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PURIFICATION AND PROPERTIES OF GALACTOLIPASE

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SUMMARY

1. Isolation and purification of the mono- and digalactolipase activity from runner-bean leaves (*Phaseolus multiflorus* Lam.) resulted in one protein which contained both galactolipase activities.

2. The ratio of the specific activities of the monogalactolipase activity to the digalactolipase activity was 2:1.

3. The isoelectric point of the protein with both galactolipase activities is pH 7.0.

4. After incubation no detectable amounts of a lyso compound of the galactolipids were observed in the reaction mixtures.

5. Experiments concerning the enzyme stability as affected by temperature and storage demonstrated that galactolipase from runner-bean leaves is quite stable.

6. The Michaelis–Menten constants for the mono- and digalactolipase activities are 0.65 mM and 0.31 mM, respectively.

7. The molecular weight of galactolipase is 110 000.

8. Galactolipase is activated by the addition of strong reductants.

9. Galactolipase is completely inhibited by cysteine.

INTRODUCTION

Monogalactosyl diglyceride and digalactosyl diglyceride, were identified for the first time by CARTER *et al.*[1–3], in wheat flour and have been found by several authors in photosynthetic plant tissues[4–8] as well as in photosynthetic bacteria[9]. The galactosyl diglycerides form the largest lipid fraction of the chloroplast[10]. The galactolipid content of the chloroplasts is related to chlorophyll content. WINTERMANS[5] found more galactolipids in green leaves of *Sambucus nigra* and *Phaseolus vulgaris* than in yellow leaves. ROSENBERG[11–13] demonstrated that in cells of *Euglena gracilis* the appearance or disappearance of chlorophyll is accompanied by the simultaneous appearance or disappearance of the galactosyl diglycerides in a relatively fixed ratio. He suggested that the galactolipids may have a possible function with respect to the localization of the porphyrin structures of the chlorophyll by forming a stable lock and key fit between the phytol chains of the chlorophyll molecules and the fatty acyl chains of the galactolipids.

CHANG AND LUNDIN[14] found a stimulatory effect of the galactolipids on the rate of cytochrome *c* photoreduction by intact spinach chloroplasts. Moreover, it was found by FERRARI AND BENSON[15] and by KATES[7] that the galactolipids have a high rate of turnover. Together, the findings in the literature seem to indicate that these galactolipids are important for the photosynthetic apparatus of the plants.

The galactolipids apparently need specific enzymes for their hydrolysis[16,17]. SASTRY AND KATES[16] have tested several plant species for the presence of galactolipase activity and found that different Phaseolus species are able to hydrolyze mono- and digalactosyl diglyceride. On the basis of their data, they assume that two different enzyme systems exist for the hydrolysis of the two galactosyl diglycerides. Our earlier results with young spinach leaves[18], however, did not support the hypothesis of the two-enzyme system for the hydrolysis of the galactolipids. The findings in this paper suggest that there is only one enzyme which can act on both galactolipid substrates. Some properties of this enzyme are described.

MATERIALS AND METHODS

Plant material

Spinach plants (*Spinacia oleracea* L.) were grown in the greenhouse or obtained from the local market. Runner-bean plants (*Phaseolus multiflorus* Lam.) were grown in a greenhouse with a day temperature of 25–26° and a night temperature of 21–22°, under a 400-watt Philips HPL lamp. Primary leaves were harvested 14–16 days after sowing.

Substrates

Mono- and digalactosyl diglyceride were extracted from spinach leaves by the method of BLIGH AND DYER[19]. These two galactolipids were isolated and separated in two different ways as described previously[18,20]. The majority of the pigments present in the mono- and digalactosyl diglyceride fractions could be removed by column chromatography with silica gel without binder (Machery, Nagel and Co., Düren, Germany) as absorbent under the influence of the applied centrifugal force. Particle size of the silica gel was 5–25 μ. This method is described by MEYER, EID AND VERDUIN[21]. Columns of 3.5 cm × 1.5 cm were used. Stepwise elution was carried out with 30 ml of the following solvent systems: chloroform, chloroform–acetone (10:1, v/v), chloroform–acetone (2:1, v/v) for the monogalactosyl diglyceride fraction and chloroform–acetone (2:1, v/v), chloroform–acetone (1:2, v/v), and acetone for the digalactosyl diglyceride fraction.

Pigments with the same or nearly the same R_F value as both galactolipids on a thin-layer chromatogram developed in Solvent system a or b (see: *Thin-layer chromatography*) could not be removed.

After purification of each of the two galactolipid fractions by this method the chloroform–acetone (2:1, v/v) fraction contained monogalactosyl diglyceride, and the acetone fraction digalactosyl diglyceride. Both galactolipid fractions were stored under a nitrogen atmosphere at −20°.

