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PURIFICATION AND SEPARATION OF α- AND β-GALACTOSIDASES FROM SPINACH LEAVES

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SUMMARY

α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) and β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) were extracted from spinach leaves and separated from each other by chromatography on DEAE-cellulose and by Sephadex gel filtration. The α-galactosidase was purified 15–30-fold and β-galactosidase over 1000-fold. Using *p*-nitrophenyl galactosides, α-galactosidase had an optimal pH at 5.3 and a K_m of 0.3 mM; it hydrolyzed melibiose at 1.5% of the rate of hydrolysis of the *p*-nitrophenyl α-galactoside. This enzyme still had phosphatase, sulfatase and esterase activities. β-Galactosidases had an optimal pH at 4.2 and a K_m of 0.4 mM. It hydrolyzed lactose at 2% of the rate of hydrolysis of *p*-nitrophenyl β-galactoside. The enzyme was free of α- or β-glucosidase, phosphatase or esterase; it still had considerable sulfatase activity.

Monogalactosyldiglyceride, adsorbed onto celite was hydrolyzed by the β-galactosidase at a 250-fold lesser rate than that of *p*-nitrophenyl β-galactoside. Neither enzyme hydrolyzed digalactosyldiglyceride from *Anacystis nidulans*.

INTRODUCTION

α- and β-galactosidases are widely distributed in nature. They have been observed in bacteria, molds, yeast, animals and plants[1]; the distribution in animal organs has been studied by CONCHIE *et al.*[2]. β-Galactosidase was extracted and purified from bacteria[1,3], germinating seeds[4] and animal organs[5–7]. α-Galactosidase was extracted and purified from bacteria[8], sweet almonds[9], rat uterus[10] and germinating seeds[4,11]. The enzymes in bacteria have an optimal activity close to pH 7, while those in mammalian or plant tissue have optima at acid pH values. The β-galactosidase of mammalian tissue resides in lysosomes[12]; the presence of these or similar subcellular particles

Abbreviation: PHMB, *p*-hydroxymercuribenzoate.

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(*i.e.* "spherosomes") in plant tissue has been suggested but has not yet been fully confirmed[13–15].

In this work, α- and β-galactosidases were isolated from spinach leaves which are commercially available. The two enzymes were separated from each other; α-galactosidase was purified about 30-fold and the β-galactosidase was purified over 1000-fold. Preliminary experiments were performed on the hydrolysis of the plant galactolipids by these enzymes.

EXPERIMENTAL PROCEDURE

Assay of enzymatic reactions

Incubation mixtures in volumes of 0.2 ml contained the nitrophenyl derivative, buffer and enzyme, as described in the legends to the tables and figures. The reaction was terminated and the product determined as follows. When purified enzyme preparations were employed, 1 ml of 0.25 M glycine–carbonate buffer (pH 10) or 1 ml of 0.125 M sodium tetraborate was added and the yellow color of *p*-nitrophenol, released from nitrophenyl derivative by the enzyme, was read at 420 nm. When crude enzyme preparations were used, 0.5 ml of 5% trichloroacetic acid was added and the precipitate was removed by centrifugation. 0.2 ml of 1 M NaOH was added to the supernatant, followed by 1 ml of 0.25 M glycine–carbonate buffer and the color was read at 420 nm. (The values used as molar extinction coefficients of *p*-nitrophenol and *o*-nitrophenol were 15 000 and 5300, respectively). The quantities of enzymes or cell fractions added to the assay mixtures were adjusted to produce a color of 0.1–0.6 *A* unit.

When disaccharides or glycolipids were used as substrates, the reaction was assayed by determining the quantity of galactose released as follows. After incubation, the mixtures (in volumes of 0.5 ml) received 0.1 ml of a 1 M solution of Tris buffer (pH 8.9), followed by 0.45 ml of a mixture containing 0.1 M Tris (pH 8.6), 4 mM GSH and 0.4 mM NAD^+. The absorption at 340 nm was recorded and 5 μl of galactose dehydrogenase (5 mg/ml suspended in an ammonium sulfate solution from Boehringer, Mannheim) were added. After 1 h at room temperature the absorption at 340 nm was again recorded. The amount of galactose was equivalent to the NADH formed (molar extinction coefficient of NADH: 6.3 *A* units/μmole).

