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Purification and characterization of a soluble lipolytic acylhydrolase from Cowpea (*Vigna unguiculata* L.) leaves

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Abstract

With the use of [^{14}C]monogalactosyl diacylglycerol as substrate for enzymatic test, a lipolytic acylhydrolase (EC 3.1.1.26) was purified 2053-fold with a yield of 2.0% from soluble leaf extract of *Vigna unguiculata* L. cv. EPACE-1. The procedure involved ammonium sulfate precipitation, Q-Sepharose Fast Flow chromatography, gel filtration on Sephacryl 300 HR and chromatofocusing on Mono P, followed by a semi-preparative electrophoresis on polyacrylamide gel. The purified enzyme had a molecular mass of about 80 kDa as determined by gel filtration. On SDS-PAGE, it showed a single band corresponding to a molecular mass of 40 kDa. The isoelectric point of the enzyme was estimated to be 5.0–5.1 by isoelectric focusing and chromatofocusing. The K_m value was 0.119 mM for monogalactosyl-diacylglycerol. The hydrolytic activity of the enzyme on different substrates was determined: the relative rates were digalactosyl-diacylglycerol > monogalactosyl-diacylglycerol > phosphatidylcholine > phosphatidylglycerol. For all substrates the products of hydrolysis were free fatty acids. Triacylglycerols were not hydrolysed. The enzyme was activated by calcium but was not calcium-dependent. Experiments concerning the enzyme stability as affected by temperature and pH demonstrated that it was quite stable.

Keywords: Acylhydrolase, lipolytic; Galactolipase; Phospholipase; Cowpea

1. Introduction

In plants, membrane lipids have been found to play an essential role in the maintenance of the cell integrity and functioning, especially under harsh environmental conditions like heat, cold, salinity or water deficit (see Refs. [1–4]). Plants are able to adapt to these unfavourable surroundings thanks to a particular composition of their membrane lipids or thanks to a capacity to modify them in order to render them more resistant. However, when the stress becomes too harmful, homeostasis could no more be maintained and misfunctionings occur in the cell metabolism.

Decreases in polar lipid content have been observed during senescence [5–7] and under various stresses like heat [8], cold [9,10], and water deficit [11–14]. These variations are mainly due to activation of lipolytic enzymes, particularly phospholipases and galactolipases.

Unlike plant phospholipases D and C, very few reports exist on plant galactolipases and non specific acylhydrolases which remove fatty acids from galactolipids, even though the latter are the most abundant of lipids in plants, found in high concentrations in the chloroplasts.

Under stressful conditions, galactolipids, in particular monogalactosyl-diacylglycerol (MGDG) and digalactosyl-diacylglycerol (DGDG), are rapidly hydrolysed. The presence of galactolipase activities has been demonstrated in green leaves and in isolated cell organelles [15–20]. The products of lipolytic enzyme degradation inhibit several physiologic functions [21,22]. Endogenous galactolipases thus play an important role in leaf metabolism.

Abbreviations: CAPS, 3-[cyclohexylamine]-1-propanesulfonic acid; CHES, 2-[N-cyclohexylamine]ethanesulfonic acid; DGDG, digalactosyl-diacylglycerol; DTT, DL-dithiothreitol; FA, fatty acid; kDa, kilodaltons; IEF, isoelectrofocusing; LAH, lipolytic-acylhydrolase; MES, morpholinoethane sulfonic acid; MGDG, monogalactosyl-diacylglycerol; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PG, phosphatidylglycerol; pI, isoelectric point; PMSF, phenylmethane sulfonyl fluoride; PVP, polyvinyl pyrrolidone; TAG, triacylglycerols; TLC, thin-layer chromatography.

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Attempts for purifying galactolipases and non specific lipolytic acylhydrolases are found in literature [23–31]. Except the work of Burns, Galliard and Harwood [28], who have separated two distinct hydrolases from leaves of *Phaseolus multiflorus*, one with galactolipase activity, the other with phospholipase activity, in all reports, a unique enzyme has been isolated, which appeared to be a non-specific acylhydrolase, acting on galactolipids as well as on phospholipids. However, results concerning the other characteristics of this non-specific lipolytic-acylhydrolase (LAH) were contradictory: in particular, its molecular mass was found to vary from 40 kDa [31] to 110 kDa [23]; its isoelectric point from 4.4 [30] to 7.0 [23].