Enzyme extraction

After harvesting, the primary leaves of *Phaseolus multiflorus* (approx. 300 g)

were immediately frozen in liquid nitrogen, kept at 4° for at least 1 h, and subsequently homogenized in an Ato-mixer with 550 ml distilled water for 2 min. The homogenate was filtered through two layers of cheese-cloth, and the filtrate was centrifuged at 20 000 × *g* for 20 min in a M.S.E. high-speed 18 centrifuge to remove starch and chloroplast fragments. The residues obtained after centrifugation and cheese-cloth filtration were pooled and homogenized again with 150 ml 0.01 M phosphate buffer at pH 7.0. This second homogenate was filtered through two layers of cheese-cloth and the filtrate centrifuged at 20 000 × *g* for 20 min. The residue was discarded, and the supernatants of the first and second centrifugation were pooled and centrifuged for 1 h at 105 000 × *g* in a Spinco L-2 65 B or a Martin Christ Omega ultracentrifuge to remove microsomal particles. The clear supernatant (approx. 900 ml) was dialyzed in cellulose tubes (Cenco, Breda, The Netherlands) against 5 l of 0.01 M phosphate buffer at pH 7.0 for 18 h. Solid $(NH_4)_2SO_4$ was added to the dialysate, and the fraction precipitating between 25 and 70% saturation was collected by centrifugation at 20 000 × *g* for 20 min. The precipitate was suspended in 15 ml of 0.01 M phosphate buffer at pH 7.0 and dialyzed against 5 l of the same buffer for 3.5 h. The enzyme preparation is then clear.

Gel filtration

The gel was prepared by swelling of about 35 g Sephadex G-200 (Pharmacia, Uppsala, Sweden) in 1500 ml of 0.01 M phosphate buffer at pH 7.0 for at least 3 days at room temperature or 5 days at 4°. The slurry was degassed, cooled to 4°, and poured into a column with inner dimensions of 90 cm × 4 cm. After the $(NH_4)_2SO_4$ precipitation, the dialysate was applied to the column. Elution was carried out with 0.01 M phosphate buffer at pH 7.0. The flow rate was 2.2 ml/h per cm^2, and fractions of 14 ml were collected. Absorbances at 280 nm were read from a Zeiss PMQ II spectrophotometer.

Ion-exchange chromatography

DEAE-cellulose (DE-32, microgranular, standard) was purchased from Whatman and pretreated according to the manufacturer's instructions, *i.e.*, 7 g of DEAE-cellulose was suspended in 0.01 M phosphate buffer at pH 7.0. The slurry was poured into a column with inner dimensions of 10 cm × 2 cm or 7 cm × 2 cm. Elution was carried out at 4° with 0.05 M phosphate buffer at pH 7.0 followed by elution with a linear salt gradient 0.0–0.5 M NaCl in the same buffer. The flow rate was 12.4 ml/h per cm^2. Fractions of 6.7 ml were collected.

Acylester determination

The procedure of SNYDER AND STEPHENS[22] modified by RENKONEN[23] with methyl stearate (Applied Science Laboratories, State College, U.S.A.) as standard was used. Absorbances at 530 nm were read from a Zeiss PMQ II spectrophotometer in 1-cm glass cuvettes.

Enzyme assay

The enzyme activity was tested as described previously[16] with an aliquot of the enzyme preparation equivalent to 0.2 g leaf in 0.5 M phosphate buffer at pH 7.0 for monogalactosyl diglyceride and at pH 5.6 for digalactosyl diglyceride. The enzyme

activity is expressed as μequivalent reduction of acylester/h incubation at 30° in %, after correction for enzyme blanks. Specific activity is expressed as μequivalent reduction acylester/mg protein per h incubation at 30°. When the column fractions were tested for galactolipase activity, 1-ml aliquots were used. The enzyme activity is then expressed as $\Delta A_{530\ nm} = A_{530\ nm}$ (substrate blank) $- A_{530\ nm}$ (incubated sample). All tests were carried out in duplicate.

Protein determination

Protein was determined according to the method of LOWRY *et al.*[24], after precipitation with 10% trichloroacetic acid. Crystalline bovine albumine, Cohn Fraction V (Calbiochem, Los Angeles, U.S.A.), was used as standard.

Disc electrophoresis

The method of ORNSTEIN[25] and DAVIS[26] was followed. Polyacrylamide (15%) gels of 7.5 cm × 1.4 cm without an upper gel were used. Samples of 2.5 ml containing 2–5 mg protein in 25% sucrose were applied to the gels. Runs were made at 4° with 0.05 M Tris–glycine buffer at pH 8.3 and 20 mA per gel. The areas with galactolipase activity were cut out, homogenized in a Potter–Elvehjem homogenizer with 0.01 M phosphate buffer at pH 7.0, and stored overnight at 4°. The homogenate was centrifuged for 20 min at 30 000 × *g*, the residue was washed with the same buffer, and subsequently the supernatants were pooled and dialyzed overnight against 5 l of 0.01 M phosphate buffer at pH 7.0.

