studies it became apparent that the β-galactosidase activity associated with the chloroplasts increased as the leaf aged. For example, chloroplasts from 7-d old leaves accounted for 25% of the cellular activity, whereas in 9-d old leaves the proportion increased to 56%. Regardless of leaf age, the vacuoles contained about 20% of the leaf activity. The distribution of the other hydrolases followed the pattern established previously for wheat leaves (20). The enzyme α-galactosidase (using *o*-nitrophenyl-α-galactoside as substrate), which is presumed to participate in disassembly of the galactolipids (Fig. 1), was not detected in the chloroplast and appears to be predominantly a vacuolar enzyme.

Properties of the β-Galactosidases. *pH-Optimum.* The effect of pH on β-galactosidase activity of protoplasts, vacuoles, and chloroplasts against the synthetic substrate *p*-nitrophenyl-β-galactoside is shown in Figure 2. The chloroplast enzyme exhibited a sharp pH optimum at 4.8 whereas activity of the vacuole enzyme was optimal in the range pH 3.5 to 4.3.

Thermal Stability. The effect of preincubation temperature on the stability of the chloroplast and vacuole enzymes is shown in Figure 3. The chloroplast enzyme appears to be much more stable than the vacuole enzyme. However, this observation should be considered with caution. For example, the stability of enzymes can be improved markedly by the addition of exogenous protein; the enzyme nitrate reductase is an excellent example of this phenomenon (17). The high stability of the chloroplast β-galactosidase may therefore simply reflect the high concentration of protein, mostly the enzyme ribulose bisphosphate carboxylase, in this preparation. This hypothesis is supported to some extent as the stability of the vacuole β-galactosidase is improved dramatically by the addition of 1% BSA. However, even with this treatment the vacuole enzyme is still not as stable as the chloroplast enzyme.

Effect of Inhibitors. Both the vacuolar and chloroplast β-galactosidase possess an essential sulfhydryl group, as witness the response to 4-hydroxymercuric benzoic acid (Table II). The enzymes do differ markedly in their response to D-galactose and D-galactono-1,4-lactone; the chloroplast enzyme is insensitive to these compounds, whereas activity of the vacuolar enzyme is significantly reduced.

Substrate Specificity. Table III summarizes the activity of the chloroplast and vacuolar enzymes against a range of substrates. In each case, activity was assessed by measuring the galactose released and is related to activity with the substrate *o*-nitrophenyl-

Table I. *Comparison of the Distribution of β-Galactosidase and Other Hydrolases between the Vacuole and Chloroplasts of Wheat Leaf Mesophyll Protoplasts*

Enzyme	Protoplast	Vacuole	Chloroplast	Relative Activity	
				Vacuole[a]	Chloroplast[b]
	nmol h⁻¹ 10⁶ protoplasts⁻¹	*nmol h⁻¹ 10⁶ vacuoles⁻¹*	*nmol h⁻¹ 180 × 10⁶ chloroplasts⁻¹*	*% of protoplast activity*	
α-Mannosidase	628	355	5	56	1
β-*N*-Acetylglucosamidase	562	312	16	55	3
Acid phosphatase	83 × 10³	46 × 10³	6	55	1
Phosphodiesterase	707	247	37	35	5
Carboxypeptidase	2137	2184	ND[c]	102	0
α-Galactosidase	54	26	ND	48	0
β-Galactosidase	149	23	76	15	51

[a] Assuming one vacuole per protoplast.
[b] Assuming 180 chloroplasts per protoplast (19).
[c] ND, not detectable.

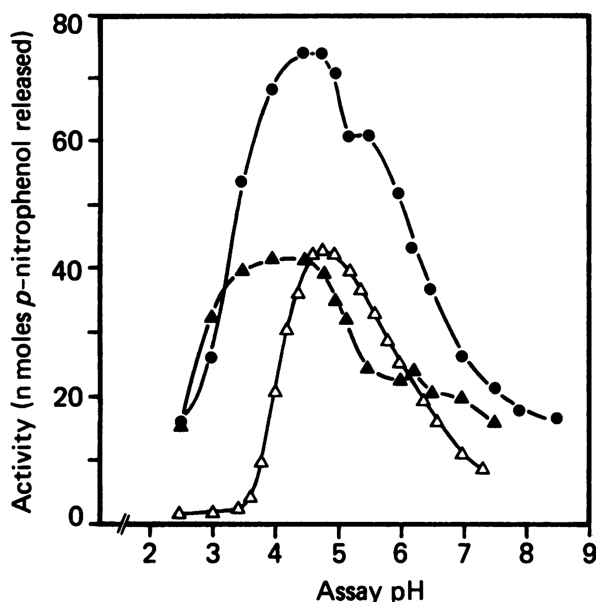

FIG. 2. pH-Response curve of β-galactosidase. Protoplasts (●), chloroplasts (△), and vacuoles (▲) were prepared as described in "Materials and Methods" and activity was determined with *p*-nitrophenyl-β-galactoside as substrate.