In most reports, the enzyme was only partially purified [24–26,28], and in those where the authors claimed to have the pure enzyme, the in situ enzymatic test after polyacrylamide gel electrophoresis, was a measure of esterase activity [27,29–31].

Surprisingly, since the late work of Matsuda and Hirayama in 1970 [32], no recent report on purification of plant galactolipases or lipolytic acyl hydrolases was found in literature, despite the great development of interest in the field of stress and senescence.

In the present study, we have purified a LAH from solubl. extracts of Cowpea (*Vigna unguiculata* L.) leaves and determined some of its biochemical properties.

2. Materials and methods

2.1. Plant material

Experiments were performed using a cultivar of *Vigna unguiculata* (L.) Walp, EPACE-1, originating from Brazil. The plants were grown in a greenhouse, in pots of diameter 9 cm, in a mixture of vermiculite and peat, under temperature controlled conditions (22°C during the day and 19°C at night), as previously described [33]. They were used for experiments when 5-weeks old, with 3 fully-expanded mature leaves and 2 young leaves.

2.2. Preparation of substrates for enzymatic assays

The substrates used in this work: MGDG, DGDG, PG, PC and TAG were prepared from young leaves (4th and 5th leaves, counting from the cotyledons) of the same plants described above. The leaf lipids were radioactively labelled with [^{14}C]acetic acid, sodium salt (CEA, France, specific activity 1.85 GBq.mmol^{-1}) and extracted in chloroform/methanol/water following Allen and Good [34]. Lipid classes were separated by thin-layer chromatography on silica-gel plates (Merck G-60), using the solvent systems proposed by Lepage [35] and Mangold [36]. Lipids were visualized under UV, scraped off and eluted from silica gel. Details of the methods are described in [19]. The fatty acid composition of the different lipid substrates was determined by gas chromatography and the radioactivity incorporated in fatty acids was counted after separating them following their degree of unsaturation on activated silica-gel containing silver nitrate, as described in [37].

2.3. Assay for galactolipase activity

[^{14}C]MGDG, prepared from Cowpea leaves as described in the previous paragraph, was used as substrate in routine assays for galactolipase activity. The substrate (550 Bq, 65 nmol) was dried under a stream of nitrogen and dissolved in 50 μl methanol containing 0.4% Triton X-100 (w/v). This mixture was brought to a final volume of 150 μl by adding 0.1 M MES buffer (pH 6.0). Emulsification was ensured by sonication at room temperature for 15 min (Sonicator W-10, Sonica, NY).

The enzyme assay mixture consisted of the substrate preparation described above (150 μl) and 200 μl enzyme solution containing 5 mM $CaCl_2$. After 3 h of incubation in a waterbath at 30°C, the reaction was stopped. The products resulting from enzymatic degradation were separated by TLC on silica-gel plates as described by El-Hafid et al. [19]. Radioactivity of the free fatty acids band was measured using a liquid scintillation analyzer (Packard 1600CA).

2.4. Extraction of leaf-soluble enzyme

Fresh leaves (300 g) frozen in liquid nitrogen were homogenized in 1000 ml extraction buffer: 0.1 M MES NaOH, pH 6.5 containing 10 mM DTT, 1 mM cystein, 5 mM sodium metabisulfite, protease inhibitors (0.5 mM PMSF, 0.5 mM benzamidine, 1 μM pepstatin A) and 10% glycerol (v/v), using a mortar and pestle. 2% insoluble PVP (w/v) was added just before homogenization.

The homogenate was filtered through a layer of monyl (64 μm pore size) and centrifuged at 39 000 × *g* for 30 min (Beckman J2-21 M/E). The resulting supernatant was acidified to a pH of 5.1 with 1 M HCl, and the precipitated membranes of the different organelles were sedimented by centrifugation under the same conditions described above. The pH of the supernatant resulting from this centrifugation was increased to 6.5 and corresponded to the crude extract.