Disc electrophoresis at pH 7.0 was carried out by a simplified modification of the method of FELBERG AND SCHULTZ[27]. A solution of 15% acrylamide was poured into tubes of 10 cm × 1.4 cm to a height of 4 cm, according to the procedure of ORNSTEIN[25] and DAVIS[26]. The solutions in the tubes were then polymerized at 39°. After polymerization the gels were cooled to 4°. The samples, in 30% sucrose, were placed on the gels. A second 15% acrylamide solution was then carefully applied on the samples. This second solution contained no potassium ferricyanide. The solution was now able to polymerize at 4°. Runs were made with 0.05 M Tris–glycine–HCl buffer at pH 7.0 and 20 mA per gel. When the runs were finished after 1.5 h, the sample zones were pipetted out with a syringe, pooled and dialyzed against 5 l of 0.01 M phosphate buffer at pH 7.0.

If necessary the gels were stained with 1% Amidoblack in 7% acetic acid for 1 h and destained electrophoretically with 2% acetic acid.

When a gel was tested for galactolipase activity it was cut into 2-mm slices. Each slice was cut into smaller pieces which were put into tubes with 2 ml of 0.01 M phosphate buffer at pH 7.0 for monogalactosyl diglyceride and at pH 5.6 for digalactosyl diglyceride. The tubes were stored overnight at 4°. For the galactolipase activity test 1.2 ml of the phosphate buffer was used.

Thin-layer chromatography

Thin-layer chromatography was performed on silica-gel plates of 20 cm × 20 cm. The plates were developed in one of the following solvent systems: a, toluene–ethyl acetate–95% ethanol (10:5:5, v/v/v)[28]; b, acetone–acetic acid–water (100:2:1, v/v/v)[29]; c, chloroform–methanol–acetone (80:16:4, v/v/v)[30]. Visible spots were obtained with the periodate–Schiff reagent[31].

Determination of molecular weight

An aliquot of the enzyme preparation equivalent to 28 g of leaves from the first DEAE-column was freeze-dried to a volume of 10 ml and mixed with 30 mg α-crystalline (mol. wt. 850 000)[32], 15 mg human hemoglobin (mol. wt. 64 450)[33], 20 mg trypsine (EC 3.4.4.4; mol. wt. 24 000)[34] and 60 μl of a suspension of lactate dehydrogenase (EC 1.1.1.27; mol. wt. 116 000)[35] in 2.2 M $(NH_4)_2SO_4$. This mixture was placed on a Sephadex G-200 column (inner dimensions, 90 cm × 4 cm) in 0.01 M phosphate buffer at pH 7.0. The column was eluted with the same phosphate buffer.

RESULTS

Enzyme isolation and purification

When the primary leaves of the runner-bean plants were homogenized with water, filtered through cheese-cloth, homogenized with phosphate buffer, and filtered again, it gave a dark green filtrate. After ultracentrifugation a light yellow supernatant was obtained, which became dark brown after precipitation with $(NH_4)_2SO_4$ and subsequent dialysis for 3 h against 5 l of 0.01 M phosphate buffer at pH 7.0.

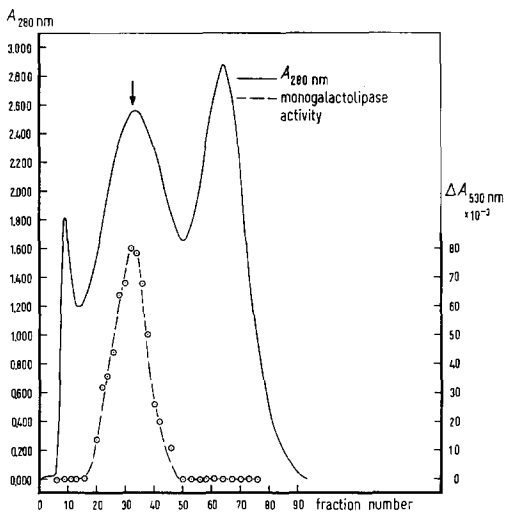


Fig. 1. Elution pattern of a Sephadex G-200 column of 90 cm × 4 cm. Absorbances were read at 280 nm in 0.5-cm quartz cuvettes. Fractions 18–48 were pooled and used for the next step in the purification procedure.

However, the $(NH_4)_2SO_4$ present was only partly removed by this dialysis. The next step in the purification procedure, gel filtration with Sephadex G-200, removed the remaining $(NH_4)_2SO_4$.

In preliminary experiments, all the fractions collected from the Sephadex column were tested for monogalactolipase activity. Since it appeared that the activity of this enzyme was confined to a single band, only this region was tested for activity in the following experiments. The elution pattern and the concurrent monogalactolipase activity of the fractions collected from the Sephadex G-200 column are shown in Fig. 1. The digalactolipase activity was tested in the pooled fractions of each peak.

The material represented by the peak marked with an arrow in Fig. 1 was also active in hydrolyzing the substrate digalactosyl diglyceride, which demonstrates that both galactolipase activities are present in this band.

The active fractions of the Sephadex column were applied to a DEAE-cellulose column of 10 cm × 2 cm. The elution pattern of the DEAE-column and the concurrent activities of both mono- and digalactolipase are shown in Fig. 2. Elution of the column with the NaCl gradient again resulted in a peak which showed mono- as well as digalactolipase activity. When the elution was finished, a dark yellow-brown band stayed on the top of the DEAE-column. This band could not be removed by increasing the salt concentration up to 3.0 M NaCl. It was also difficult to remove this substance by regeneration of the ion-exchanger. It was therefore necessary to repeat the regeneration procedure 1 or 2 times.