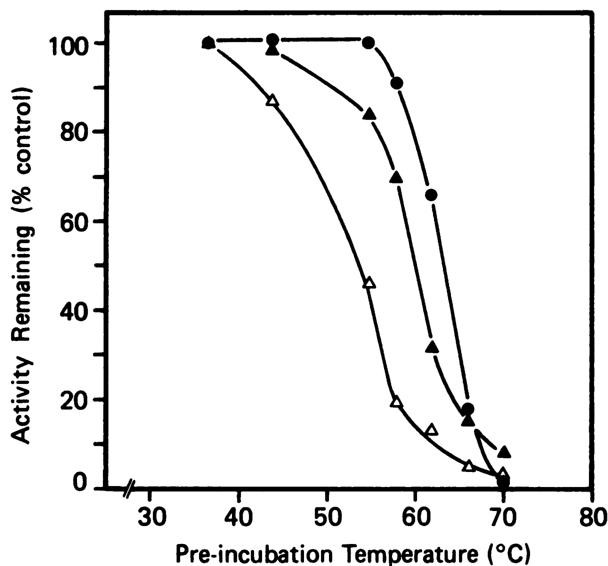

FIG. 3. Effect of preincubation temperature on β-galactosidase activity. Chloroplasts (●), vacuoles (△), and vacuoles + 1% BSA (▲) were prepared as described in "Materials and Methods". Samples of each preparation were preincubated for 10 min at each of the temperatures indicated and the residual activity determined with *p*-nitrophenyl-β-galactoside as substrate. Data is plotted as per cent control which was held at 0°C prior to assay.

β-galactoside. The reason why *o*-nitrophenyl-β-galactoside was selected in this experiment was that *p*-nitrophenol was an inhibitor of the galactose dehydrogenase used to measure the galactose released, *o*-nitrophenol was not inhibitory. The most striking difference between the enzymes was the very low activity of the chloroplast enzyme toward lactose compared to the vacuolar enzyme.

DISCUSSION

There is increasing evidence that chloroplasts are largely autonomous with respect to their senescence (5, 8, 12, 19, 20). Part of the argument in support of this hypothesis is the finding that chloroplasts contain a broad spectrum of hydrolytic enzymes, including peptide hydrolases (5, 8, 20) oxidative and peroxidative Chl-degrading enzymes (12), and galactolipase (1). The detection in chloroplasts of a β-galactosidase different from the β-galactosidase of the vacuole is further evidence consistent with this hypothesis.

Our finding that chloroplasts contain a distinctive form of β-galactosidase together with the earlier report of Anderson *et al.* (1) describing a chloroplast galactolipase is in general agreement with the scheme proposed by Sastry and Kates (16) for MGDG degradation (Fig. 1). Degradation of DGDG is not so clear since we have been unable to detect α-galactosidase activity in the chloroplast. In contrast, the vacuole seems to be an important source of this enzyme (Table I). However, our assays were based on the synthetic substrate *o*-nitrophenyl-α-galactoside and it is conceivable that the chloroplast α-galactosidase, should it exist, is unable to hydrolyze this substrate. An alternative view is that the scheme for DGDG degradation is incorrect and that the β-galactosidase is able to release digalactose (galactobiose) from digalactosylglycerol. The digalactose could then be exported and hydrolyzed by the extra-chloroplastic α-galactosidase. At present there is no evidence to allow any distinction between this scheme and that outlined in Figure 1; however, it is not usual for galactosidases to function as endo-hydrolases; rather, they seem

Table II. *Effects of Inhibitors on Chloroplasts and Vacuole β-Galactosidase Activity*

Inhibitor solutions (20 µl) were preincubated with 50 µl chloroplast or vacuole suspension for 20 min at 0°C. Residual β-galactosidase activity was determined with *p*-nitrophenyl-β-galactoside as substrate.

Inhibitor	Concentration	β-Galactosidase Activity	
		Chloroplast	Vacuole
	mM	*% control*	
4-Hydroxymercuric benzoic acid (Na salt)	1	100	56
	5	44	0
N-Ethylmaleimide	10	100	83
Iodoacetamide	10	100	82
EDTA	5	100	96
1,10-Phenanthroline	10	100	87
D-Galactose	10	100	70
	20	100	54
	0.1	100	55
D-Galactono-1,4-lactone	20	98	36

Table III. *Substrate Specificity of the Chloroplast and Vacuole β-Galactosidases*

Substrate	β-Galactosidase Activity[a]	
	Chloroplast	Vacuole
	µg galactose released/h	
o-Nitrophenyl-β-galactoside	3.73 (100)	0.92 (100)
4-Methyl umbelliferyl-β-galactoside	2.09 (56)	0.92 (100)
Phenyl-β-galactoside	1.07 (29)	2.80 (304)
5-Bromo-4-chloro-3-indoxyl-β-galactoside	1.47 (39)	0.43 (47)
6-Bromo-2-naphthyl-β-galactoside	2.20 (59)	0.87 (95)
Lactose	0.06 (2)	0.42 (46)
Monogalactosyldiacylglycerol	0.12 (4)	0.07 (8)

[a] Data in parentheses is β-galactosidase activity as a percentage of activity against *o*-nitrophenyl-β-galactoside.

to always be exo-hydrolases (18).

In the case of each of the hydrolytic enzymes listed above, activity is readily detected in chloroplasts isolated from leaves showing no readily assessable symptoms of senescence. The enzymes therefore appear to be normal constituents of nonsenescing chloroplasts. Modulation of the enzymes therefore becomes of paramount importance. Because of the sequential role of the galactolipase, α- and β-galactosidase in the degradation of the thylakoid galactolipids MGDG and DGDG (Fig. 1), it is most likely that control will be imposed at the level of galactolipase. It is interesting to extend this speculation and consider a recent study of the latent oxidative Chl-degrading enzyme detected in barley thylakoids (12). This enzyme is activated *in vitro* by linolenic acid and other long-chain, polyunsaturated fatty acids. Linolenic acid and other analogous C_{18} polyunsaturated fatty acids are the major constituents of thylakoid galactolipids (6). Therefore, modulation of galactolipase activity may impact directly on the regulation of Chl degradation.

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