Protein determination

Because of difficulties encountered in the determination of protein content of crude or partially purified plant preparations, three separate methods were employed. These were biuret[16], Folin's methods as modified by LOWRY *et al.*[17], and measurement of absorption at 280 nm, with corrections for nucleic acid content[18]. The values of the first two methods were fairly close, with variations up to 25%. The values obtained with the ultraviolet absorption method were up to 100% higher than those of the other two procedures. For very dilute protein solutions (*i.e.* purified β-galactosidase), the following modification of the procedure of LOWRY *et al.*[17] was employed. Protein solutions were adjusted to 1 ml with water. 0.2 ml of 10% Na_2CO_3 in 0.5 M NaOH was added, followed by 4 μl of 2.5% $CuSO_4$ in 5% sodium citrate. After 10 min, 0.1 ml of Folin's reagent was added and after 30 min the color was read at 750 nm.

Chemicals

Nitrophenyl derivatives listed in Table III were purchased from Sigma. Because of the sensitivity of the enzymes to minute quantities of heavy metals, all chemicals used were of analytical reagent grade. Enzyme grade ammonium sulfate (Mann) and glass redistilled water were employed. Monogalactosyldiglyceride was a gift of Professor A. ROSENBERG of the Hershey Medical School; digalactosyldiglyceride was a gift of Professor F. ALLEN of Pomona College.

Plant material

Spinach was obtained at the local supermarkets; it frequently had been in cold storage for 2–4 days prior to purchase. Several strains of spinach were encountered during the course of this work and somewhat variable results were obtained with various batches of spinach, especially at the stage of ammonium sulfate fractionation. It is therefore recommended that this step be investigated prior to enzyme purification to determine the degree of ammonium sulfate saturation optimal for partial separation of the two enzymes or for the highest increase of specific activities. Most results reported in this paper were obtained using spinach grown in southern California in May and June.

Subcellular fractionation of spinach leaves

Spinach leaves were homogenized with 2.5 vol. of a mixture of 0.5 M sucrose, 0.1 M Tricine and 0.02 M ascorbic acid; the pH of this mixture had been adjusted to 8.0. In Expt. 1 of Table II, 100 g of washed leaves were blended at high speed for 20 sec with 250 ml of the homogenizing medium. Debris was removed at 200 $\times$ g for 2 min, chloroplasts were precipitated at 6000 $\times$ g (2 min), mitochondria at 20 000 $\times g$ (20 min) and microsomes at 100 000 $\times g$ (60 min). Each of the subcellular fractions was suspended in 50 mM sodium phosphate buffer (pH 7). In Expt. 2, 50 g of washed leaves were mixed with sand and ground in a mortar and pestle with 125 ml of the homogenizing medium. The mixture was filtered through 4 layers of cheesecloth and debris was removed at 300 $\times g$ (3 min). Chloroplasts, mitochondria and microsomes were sedimented and suspended as in Expt. 1.

RESULTS

Purification of α- and β-galactosidases (Table I, Figs. 1–3)

Spinach was washed with water, the leaves and midribs were collected and blotted. 586 g were blended for 1 min at high speed with 586 ml of 50 mM sodium phosphate buffer (pH 7.0). The homogenate was centrifuged for 5 min at 10 000 $\times g$ and the supernatant ("extract") was adjusted to pH 3.8 with 38 ml of 1 M phosphoric acid. The precipitate obtained was removed by centrifugation at 16 000 $\times g$ for 5 min and the supernatant was left overnight at 4°. It was again centrifuged as before and the supernatant was retained. Solid ammonium sulfate was added to the supernatant; protein which precipitated at 40, 50 and 65% saturation was collected by centrifugation and dissolved in 20 mM sodium phosphate (pH 6.5). The solutions were dialyzed overnight against the same buffer, then clarified by centrifugation and stored at −20° until further used.

TABLE I

PURIFICATION OF α- AND β-GALACTOSIDASES

Incubation mixtures in volumes of 0.2 ml contained 100 μmoles of sodium acetate (pH 5), 1 μmole of *p*-nitrophenyl α- or β-galactoside and enzyme. They were incubated for 10 min at 37° and the *p*-nitrophenol was determined as described in EXPERIMENTAL PROCEDURE. Protein was determined according to LOWRY *et al.*[17].