2.5. Purification

Step 1: Ammonium sulfate precipitation

Ammonium sulfate was added to the crude extract to a concentration of 80% saturation. The mixture was left stirring for 12 h at 4°C. The precipitated protein was centrifuged at 27000 × *g* for 15 min. The sediment was resuspended in a limited volume of buffer A (10 mM Tricine (pH 8.0), 0.5 mM DTT, 0.1 mM cystein, 0.5 mM sodium metabisulfite, 10% glycerol (v/v), and protease inhibitors as in the extraction buffer). Undissolved material was removed by centrifugation under the same conditions

Step 2: Desalting

The clear yellow supernatant resulting from step 1 was desalted on a Sephadex G-25 column (2.5 × 100 cm, Pharmacia, Uppsala, Sweden), equilibrated with buffer A. The protein was eluted at a flow rate of 1.3 ml.min^{-1} using the same buffer. The protein peak was collected and assayed for enzymatic activity.

Step 3: Ion-exchange chromatography

Using an FPLC (Fast Protein Liquid Chromatography, Pharmacia) system, the protein fraction was applied at a flow rate of 5.3 ml.min^{-1}, to a Q-Sepharose Fast Flow column (2.6 × 15 cm, Pharmacia), equilibrated with buffer A. The column was washed, and bound proteins were eluted by a NaCl gradient of 0–1 M NaCl in the same buffer. Fractions were collected every 2 min, and then assayed for enzymatic activity.

Step 4: Gel filtration

The active fractions obtained from the preceeding step were pooled and concentrated. The concentrated protein solution (4 ml) was then applied, at a flow rate of 1 ml.min^{-1} using a peristaltic pump, to a Sephacryl HR-300 column (2.6 × 100 cm, Pharmacia), equilibrated with buffer A. Elution was continued at the same flow rate; fractions of 4 ml were collected. The enzymatic activity of fractions was assayed. The column was calibrated using the same separation procedure. Blue dextran marked the void volume, α-amylase (250 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa), were used as molecular weight markers.

Step 5: Chromatofocusing

The active fractions resulting from step 4 were pooled and applied at a flow rate of 0.5 ml.min^{-1} to a Mono-P column (5 × 20 cm, Pharmacia), equilibrated with 25 mM Bis-Tris buffer (pH 7.1). Chromatofocusing was carried out with 35 ml 10% (v/v) Polybuffer 74-HCl pH 4.0 (Pharmacia). Fractions of 0.5 ml were collected and the enzymatic activities were measured.

Step 6: Semi-preparative polyacrylamide gel electrophoresis

Native-PAGE of the enzyme after chromatofocalisation was performed using a Bio-Rad apparatus (Mini Protean II). The gels were polymerized with concentrations of monomer from 7.5 to 12.5%, with a spacer gel of 4%. Electrophoresis was run with 25 mM Tris-glycine buffer (pH 8.8), at constant current of 20 mA. Bromophenol blue was used as the tracking dye. Test bands of the gel were stained with the Bio-Rad silver nitrate staining kit. By comparison, the colourless bands obtained, were cut up and carefully crushed in buffer A. Cold diffusion for 24 h followed by centrifugation at 15000 × g for 10 min allowed the precipitation of acrylamide. The supernatants were collected and their enzymatic activity tested.

2.6. Analytical polyacrylamide gel electrophoresis

Analytical native and SDS-PAGE were performed using the PHAST system (Pharmacia). The gels used were ready-for-use Phast gels 10–15 for isoelectric focusing. Phast gels 4–6.5 were chosen. The protein bands were stained with the silver nitrate staining kit (Pharmacia).

2.7. Protein determination

Protein determination was carried out using the Bio-Rad dye reagent based on the method of Bradford [38], with bi-crystalline bovine serum albumin as the standard.