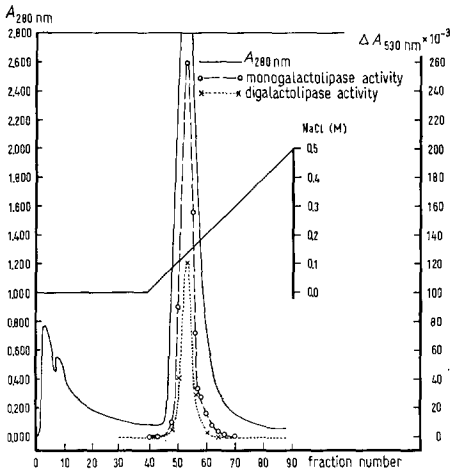


Fig. 2. Elution pattern of a DEAE-cellulose column of 10 cm × 2 cm. Absorbances were read at 280 nm in 0.5-cm quartz cuvettes. Fractions 47–59 were pooled and subsequently dialyzed.

The fractions of the galactolipase active peak, which were yellow-brown, were pooled and dialyzed against 5 l of 0.01 M phosphate buffer at pH 7.0 overnight to remove salt. The volume of the galactolipase fraction from the DEAE-column after dialysis was 115 ml. The whole fraction was then placed on 15% polyacrylamide gels. It was necessary to use 45–50 gels. Electrophoresis was carried out at pH 8.3 with 6 gels running at the same time. Electrophoresis lasted approx. 100 min.

According to the literature, gels of 7.5% polyacrylamide are usually used. In our case, however, it was found that with gels of 15% polyacrylamide a better resolution of the protein over the gel was obtained.

The front of the gels was marked by a yellow-brown band originating from the sample. This band moved through the gel with the same or nearly the same velocity as bromo phenol blue. After staining with Amidoblack and destaining with acetic acid, the front band appeared as a sharp blue band. After the electrophoresis the whole gel was slightly yellow-brown colored. The distribution of the monogalactolipase activity on the gel is shown in Fig. 3. This figure demonstrates that the mono-

galactolipase activity corresponds to a pronounced dark band in the gel. The area of the gel with the monogalactolipase activity also contained the digalactolipase activity This was demonstrated by cutting out the area with monogalactolipase activity and homogenizing it with 5 ml of 0.01 M phosphate buffer at pH 5.6, followed by centrifu gation at 30 000 × *g* for 20 min. The supernatant was then tested for digalactolipase activity. The remaining parts of the gel were inactive on both substrates. It was found that both galactolipase activities were always located at 1/3 of the distance covered by the front. On each side of this location, 4–5 mm of the gel was cut out and used for the next step in the purification procedure.

The supernatant of the homogenized and subsequently centrifuged gel parts was applied to a second DEAE-cellulose column of 7 cm × 2 cm. The elution pattern of this column is shown in Fig. 4. The first peak of the column which was eluted with 0.05 M phosphate buffer at pH 7.0 contained gel material which was also extracted

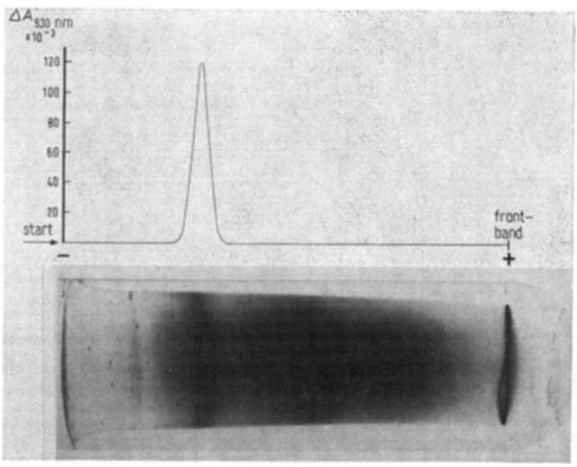


Fig. 3. Distribution of the monogalactolipase activity on 15% polyacrylamide gel. The activity of the enzyme was tested as described in the text. A sample of 0.5 mg protein of the galactolipase active fraction from the first DEAE-cellulose column was placed on the gel.

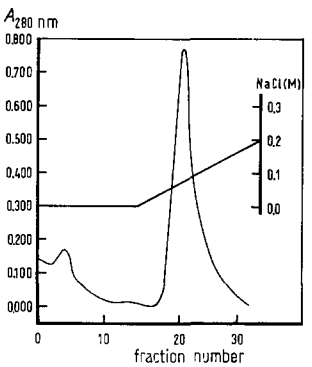


Fig. 4. Elution pattern of a DEAE-cellulose column of 7 cm × 2 cm. Absorbances were read in 1-cm quartz cuvettes at 280 nm. Fractions 1–11 contained the gel material. Fractions 19–27 were used for the electrophoresis at pH 7.0.

from the gel together with the two galactolipase activities. However, the eluate was still colored a light yellow-brown. After elution a yellow-brown band which had a much lower intensity than in the case of the first DEAE-column, remained on the top of the column. Regeneration of the ion-exchanger easily removed the yellow-brown substance.