Fraction	*Volume (ml)*	*Protein (mg)*	*Activity (μmoles/h)*		*Specific activity (μmoles/mg/h*		*Purification (-fold)*	
			α	β	α	β	α	β
Extract	670	7500	3135	7770	0.42	1.04	1.0	1.0
pH 3.8 supernatant	650	1400	2535	5250	1.80	3.75	4.3	3.6
Ammonium sulfate								
40–50% satn.	32	70	109	1926	1.56	27.50	3.7	26.4
50–65% satn.	22	118	536	234	4.54	1.98	10.9	1.9
α-Galactosidase	92	30	184		6.13		14.7	
β-Galactosidase								
DEAE-cellulose effluent	85	15		1166		77.7		75.0
Sephadex G-150 effluent								
Fraction 1	39	0.37		402		1086		1044
Fraction 2	78	4.7		195		42		41

Isolation of α-galactosidase

10 ml of the fraction which precipitated between 50 and 65% saturation with ammonium sulfate were used for further purification of α-galactosidase. This volume contained 54 mg protein as determined according to the method of LOWRY *et al.*[17], 38 mg according to the biuret procedure and 104 mg according to absorption at 280 nm (see *Protein determination*). Ammonium sulfate was added to 70% saturation, the precipitate thus obtained was dissolved in 1.5 ml of 50 mM sodium phosphate buffer (pH 7) and filtered through a column (38 cm × 3 cm) of Sephadex G-150 (40–120 μ, from which fine particles had been removed by repeated mixing with buffer and decantation), previously equilibrated against the same buffer. 5-ml fractions were collected at a rate of about 1 ml/min. Fractions 26–34 (Fig. 1) were combined and

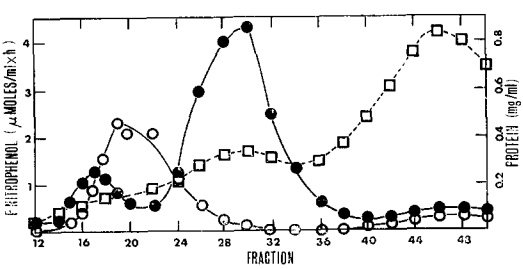


Fig. 1. Filtration of enzymes through Sephadex G-150. For details, see text. Assay conditions were similar to those in Table I except that 0.1 ml of each fraction was used; the reaction was terminated by the addition of 1 ml of 0.25 M glycine–carbonate buffer (pH 10). Protein was determined according to WARBURG AND CHRISTIAN[18]. ●—●, α-galactosidase; ○—○, β-galactosidase; □— — —□, protein.

stored at −20°. This procedure yielded an enzyme purified about 15-fold (Table I). The specific activity at the peak was 13 μmoles *p*-nitrophenol released by 1 mg protein in 1 h (about 33-fold purified).

Fig. 1 shows that β-galactosidase precedes α-galactosidase on the Sephadex column; however, some overlap occurred. A complete separation between the two enzymes could be obtained if smaller amounts of protein were filtered through the Sephadex column. Fig. 1 also shows a small peak of α-galactosidase, eluted prior to the β-galactosidase. This is most probably due to column overload and was not present when smaller amounts of protein were filtered through the column.

Isolation of β-galactosidase

17.5 ml of the fraction which precipitated between 40 and 50% saturation with ammonium sulfate (38.5 mg protein according to LOWRY *et al.*[17], 30 mg according to the biuret procedure and 55 mg according to absorption at 280 nm, see *Protein determination*) were diluted with an equal volume of water (final phosphate concentration 10 mM) and absorbed onto a column of DEAE-cellulose (30 cm × 3 cm) previously equilibrated with 10 mM sodium phosphate (pH 7). After all 35 ml had been absorbed, the column was eluted with 50 mM NaCl in 10 mM sodium phosphate (pH 7). Fractions of 100 drops (about 6 ml) were collected. Fractions 19–26 (Fig. 2) were combined, ammonium sulfate was added to 70% saturation, and the suspension was stored overnight at 4°. The precipitate was collected by centrifugation, dissolved in 2 ml of 20 mM sodium phosphate (pH 7) and filtered through a column (40 cm × 3 cm) of Sephadex G-150, previously equilibrated with the same buffer (Fig. 3). 4-ml fractions were collected at a rate of 0.4 ml/min. Fractions 19–23 were combined and will be designated as Fraction 1. Fractions 15–18 and 24–27 were also combined and will be designated as Fraction 2. Both fractions were stored at −20°.