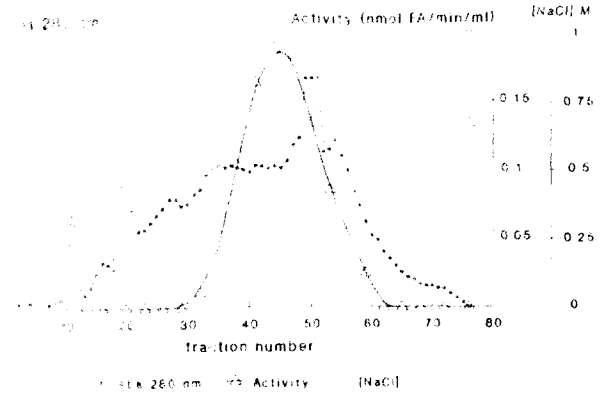
3. Results

3.1. Purification of the enzyme

Proteins in the crude extract, precipitated by ammonium sulfate were applied to a Sephadex G-25 column (2.6 × 100 cm). This step allowed the removal of salts, small molecules and undesirable material as pigments and phenols. It could also be remarked that a slight increase in the

Table 1
Purification of a soluble MGDG-lipolytic-acylhydrolase from Cowpea leaves

Step	Protein (mg)	Total activity (nmol FA.min^{-1})	Specific activity (nmol FA.min^{-1} mg protein^{-1})	Yield (%)	Purification (-fold)
$(NH_4)_2SO_4$	381.8	157.3	0.412	100	
G-25	333.6	167.8	0.503	107	1.22
Q-Sepharose	25.97	36.05	1.388	22.9	3.37
Sephacryl 300	1.30	17.9	13.76	11.4	33.4
Mono-P	0.152	3.92	25.71	2.5	63
Native-PAGE	0.03	3.25	108.3	2.0	263



1. Elution profile of protein (absorbance at 280 nm) and MGDG-hydrolase activity on a Q-Sepharose fast flow column. The enzyme on, obtained after ammonium sulfate precipitation and desalted on adex G-25, was loaded on a Q-Sepharose fast flow column (2.5 × 15 at a flow rate of 3 ml min^{-1}. Bound proteins were eluted with a linear ent from 0 to 1 M NaCl. Fractions of 10.6 ml were collected and ayed to enzyme assay.

d of total enzymatic activity was recovered after this (107 %, Table 1), probably due to the elimination of a mol. wt. weight inhibitor. The resulting fraction was chromatographed on a Q-Sepharose **column.** As shown ig. 1 the enzyme eluted between fractions 33 and 58. maximum activity, according to enzyme assay, corre-nded to the fractions separating between 0.35 and 0.43 M NaCl gradient Fractions separating within this lien ing were collected and pooled Up to this point, 37-fold purification was achieved (Table 1).

he pooled fractions were concentrated and further ie on gel filtration column (Sephacryl 300 HR, ii ind ed

the fractions (22 to 33) having MGDG-acylhydrolase ity (Fig 2) were collected and subjected to chromato-osing (Mono-P, Pharmacia) using pff 4–7 ampholytes pre ented in Fig 3. the maximum activity, situated ween fractions 70 and 90, corresponded to an isoelectric II. of 5.0 The protein recovery was low (0.04%), a fold purification of the LAH from the homogenate was ieved, with a yield of 2.5% (Table 1).

SDS-PAGE of the pooled active Mono-P fractions still ealed the existence of several bands (2 major and one ior results not shown). Attempts to further purify the zyme using several chromatographic methods ydroxylapatite, Bio-Rad; Mono-Q, Pharmacia; Octyl-pharose Pharmacia) did not allow the separation of se bands.

A semi-preparative Native-PAGE elecrophoretic step s therefore included in the purification process. After tion of the proteins from the gel, only one of the bands owed galactolipase activity whilst none was detected for ers.

The pI it of the active band was tested by SDS-PAGE: ly one band of MW = 40000 was visible (Fig. 4). IEF e revealed one unique band, at pI = 5.0 (results not wn). This ultimate step led to a 263-fold purification of