The best way to remove the colored substance from the NaCl eluate was by disc electrophoresis at pH 7.0. It was found that at this pH the colored substance moved to the anode as a sharp band, whereas neither galactolipase activity moved in any direction. Thus this experiment showed that the isoelectric point for both galactolipase activities is pH 7.0. The sample zone is then colorless. Dialysis of the pooled sample zones, after the electrophoresis at pH 7.0 against 5 l of 0.01 M phos-

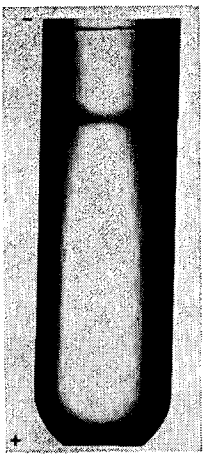


Fig. 5. Purified galactolipase preparation. The sample contained 3 ml 0.01 M phosphate buffer at pH 7.0, 25% sucrose and 50 μg protein. The electrophoresis was carried out with 0.05 M Tris–glycine buffer at pH 8.3 and 20 mA.

phate buffer to remove sucrose and the Tris buffer of the electrophoresis, was the last step in the purification procedure for both galactolipase activities.

An electropherogram of the purified enzyme preparation on a gel of 15% polyacrylamide is shown in Fig. 5. It shows one single band which contains both the mono- and the digalactolipase activity. A second criterion for the purity of the enzyme was electrophoresis on a gel of 15% polyacrylamide at pH 7.5. At this pH we also found a single band which moved through the gel more slowly than at pH 8.3. A third criterion for the purity of the enzyme was electrophoresis on a gel of 15% polyacrylamide at pH 8.3 containing 5 M urea. After staining with Amidoblack and destaining we also found a single band.

TABLE I

PROTEIN CONTENT AND SPECIFIC ACTIVITIES AT EACH STEP IN THE PURIFICATION PROCEDURE OF THE GALACTOLIPASE ACTIVITIES

The values in this table are averages of the results of 2–4 experiments.

Purification procedure	*Protein/300 g leaf*		*Specific activity*		*Relative specific activity*	
	mg/fraction	*%*	*GDG**	*GGDG***	*GDG**	*GGDG***
First cheese-cloth filtration	3970					
Second cheese-cloth filtration	1020	4990	100	0.18	0.09	1.0	1.0
Pooled supernatants after centrifugation at 20 000 × *g*	2050	41	0.20	0.10	1.1	1.1
Supernatant after ultracentrifugation	1050	21	0.25	0.12	1.4	1.3
Precipitate between 25–70% $(NH_4)_2SO_4$ saturation	747	14.5	0.43	0.25	2.4	2.8
Sephadex G-200 column	266	5.3	1.54	0.80	8.6	8.9
First DEAE-column	105	2.1	2.68	1.50	14.8	16.7
Second DEAE-column	7.4	0.15	3.25	1.62	18.1	18.0
Disc electrophoresis at pH 7.0	1.0	0.02	10.20	3.80	56.7	42.2

* GDG, monogalactosyl diglyceride
** GGDG, digalactosyl diglyceride

The protein contents of the galactolipase active fraction at each step in the purification procedure as well as the specific and relative specific activities of both mono- and digalactolipase on the substrates mono- and digalactosyl diglyceride are compiled in Table I. The data show that when the residue obtained after homogenizing with water and filtering with cheese-cloth is again homogenized with buffer, an additional 25% of the protein can be extracted. It is also shown that the recovery of the enzyme with both galactolipase activities is low, namely 0.02% of the total protein extracted. This corresponds to approx. 3 μg enzyme/g leaf fresh weight.

It was found that the specific activity of the enzyme in the pooled and centrifuged cheese-cloth filtrates towards the substrate monogalactosyl diglyceride is two times the specific activity of the enzyme towards digalactosyl diglyceride. At each step of the purification procedure the ratio of the specific activities towards monogalactosyl diglyceride and digalactosyl diglyceride is the same, namely 2:1. Subsequently, the relative specific activities increase by the same factor for both galactolipase activities at each step of the purification procedure except the last, when the increase in relative specific activity is not the same for both galactolipase activities.

After the last step in the purification of this enzyme the monogalactolipase activity is purified 57-fold and the digalactolipase activity 42-fold with regard to the pooled cheese-cloth filtrates.