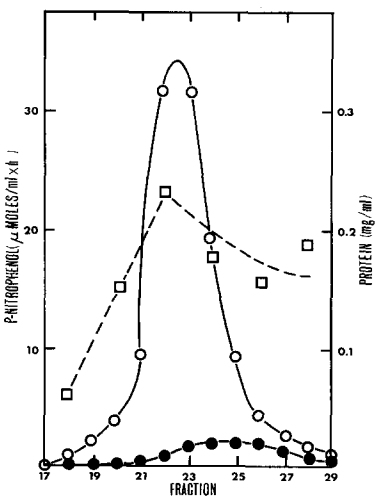


Fig. 2. Chromatography on DEAE-cellulose. Conditions were similar to those of Fig. 1, except that 0.12 ml of each fraction was used to determine α-galactosidase and 0.02–0.1 ml to determine β-galactosidase activity. ●—●, α-galactosidase; ○—○, β-galactosidase; □— — —□, protein.

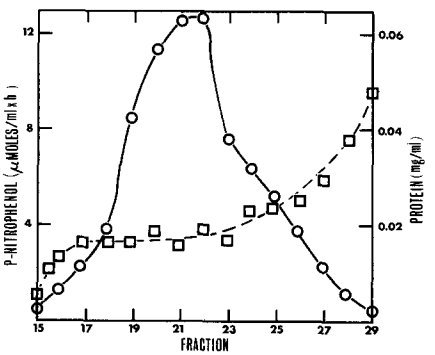


Fig. 3. Filtration of DEAE-cellulose effluent through Sephadex G-150. Conditions were similar to those of Fig 1, except that 0.05 ml of each fraction was used. ○—○, β-galactosidase; □— — —□, protein.

As seen in Fig. 3, β-galactosidase filtered through the Sephadex G-150 column prior to the protein peak and had a very low protein content (about 10–20 μg/ml). Protein determinations of the fractions shown in Fig. 3, based on absorption at 280 nm, were therefore somewhat inaccurate. The protein content of the combined fractions was determined by a semi-micromodification of the method of LOWRY *et al.*[17] as described in EXPERIMENTAL PROCEDURE. Fraction 1 of β-galactosidase had a specific activity of over 1000 μmoles *p*-nitrophenol released per mg protein in 1 h. This represents a purification of over 1000-fold over the enzyme activity in the homogenate less debris.

α-Galactosidase (at a concentration of 330 μg/ml) retained full activity for 1 month when stored at −20°. β-Galactosidase (Fraction 1, 10 μg/ml) lost 50% of its activity when stored for 1 month at −20°.

TABLE II

SUBCELLULAR FRACTIONATION OF SPINACH LEAVES

Conditions were the same as in Table I, except that the tubes were incubated for 15 min. Values are expressed as μmoles of *p*-nitrophenol released in 1 h. For fractionation procedure of the spinach leaves, see text.

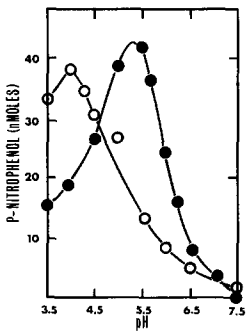
Fraction	*α-Galactosidase*		*β-Galactosidase*	
	Expt. 1	*Expt. 2*	*Expt. 1*	*Expt. 2*
Chloroplasts	0	2.3	0	4.8
Mitochondria	5.6	5.0	9.6	10.2
Microsomes	8.4	19.3	13.1	37.9
100 000 × *g* supernatant	105.5	88.5	307.2	285.7

Subcellular localization of the enzymes in spinach leaves (Table II)

To determine the localization of α- and β-galactosidases in subcellular fractions of spinach leaves, these were homogenized and fractionated as described in EXPERIMENTAL PROCEDURE. Table II shows the results of subcellular distribution of the enzymes. It is evident that most of the activity was present in the 100 000 × *g* supernatant and only a small portion of the total activity was located in the other subcellular fractions. In Expt. 1, 11.7% of α- and 7% of β-galactosidase were present in particulate fractions. In Expt. 2, where a more gentle disruption of the leaves was employed, 23% of a α- and 16% of β-galactosidase activity were present in particulate fractions.