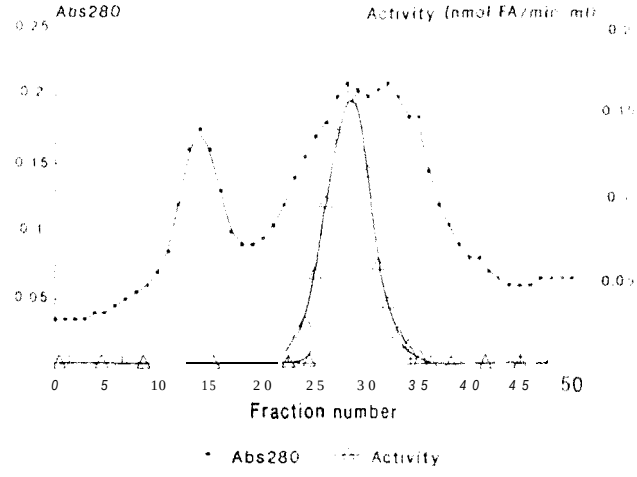


Fig. 2. Elution profile of protein (absorbance at 280 nm) and MGDG-acylhydrolase activity on a Sephacryl 300 HR column. The pooled fractions from the Q-Sepharose column were concentrated and loaded onto a Sephacryl 300 HR column (2.6 × 100 cm) at a flow rate of 1 ml min^{-1}. Fractions of 4 ml were collected and subjected to enzyme assay.

the enzyme from the homogenate of EPACE-1 leaves with a yield of 2% (Table 1).

3.2. *Characteristics of the purified enzyme*

Molecular weight

Gel filtration on Sephacryl 300 HR (step 3 in the purification scheme) gave an estimate of the molecular mass of the enzyme. The elution point of the enzyme and those of the reference standards plotted against the logarithms of their molecular masses are shown in Fig. 5. The estimated molecular mass of the enzyme as determined by gel filtration was found to be about 80 kDa. SDS-polyacrylamide electrophoresis of the purified enzyme, as shown in Fig. 4, revealed one band of about 80 000

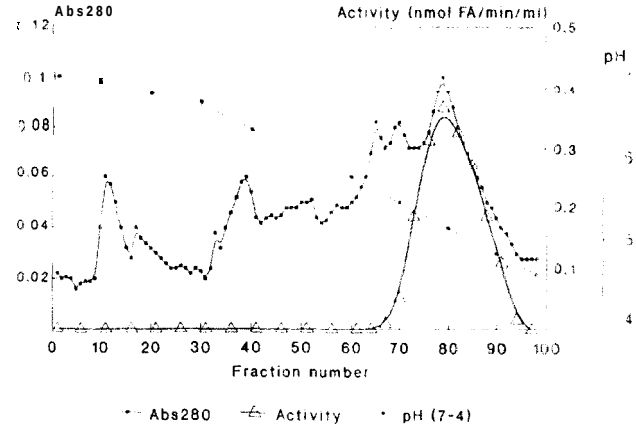


Fig. 3. Elution profile of protein (absorbance at 280 nm) and MGDG-acylhydrolase activity on a Mono-P column. The active fractions from the Sephacryl 300 HR column were subjected to chromatofocalisation on a Mono-P column (5 × 20 cm) using ampholytes for the pH range of 4–7. Fractions of 0.5 ml were collected at a flow rate of 0.5 ml min^{-1} and were assayed for enzymatic activities.

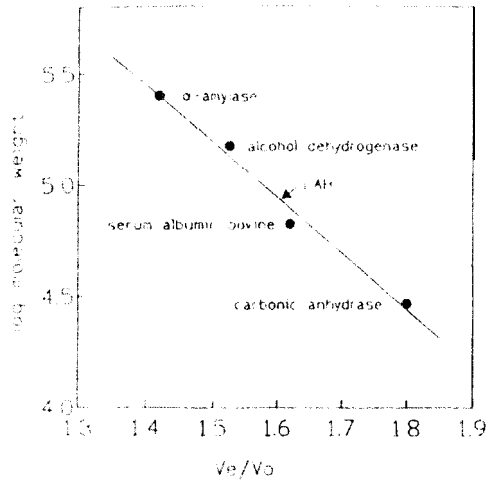


Fig. 4. Determination of the molecular mass of the purified enzyme by gel filtration on a Sephacryl 300 HR column. The enzyme solution was applied to a column (2.6 × 100 cm) of Sephacryl 300 HR at a flow rate of 1 ml.min^{-1} and fractions of 4 ml were collected. Void volume was determined with blue dextran. The ratio of elution volume (V_e) to void volume (V_o) was plotted against the logarithm of molecular masses for the marker proteins: α-amylase (250 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). The arrow indicates the elution position of the MGDG-acylhydrolase.

molecular weight. These observations demonstrated that the Cowpea leaf MGDG-acylhydrolase is a dimer.