Stability experiments

When the incubation mixtures were heated in a water bath just before the incubation, it was found that the galactolipase activities decrease, as is shown in Fig. 6. After 10 min at 60° there is about 70–75% of the monogalactosyl diglyceride-hydrolyzing activity left as compared with the control sample. In the case of di-

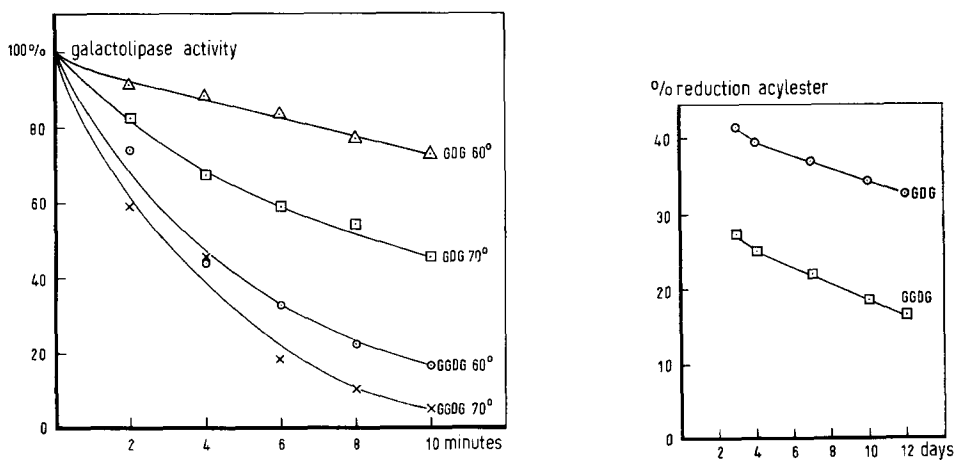


Fig. 6. Effect of different temperatures on the galactolipase activities. The reaction mixtures contained approximately 1 μmole monogalactosyl diglyceride (GDG) or digalactosyl diglyceride (GGDG).

Fig. 7. Decrease in mono- and digalactolipase activity with time during storage at 4°. The reaction mixtures contained approx. 1 μmole monogalactosyl diglyceride (GDG) or digalactosyl diglyceride (GGDG).

galactolipase activity there remained only 16% of the original activity after heating for 10 min at 60°.

Heating of the incubation mixtures at 70° resulted in a stronger decrease of both galactolipase activities, as expected. However, after 10 min at 70° there is still some activity left.

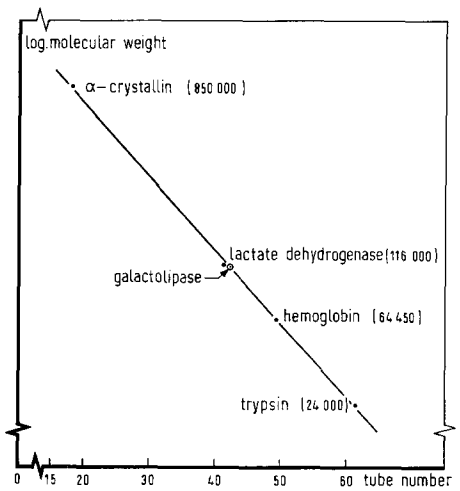
The enzyme preparation after the $(NH_4)_2SO_4$ precipitation and the subsequent dialysis was stored at 4°. At different times a sample was taken and tested for both galactolipase activities at their respective pH optima. The results from this experiment are shown in Fig. 7. The curves show that there is no strong decrease in either galactolipase activity during the storage of this enzyme preparation at 4°. At the same time it was found that after 4 weeks, the monogalactolipase activity was reduced to approx. 60% and the digalactolipase activity to approx. 53% of the original activity.

Determination of Michaelis–Menten constants

Mono- and digalactosyl diglyceride were incubated at 30° at their respective pH optima in varying concentrations. When plotted according to LINEWEAVER AND BURK[36] a linear relation between $1/v$ and $1/S$ is obtained. In the case of the monogalactolipase activity $1/v_{max}$ is 3.54, which gives a calculated K_m value of 0.65 mM. In the case of the digalactolipase activity $1/v_{max}$ is 2.55, and the K_m value 0.31 mM. The Michaelis–Menten constants demonstrate that the enzyme has a greater affinity for the digalactolipid than for the monogalactolipid.

Determination of molecular weight

The approximate molecular weight of galactolipase from runner-bean leaves



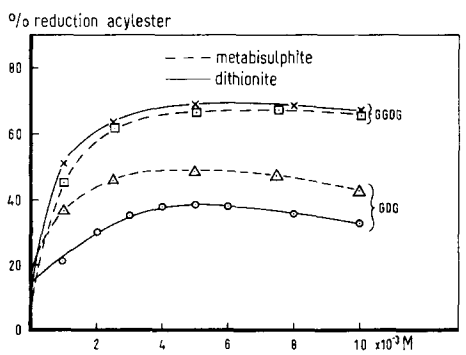


Fig. 8. The determination of the molecular weight of galactolipase carried out by means of a Sephadex G-200 column and 4 reference proteins.