General properties of the enzymes

The optimal pH of hydrolyses were 5.3 with α-galactosidase and 4.2 with β-galactosidase (Fig. 4). The K_m values were 0.3 mM for α- and 0.4 mM for β-galactosidase (Fig. 5). When heated at pH 5, for 10 min at 43°, α-galactosidase lost 50% of its activity; the corresponding temperature for β-galactosidase was 49° (Fig. 6). Curves similar to those of Fig. 6 were obtained when the enzymes were heated in the presence of buffer at pH 7. This is dissimilar to the behavior of β-galactosidase of salmon liver, where the enzyme was more stable at acid than at neutral pH (ref. 19).



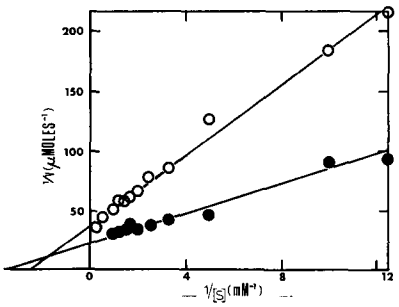
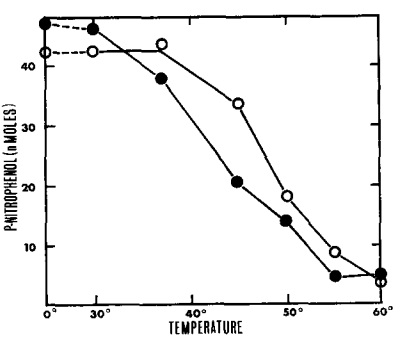


Fig. 4. Dependence of the rates of hydrolysis on pH. Incubation mixtures in volumes of 0.2 ml contained 0.06 ml of a molar solution of sodium phosphate and citrate buffers at the appropriate pH values, 1 μmole of substrate, 20 μg of α-galactosidase or 3.6 μg of β-galactosidase (Fraction 2). After 15 min at 37°, 1 ml of glycine–carbonate buffer was added and the color was read at 420 nm. ●—●, α-galactosidase; ○—○, β-galactosidase.

Fig. 5. Dependence of the rates of hydrolysis on substrate concentration. Incubation mixtures in volumes of 0.2 ml contained 40 μmoles of acetate buffer (pH 5.0), 16 μg of α-galactosidase or 3 μg of β-galactosidase (Fraction 2) and appropriate concentration of *p*-nitrophenyl α- or β-galactoside. Incubation and termination were same as in Fig. 4. ●—●, α-galactosidase; ○—○, β-galactosidase.

Effect of inhibitors

Galactose, a product of the hydrolytic reaction, inhibited substrate hydrolysis by both enzymes (Fig. 7). 1.6 μmoles of galactose caused a 50% inhibition of the reaction catalyzed by α-galactosidase, whereas 20 μmoles of galactose were needed



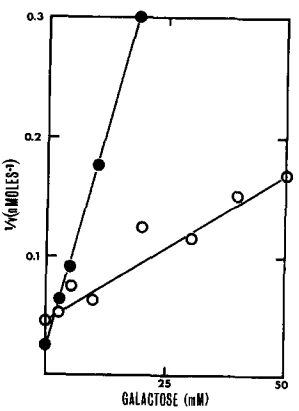


Fig. 6. Thermal stability of the enzymes in the absence of substrates. 0.1 ml of enzyme (33 μg of α- or 6 μg of β-galactosidase, Fraction 2) was mixed with 0.03 ml of 0.33 M acetate buffer (pH 5) and placed in a bath preheated to the specified temperature. After 10 min the tube was placed in ice. 0.06 ml of 1 M acetate buffer (pH 5) and 0.02 ml of 0.05 M substrate were added and the tubes were incubated for 15 min at 37°. 1 ml of 0.25 M glycine–carbonate buffer was added and the color was read at 420 nm. ●—●, α-galactosidase; ○—○, β-galactosidase.