Effect of pH

Under the conditions of routine assay, the purified enzyme shows activity at all the pHs tested, with a maximum activity at pH 5.0 (Fig. 6).

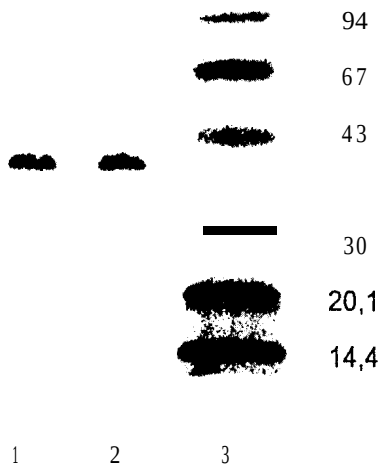


Fig. 5. SDS-polyacrylamide gel electrophoresis of the purified MGDG-acylhydrolase from Cowpea leaves. The active protein band from the preparative Native-PAGE was eluted and subjected to SDS-PAGE (see Section 3) and stained with silver nitrate. Lane 1 and 2, purified enzyme; lane 3, standards. Molecular mass (kDa) is indicated on the right.

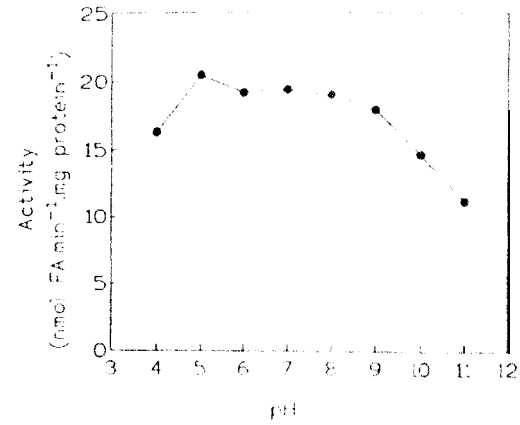


Fig. 6. Effect of pH on the activity of MGDG acyl hydrolase. The purified enzyme was incubated for 3 h at 30°C in the following buffers: 0.1 M sodium acetate (pH 3.0, 4.0, 5.0), 0.1 mM MES (pH 6.0), 0.1 M Tris-HCl (pH 7.0), 0.1 M Tricine (pH 8.0), 0.1 M CHES (pH 9.0), 0.1 M CAPS (pH 10.0, 11.0).

Thermal stability of the enzyme

When the enzyme was heated for 10, 30, and 60 min in a waterbath at different temperatures, it was shown that it exhibits a rather high resistance to heat inactivation, being maximally active after 10 min incubation at 50°C. (Fig. 7). At more longer incubation times and higher temperatures however, the activity of the enzyme began to fall.

Effect of calcium

Practically no effect on the enzymatic activity was observed at calcium concentrations of 1 mM and 5 mM. An increase in activity was observed from a concentration of 50 mM, reaching the maximum at a concentration of 500 mM.

Effect of substrate concentration

The curve relating the acylhydrolase activity and different MGDG concentrations (Fig. 8A) indicates that the

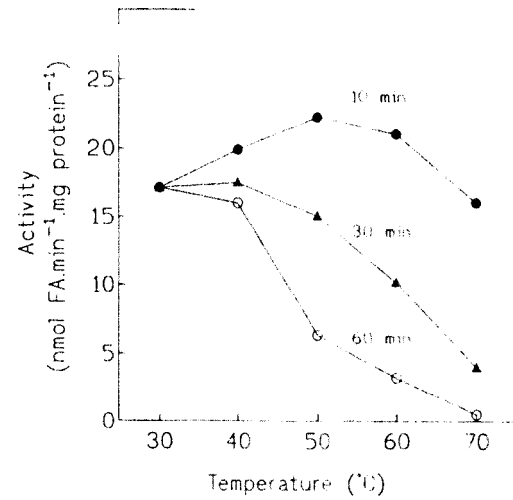
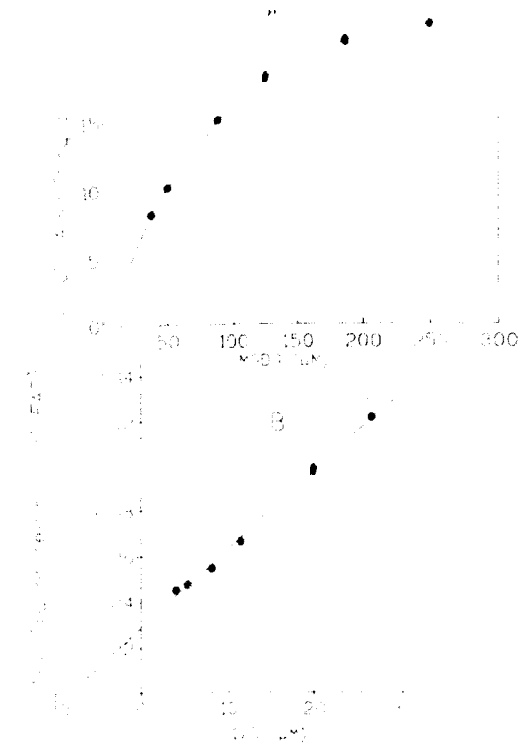