Fig. 9. Effect of the reductants sodium dithionite ($Na_2S_2O_4$) and sodium metabisulphite ($Na_2S_2O_5$) on both galactolipase activities. GDG, monogalactosyl diglyceride and GGDG, digalactosyl diglyceride.

was determined by means of a Sephadex column, as described in METHODS AND MATERIALS. In Fig. 8 are plotted the number of the tubes with the different protein peaks against the logarithm of the molecular weight of the reference proteins. This figure shows that the molecular weight of galactolipase differed slightly from the molecular weight of lactate dehydrogenase. The molecular weight calculated from this curve for galactolipase from runner-bean leaves was 110 000. Since the molecular weight of galactolipase approaches that of lactate dehydrogenase, we may expect that this molecule also consists of subunits.

Activation of both galactolipase activities

Both galactolipase activities could be activated by the addition of the reductant sodium dithionite ($Na_2S_2O_4$). The concentration of this reductant is plotted against mono- and digalactolipase activity in Fig. 9. This figure shows that the highest activation of mono- as well as digalactolipase activity is reached when the dithionite concentration is between 4.0 and 7.0 mM. Dithionite concentrations higher than 7.0 mM produce a decrease in the activation of both galactolipase activities. It is also shown that the action of dithionite on the digalactolipase activity is much stronger than its action on the monogalactolipase activity.

With the reductant sodium metabisulfite ($Na_2S_2O_5$), we obtained essentially the same results as with dithionite (see Fig. 9). The only difference was that the activation of the monogalactolipase activity by metabisulfite was approx. 10% higher than the activation by dithionite.

The naturally occurring reductant ascorbic acid, which is found in relatively large amounts in plants, has no influence on either galactolipase activity.

Inhibition of both galactolipase activities

Both galactolipase activities were inhibited by the addition of two kinds of inhibitors to the incubation mixtures, *i.e.*, on the one hand, cysteine, 2-mercapto-

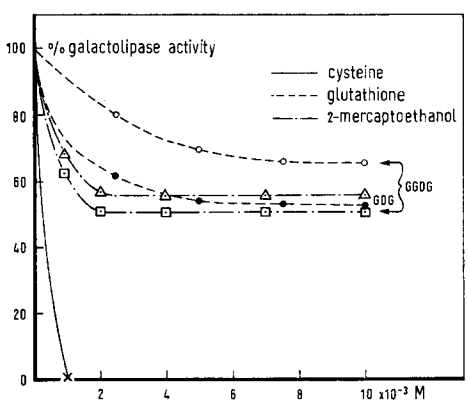


Fig. 10. Effect of reagents containing an SH-group on mono- and digalactolipase activity. Cysteine inhibits both galactolipase activities completely. GDG, monogalactosyl diglyceride and GGDG, digalactosyl diglyceride.

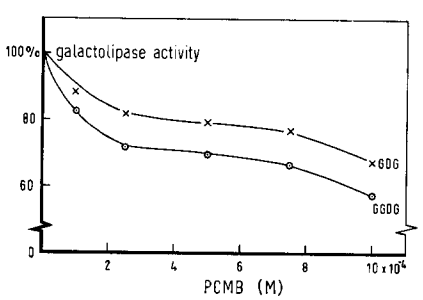


Fig. 11. Effect of PCMB on mono- and digalactolipase activity. The reaction mixtures contained approx. 1 μmole monogalactosyl diglyceride (GDG) or digalactosyl diglyceride (GGDG).

ethanol and reduced glutathione (GSH) which contain an SH-group, and *p*-chloromercuribenzoate (PCMB) which acts on the SH-groups of the protein molecule on the other hand. In Fig. 10 the concentration of the first group of inhibitors is plotted against the galactolipase activity expressed as % of the control. In Fig. 11 the galactolipase activity is plotted against the PCMB concentration.

When the enzyme preparation was treated with 2,3-dihydroxy-1,4-dithiolbutane (dithioerythritol) at pH 8.0 according to CLELAND[37] and then incubated with the galactolipid substrates, only a small inhibition, namely 13%, of both galactolipase activities at a dithioerythritol concentration of 13 mM was obtained.

DISCUSSION

SASTRY AND KATES[16] assume on the basis of their stoichiometric and chromatographic data that the following reactions are catalyzed by the galactolipase active fractions:

Monogalactosyl diglyceride → (galactosyl monoglyceride) + fatty acid → monogalactosyl glycerol + fatty acid (1)

Digalactosyl diglyceride → (digalactosyl monoglyceride) + fatty acid → digalactosyl glycerol + fatty acid (2)

In agreement with SASTRY AND KATES[16], we could not detect any lyso compound of the galactolipids in the chloroform phase after incubation of the galactolipids with a galactolipase active fraction by thin-layer chromatography in Solvent system a or c. This is apparently due to the fact that the formation of the galactosyl glycerols has a higher reaction rate than the deacylation reaction of the galactolipids to lyso compounds.

The question arises of whether there are two different enzymes, each specific for mono- or digalactosyl diglyceride, or whether there is just one enzyme which can act on both monogalactosyl and digalactosyl diglyceride. Our results suggest that in the primary leaves of *Phaseolus multiflorus* only one enzyme is present which catalyzes the hydrolysis of monogalactosyl diglyceride at pH 7.0 and digalactosyl diglyceride at pH 5.6. This one-enzyme hypothesis is based on the fact that at each step of the purification procedure the relative specific activities towards both substrates increase by the same factor. Secondly, electrophoresis of the purified enzyme preparation at two different pH's and on a 5 M urea gel results in only a single band after staining with Amidoblack and destaining. This result shows that the purified enzyme preparation probably consists of one protein which contains both galactolipase activities.