Fig. 7. Dependence of the rates of hydrolysis on galactose. Incubation mixture in volumes of 0.2 ml contained 30 μmoles of acetate buffer (pH 5), 0.4 μmole of substrate, 16.5 μg of α- or 3 μg of β-galactosidase (Fraction 2) and appropriate concentrations of galactose. After 20 min at 37°, 1 ml of 0.125 M sodium tetraborate was added and the color was read at 420 nm. ●—●, α-galactosidase; ○—○, β-galactosidase.

Fig. 8. Dependence of the rates of hydrolysis on silver nitrate and PHMB. Conditions were the same as in the experiment of Fig. 7 except that $AgNO_3$ and PHMB were used instead of galactose. A. α-galactosidase (16.5 μg). Right ordinate, values in the presence of PHMB; left ordinate, values in the presence of $AgNO_3$. B. β-galactosidase (0.3 μg, Fraction 1). ●—●, $AgNO_3$; ■– –■, PHMB.

for a similar inhibition in the case of β-galactosidase (substrate concentration with either enzyme was 2 mM).

The enzymes were inhibited by low concentrations of Ag^+ or PHMB (Fig. 8). 50% inhibition of α-galactosidase activity was obtained at $2 \cdot 10^{-7}$ M $AgNO_3$ or $6 \cdot 10^{-7}$ M PHMB (Fig. 8A). Similar inhibition of β-galactosidase was obtained at $2 \cdot 10^{-8}$ M $AgNO_3$ or $3.5 \cdot 10^{-8}$ M PHMB. Iodoacetamide or *N*-ethyl maleimide did not inhibit either enzyme even at concentrations of 10 mM. NaCl (0.1 M), $MgSO_4$ (0.01 M), $MnCl_2$ (5 mM) and $CaCl_2$ (5 mM) had no effect on either enzymatic activity.

Substrate specificity of the enzymes

Hydrolysis of nitrophenyl derivatives. Table III shows the hydrolysis of 13 derivatives of nitrophenol by the enzymes. β-Galactosidase (Fraction 1) was free of

TABLE III

SUBSTRATE SPECIFICITY OF THE ENZYMES

Incubation mixtures in volumes of 0.2 ml contained 30 μmoles of acetate buffer (pH 5), 0.25 μmole of substrate and 16.5 μg of α-galactosidase or 0.2 μg of β-galactosidase (Fraction 1). The tubes having *p*-nitrophenyl derivatives of fatty acids also contained 0.2 mg of Triton X-100 and 0.5 mg of sodium taurocholate. After 30 min at 37° 1 ml of 0.125 M sodium tetraborate was added and the color was read at 420 nm. Values are expressed as nmoles of nitrophenol released per 30 min.

Substrate	*α-Galactosidase (nmoles)*	*β-Galactosidase (nmoles)*
p-Nitrophenyl α-galactoside	47.4	0.04
o-Nitrophenyl α-galactoside	70.3	—
p-Nitrophenyl β-galactoside	1.0	53.0
o-Nitrophenyl β-galactoside	—	40.8
p-Nitrophenyl α-glucoside	0	0
p-Nitrophenyl β-glucoside	2.0	0
p-Nitrophenyl phosphate	29.6	0.5
p-Nitrophenyl sulfate	46.4	44.0
p-Nitrophenyl butyrate	15.5	1.0
p-Nitrophenyl caprylate	7.8	0
p-Nitrophenyl caproate	25.2	0
p-Nitrophenyl laurate	9.1	0
p-Nitrophenyl palmitate	4.0	0

phosphatase, glucosidase and esterase activities. The less-purified α-galactosidase preparation still had considerable phosphatase and esterase activities. Both enzymes had as high an activity with *p*-nitrophenyl sulfate as with *p*-nitrophenyl galactoside.

Hydrolysis of lactose and melibiose. Incubation mixtures (in total volumes of 0.5 ml) contained 1.5 μmoles of substrate, 30 μmoles of acetate buffer (pH 5) and 33–132 μg of α-galactosidase or 1–4 μg of β-galactosidase (Fraction 1). After 2 h at 37° the galactose that was released was determined according to EXPERIMENTAL PROCEDURE. The specific activities of the enzymes using these substrates were as follows: Melibiose was hydrolyzed by the α-galactosidase at a rate of 82 nmoles/mg in 1 h (compared with 5800 using *p*-nitrophenyl α-galactoside). Lactose was hydrolyzed by the β-galactosidase at a rate of 9.5 μmoles/mg per h (as compared to 500 using *p*-nitrophenyl β-galactoside as substrate).