Fig. 7. Thermal stability of the purified MGDG acyl hydrolase. The enzyme was incubated at various temperatures, for various duration times. Enzymatic activity was then assayed in standard conditions (3 h at 30°C, pH 6.0).

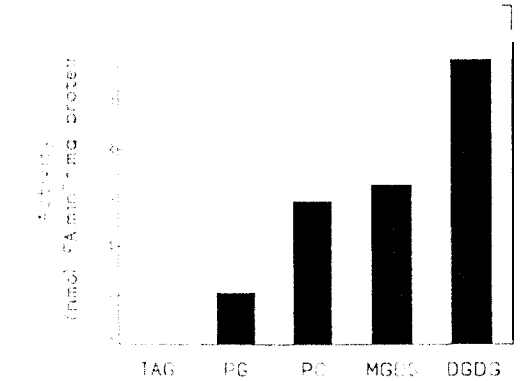


S. Effect of substrate concentration on the activity of the purified me. (A) Effect of MGDG concentration on the enzyme activity; (B) weaver–Burk plots for the hydrolysis of MGDG by the purified

me activity tended to reach a plateau beginning at 250 MGDG. The K_m value of the enzyme for this substrate was calculated to be 119 μM from a Lineweaver–k plot (Fig. 8B).

strate specificity

The purified enzyme was tested for activity towards the ous substrates shown in Fig. 9. Hydrolyzing activity obtained for the phospholipids and galactolipids tested. hydrolysis rate for DGDG was the highest (11.7 nmol



9. Substrate specificity of the purified enzyme. Substrates were acted from Cowpea leaves as indicated in Section 2. Enzymatic s were done following the standard procedure with 0.1 nmol purified me and 65 nmol of each substrate.

Table 2
Fatty acid composition (in mass%) of the polar lipids extracted from Cowpea leaves and used as substrates for LAH

	Fatty acid	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
MGDG	A	1.9		0.8	0.5	2.4	94.4
	B	8.4		3.5	3.6	5.8	78.7
DGDG	A	17.3		2.2	0.8	2.7	76.9
	B	25.7		3.7	1.3	2.1	67.1
PC	A	23.3		4.4	7.7	34.4	30.2
	B	41.7		11.2	14.1	17.4	15.6
PG	A	31.2	22.6	5.1	2.8	18.3	20.0
	B	60.7	7.6	8.4	4.1	8.8	10.4

(A) Before the enzymatic degradation; (B) after

FA.min^{-1}.mg^{-1} protein). This was followed by MGDG and PC, which showed almost equal rates (6.6 and 5.9 nmol FA.min^{-1}.mg^{-1} protein, respectively). PG showed the lowest rate (2.1 nmol FA.min^{-1}.mg^{-1} protein). On the other hand, the neutral lipid, TAG, was not affected.

Table 2 presents the fatty acid composition of the different polar lipids used as substrates, and Table 3 shows the distribution of radioactivity among the saturated (C16:0 + C18:0), mono- (C16:1 + C18:1), di- (C18:2) and tri- (C18:3) unsaturated fatty acids, prior to and after degradation by LAH. As could be seen, the lipids remaining after enzymatic reaction were less unsaturated, and the relative radioactivity of polyunsaturated fatty acids, especially linolenic acid, decreased; in other words, LAH degraded preferentially the polyunsaturated molecular species of polar lipids.