The decrease in protein content of the pooled supernatants after centrifugation at 20 000 × *g* was 59%. At the same time the relative specific activities increased by a factor of 1.1 which means that there is also a big loss of the galactolipase activities. An explanation of this phenomenon could be that the galactolipase molecules do not occur free in the cell but are bound to cell particles.

After dialysis for 48 h against distilled water of the supernatant obtained after centrifugation at 20 000 × *g*, SASTRY AND KATES[16] obtained a precipitate which contained all the monogalactolipase activity, whereas the digalactolipase activity was distributed between the precipitate and supernatant in a ratio of 7:3. These

results were arguments for their two-enzyme hypothesis. However, in our case we never obtained a precipitate, even after dialysis for more than 3 days.

The isoelectric point of both galactolipase activities was at pH 7.0. This means that in case of the monogalactolipase activity the protein molecule is neutral. At pH 5.6 the galactolipase molecule has a positive charge. It can then act on the digalactolipid. A relatively simple explanation for the double action of the enzyme would be to assume an allosteric transformation of the protein molecule under the influence of the pH. This will make the enzyme suitable for either Reaction 1 or 2, depending on the pH. If true, this allosteric transformation must be a reversible process, since after changing the pH from 7.0 to 5.6 for 7 h and then back to pH 7.0 again, the enzyme still shows monogalactolipase activity, although some loss of activity does occur.

The increase of the relative specific activity during the last step of the purification procedure is less for the digalactolipase activity than for the monogalactolipase activity. A possible explanation would be that some denaturation of the enzyme occurs which affects the allosteric transformation. Furthermore, it was found that the enzyme activity towards digalactosyl diglyceride is much more thermolabile than the activity towards monogalactosyl diglyceride. This too may be due to denaturation which also affects the allosteric transformation when the pH is altered from 7.0 to 5.6.

The experiments concerning stability on storage showed that the enzyme is reasonable stable. This is in good agreement with the results obtained with the temperature-stability experiments.

The galactolipid-hydrolyzing system from runner-bean leaves appears to be quite different from the same enzyme system in young spinach leaves. In the case of young spinach leaves, we found[18] no monogalactolipase activity and a relatively high digalactolipase activity 4 days after harvesting the leaves. During the storage of the enzyme preparation after the $(NH_4)_2SO_4$ precipitation from spinach leaves at 4°, the digalactolipase activity decreased, whereas the monogalactolipase activity showed an optimum after 10–11 days. In the case of runner-bean leaves, such an optimum in galactolipase activity was not observed.

The Michaelis–Menten constants calculated in the same way by SASTRY AND KATES[16] were 7.8 mM for the monogalactolipase activity and 1.5 mM for the digalactolipase activity. Comparison of these values with the values we found for the K_m's show that the constants we calculated are lower by a factor of approx. 10. This means that our enzyme preparation has a stronger affinity for the galactolipids than the enzyme preparations of SASTRY AND KATES[16].

It is known from the literature[38–41] that endogenous tannins or quinones and tannins formed after cell rupture inhibit many enzymes extracted from plant tissues. The activity of peptidase extracted from tobacco leaves is higher when reducing agents like sodium thioglycollate, sodium dithionite, and potassium metabisulfite are included in the extracting medium due to the reduction of quinones which inhibit the enzyme[42].

The activation of galactolipase from runner-bean leaves by sodium dithionite and by sodium metabisulfite could thus be due to the reduction of quinones which normally inhibit the enzyme. Extraction of the quinones from the enzyme preparation with light petroleum (b.p., 40–60°) or with water-insoluble polyvinylpyrrolidone (Polyclar AT) according to ANDERSON AND SOWERS[43] gave no results. In the latter

case all the galactolipase activity was bound to the polyvinylpyrrolidone and could not be removed without loss of both galactolipase activities.

Cysteine, 2-mercaptoethanol, GSH and PCMB inhibit the galactolipase activities. However, inhibitors with the SH-group, which probably act on the disulfide bridges in the protein molecule[44], show a stronger inhibition on the mono- as well as on the digalactolipase activity than PCMB. On the basis of these results, it is reasonable to assume that the SH-groups in the galactolipase molecule are less important for the galactolipase activities than the disulfide bridges. However, addition of dithioerythritol gave only a small inhibition, although it is known that this inhibitor quantitatively reduces disulfide bridges.

Even at high concentrations of 2-mercaptoethanol, GSH and PCMB, we never observed more than a 40–50% inhibition.

Both galactolipase activities were inhibited 100% by the amino acid cysteine. Since isolated chloroplasts lose their functions during storage of the chloroplast suspensions and since this loss of functions is probably partly due to the galactolipase activity[45], it should be of interest to know how chloroplasts will behave when isolated in the presence of cysteine.

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