Hydrolysis of plant galactolipids. The following exploratory experiments were conducted on the hydrolysis of mono- and digalactosyl diglycerides by the galactosidases. After several trials using various detergents, the following procedure was employed. 0.15 mg each of monogalactosyldiglyceride (from *Euglena gracilis* Z) or digalactosyldiglyceride (from *Anacystis nidulans*) were dissolved in a mixture of chloroform and methanol (2:1, v/v) and pipetted into test tubes containing 30 mg of acid-washed Celite 545 (ref. 20); the solvents were then evaporated by heating under nitrogen. 50 μmoles of sodium acetate (pH 5), 66–132 μg of α-galactosidase or 1–4 μg of β-galactosidase (Fraction 1) or a mixture of 33 μg of α- and 1 μg of β-galactosidase, and water to make up a vol. of 0.5 ml were added and the tubes were shaken for 2 h at 37°. Galactose that was released was determined as described in EXPERIMENTAL PROCEDURE except that the celite was removed by centrifugation prior to the addition of galactose dehydrogenase. Monogalactosyldiglyceride was hydrolyzed by β-galactosidase at a rate of 2.1 μmoles/mg per h (as compared with 500 when *p*-nitrophenyl β-galactoside was used). The digalactosyldiglyceride was not hydrolyzed under the conditions of this experiment.

DISCUSSION

This paper describes the partial purification and properties of α- and β-galactosidases of spinach leaves. These two enzymes were fully separated from each other, thus permitting investigation of the cleavage of glycosides with either α- or β-galactosidic linkages in the presence of each other. For purification of β-galactosidase, advantage was taken of the observation that this enzyme was loosely bound to DEAE-cellulose and could be eluted from this ion-exchange column with a salt concentration as low as 0.05 M NaCl. When the eluted protein was further applied to a Sephadex G-150 column, the peak of enzymatic activity filtered through prior to the first protein peak. These two procedures, combined with previous removal of protein which precipitates at pH 4 and ammonium sulfate fractionation, resulted in a high purification of this enzyme in four relatively simple steps. The enzyme thus obtained had a specific activity of about 1000 μmoles/mg protein per h with *p*-nitrophenyl β-galactoside as substrate. Lactose was hydrolyzed at one fiftieth of this rate. α-Galactosidase was less purified, the specific activity was 6–12 μmoles per mg protein per h, using *p*-nitrophenyl α-galactoside; melibiose was hydrolyzed at one seventieth of this

rate. In contrast to the β-galactosidase, this enzyme still had considerable phosphatase and esterase activity.

Both enzymes were inhibited by very low concentrations of Ag^- or PHMB. The fact that iodoacetamide and *N*-ethyl maleimide were not inhibitory even at a 10 mM concentration suggests that the effects of the heavy metals might not be on an SH group.

Both enzymes exhibited optimal activities at low pH values (5.3 for α- and 4.2 for β-galactosidase). They are thus similar to the corresponding enzymes of animal tissues which are found in lysosomes[12]. However, when spinach leaves were blended and the homogenate submitted to subcellular centrifugation, 88% of α-galactosidase and 93% of β-galactosidase were found in the 100 000 × *g* supernatant. When the tissue was disrupted by grinding with sand in a mortar rather than blending, a somewhat higher proportion was found in particulate fractions (23% of α- and 16% of β-galactosidase). This suggests that if these enzymes are indeed located in lysosome-like particles, the membrane of these organelles must be very fragile and easily disrupted, releasing the enzymes into the cell sap even when gently ground in a mortar with an isotonic buffered medium. In two batches of spinach additional galactosidases, with optimal activity at a neutral or slightly alkaline pH, were observed in the mitochondria or chloroplasts. However, their activity was low and they could not be detected in most batches of spinach used.