4. Discussion

In the present study, we have purified a MGDG-lipolytic acylhydrolase from soluble extracts of Cowpea leaves, and determined its biochemical characteristics.

The purified enzyme exhibited no activity towards TAG, on the other hand it deacylated phospholipids as well as

Table 3
Distribution of the radioactivity (in% of the total radioactivity) among the fatty acids of the polar lipids extracted from Cowpea leaves and used as substrates for LAH

	double bond	:0	:1	:2	:3
MGDG	A	1.6	0.3	5.2	93
	B	8.1	0.9	4.8	86.2
DGDG	A	7.2		7.0	85.5
	B	33.5	1.4	6.6	58.5
PC	A	22.1	2.3	28.9	46.7
	B	65.7		20.0	14.3
PG	A	25.1	3.0	40.3	31.6
	B	86.7		10.0	3.3

(A) Before the enzymatic degradation; (B) after. :0 = saturated fatty acids (C16:0 + C18:0), :1 = monounsaturated (C16:1 + C18:1), :2 = diunsaturated (C18:2), :3 = triunsaturated (C18:3).

galactolipids, with a preference for the polyunsaturated molecular species: it was therefore similar to the LAH first described by Galliard [24] in potato tubers. Similar results were obtained by Matsuda et al. on Bean and Potato leaves [29,30] and by Hirayama et al. on potato tubers [27]. These authors suggest that the active site was the same for MGDG and PC [27].

The enzyme was shown to have a native molecular mass of 80 kDa. This value was similar to those found by Burns et al. [28] on *Phaseolus multiflorus* leaves (70–90 kDa) and by Matsuda et al. [30] on leaves of *Phaseolus vulgaris* (90 kDa), but differ noticeably from those found by Helmsing [23] on *Phaseolus multiflorus* leaves (110 kDa) and by Anderson et al. [26] on *Phaseolus vulgaris* chloroplasts (55 kDa). By SDS-PAGE, we showed that the enzyme was a dimer of 2 polypeptides of 40 kDa each. Curiously, no report on the subunits of the LAH or of the galactolipase could be found in literature.

The isoelectric point of the enzyme was at pH 5.0. Apart from Helmsing's report of a $pI = 7$ [23], the LAHs described in literature seemed generally to be acidic proteins, with a pI ranging from 4.4 [30] to 5 [27]. In Rice bran, Matsuda and Hirayama [31] demonstrated the existence of multiple isomeric forms of the enzyme.

The activity of the enzyme was also found to be maximal at rather acid pH: 5–6, a value consistant with those reported by other authors.

Our experiments on the thermostability of the enzyme indicated that it is relatively heat-resistant. Helmsing [23], Matsuda and Hirayama [29] also found a good resistance to high temperatures of their acylhydrolases, especially at acidic pH. Moreover, Anderson et al. [26] suggested the existence, in Bean leaves, of a heat-labile inhibitor of the enzyme since they observed an increase in activity when the leaf extract was subjected to 65°C for 2 min. This could explain the increase in activity that we obtained after gel filtration of the crude enzyme, the inhibitor being probably separated on the Sephadex G-25 column.

The enzyme was slightly activated by $CaCl_2$, but was not calcium-dependent, at the difference of phospholipases A from animal sources.

In the present study, we have purified the MGDG-acylhydrolase from the particle-free extract of Cowpea leaves. However, MGDG-acyl hydrolytic activities have been found in the chloroplast membranes [16,21,27]. It will be of great interest to precise the eventual differences or similarities between them. Further work on this problem is in progress in our laboratory.

Recently [39], it was demonstrated that the major storage protein of Potato tubers, patatin, has an acyl hydrolase activity towards polar lipids. However, patatin was not found in leaves [40], and it has a specific activity with galactolipids 30-fold less than with phospholipids [40]. Then, the molecular identity between patatin and leaf LAH still remains ambiguous.

On the other hand, our purification scheme leading to the availability of the pure leaf enzyme makes possible more detailed studies on the sub-cellular localization of the enzyme and on the mechanisms of its regulation that may provide a more comprehensive understanding of the process of membrane lipid degradation in plants.

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