The fact that chloroplasts had no galactosidase activity (Table II) was of special interest. Most of the plant glycolipids are located in the chloroplasts, it might therefore have been expected that enzymes hydrolyzing them might be associated with this organelle. Work from other laboratories[21–24] has shown the presence in runner bean leaves of deacylases that split off the fatty acids from mono- and digalactosyldiglycerides. The results in this paper, which are still of a preliminary nature, show that the β-galactosidic linkage of monogalactosylglyceride[25] was cleaved by the β-galactosidase. The rate of this cleavage was about 2 μmoles/mg per h, *i.e.* about 1/250 that of *p*-nitrophenyl β-galactoside. This assay was performed after 1-month storage when the β-galactosidase had already lost half of its activity. It is therefore possible that the freshly prepared enzyme had a specific activity of 4 μmoles/mg per h with monogalactosyldiglyceride as substrate. For comparison, the galactolipid deacylase of runner bean leaves, purified by procedures similar to those here employed for the isolation of the β-galactosidase[24], had a specific activity of 3.25 μequiv/mg per h and this could be further increased to 10 by disc electrophoresis.

The digalactosyldiglyceride was not hydrolyzed under the conditions used in this paper. It is not clear whether the α-galactosidase does not cleave the terminal α-galactosidic bond of the intact lipid[25] or whether the rate of hydrolysis of this substrate by the low specific activity enzyme is so slow that the method used for the determination of galactose was not sensitive enough. It should also be emphasized that the digalactosyldiglyceride that was available for this work had been prepared from Anacystis; it thus had saturated or monounsaturated fatty acids, while the similar compound in spinach has a high proportion of polyunsaturated fatty acid. It is possible that the lipid with the saturated fatty acids is less susceptible to hydrolysis by the enzyme. An alternate possibility is that the enzyme does not hydrolyze the native lipid but does split galactose off the deacylated compound (*i.e.* from monogalactosyl or digalactosyl glycerol). Further experiments are necessary to clarify the catabolism of the plant galactolipids.

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REFERENCES

1 K. WALLENFELS AND O. P. MALHOTRA, *Advan. Carbohydrate Chem.*, 16 (1961) 239.
2 J. CONCHIE, J. FINDLAY AND G. A. LEVVY, *Biochem. J.*, 71 (1959) 318.
3 R. C. HUGHES AND R. W. JEANLOZ, *Biochemistry*, 3 (1964) 1535.
4 K. M. L. AGRAWAL AND O. P. BAHL, *J. Biol. Chem.*, 243 (1968) 103.
5 S. GATT AND M. M. RAPPORT, *Biochim. Biophys. Acta*, 113 (1966) 567.
6 J. C. CAYGILL, C. P. J. ROSTON AND F. R. JEVONS, *Biochem. J.*, 98 (1966) 405.
7 D. ROBINSON, R. G. PRICE AND N. DANCE, *Biochem. J.*, 102 (1967) 525.
8 Y. LI, S. C. LI AND M. R. SHETLAR, *Arch. Biochem. Biophys.*, 103 (1963) 436.
9 O. P. MALHOTRA AND P. M. DEY, *Biochem. J.*, 103 (1967) 508.
10 R. L. COLEMAN, *Biochim. Biophys. Acta*, 159 (1968) 192.
11 P. M. DEY AND J. B. PRIDHAM, *Biochem. J.*, 113 (1969) 49.
12 C. DE DUVE, in T. HAYASHI, *Subcellular Particles*, Ronald, New York, 1959, p. 128.
13 PH. MATILE, J. B. BALZ, E. G. SEMADENI AND M. JOST, *Z. Naturforsch.*, 206 (1965) 6.
14 E. G. SEMADENI, *Planta*, 72 (1967) 91.
15 J. R. CORBETT AND C. A. PRICE, *Plant Physiol.*, 42 (1967) 827.
16 A. G. GORNALL, C. S. BARDAWALL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
17 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 196 (1951) 265.
18 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
19 S. GATT, *Science*, 164 (1969) 1422.
20 P. MORELL AND N. S. RADIN, *Biochemistry*, 8 (1969) 506.
21 P. S. SASTRY AND M. KATES, *Biochemistry*, 3 (1964) 1280.
22 R. E. MCCARTY AND A. T. JAGENDORF, *Plant Physiol.*, 40 (1965) 725.
23 P. J. HELMSING, *Biochim. Biophys. Acta*, 144 (1967) 470.
24 P. J. HELMSING, *Biochim. Biophys. Acta*, 178 (1969) 519.
25 H. E. CARTER, R. A. HENDRY AND N. Z. STANACEV, *J. Lipid Res.*, 2 (1961) 223.