Phytochemistry, 1979, Vol. 18, pp. 1793–1797. © Pergamon Press Ltd. Printed in England. 0031-9422/79/1101-1793 $02.00/0

PURIFICATION OF ACYL HYDROLASE ENZYMES FROM THE LEAVES OF *PHASEOLUS MULTIFLORUS*

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(*Received* 11 *March* 1979)

Key Word Index—*Phaseolus multiflorus*; Leguminosae; runner bean leaves; acyl hydrolase purification; substrate specificities.

Abstract—Acyl hydrolase activities have been purified from the leaves of *Phaseolus multiflorus*. The purification procedure involved heat treatment, DEAE-cellulose chromatography, Sephadex G-100 filtration and hexyl agarose chromatography. The elution pattern from hexyl agarose columns together with substrate competition experiments indicated the presence of two hydrolase enzymes. The first could hydrolyse oleoylglycerol and phosphatidylcholine while the second would deacylate glycosylglycerides and oleoylglycerol. Overall purification of both enzymes was *ca* 70-fold and the MW of the glycosylglyceride-hydrolysing enzyme was in the range 70–78 000.

INTRODUCTION

The photosynthetic tissues of higher plants are characterized by high levels of glycosylglycerides. Three compounds are important—diacylgalactosylglycerol (monogalactosyldiglyceride, MGDG), diacylgalabiosylglycerol (digalactosyldiglyceride, DGDG) and diacylsulphoquinovosylglycerol (the plant sulpholipid, SQDG)—and they are all concentrated in the chloroplast [1]. The turnover of such leaf acyl lipids is catalysed, at least in part, by the activity of acyl hydrolases. While the triacylglycerol-hydrolysing enzymes, such as castor bean lipase [2], have been isolated and studied in some detail, less is known about the acyl hydrolases which act on phospho- and glycosylglycerides. Unlike animal and microbial phospholipases, which in some cases are specific (cf. [3]), no such property has been clearly demonstrated in plants. Indeed, the current nomenclature of plant acyl hydrolases appears confusing and there are several examples to illustrate this. For example, a sulpholipase which would deacylate SQDG in a step-wise manner was also active with phospholipids [4]. Similarly, the galactolipid-hydrolysing enzyme from *Phaseolus* leaves [5, 6] actually had a higher activity towards lysophospholipids or monoacylglycerols [7].

Non-specific acyl hydrolases from potato tubers have been studied by several laboratories, e.g. [7–10]. A similar enzyme was also found in the leaves and sprouts of potatoes and the leaves of *Phaseolus vulgaris* [11]. The substrate specificities of the potato tuber enzymes were determined [8, 12] and a wide range of phospholipids, galactolipids and glycerides were hydrolysed. However, virtually no activity was found with triacylglycerols. Previous papers have described the partial purification of a sulpholipid lipase from the leaves of *Phaseolus multiflorus* [13, 14]. The characteristics of the partially-purified enzyme showed that SQDG hydrolase activity was also associated with galactolipase activity [13] and the preparation was even more active towards lysophosphatidylcholine and oleoylglycerol [14]. The enzyme, therefore, had the characteristics of the non-specific acyl hydrolase from potato tubers [8, 12]. We now report further purification of the acyl hydrolase from *Phaseolus multiflorus*. The results indicate the presence of two enzymes, one which hydrolyses oleoylglycerol and

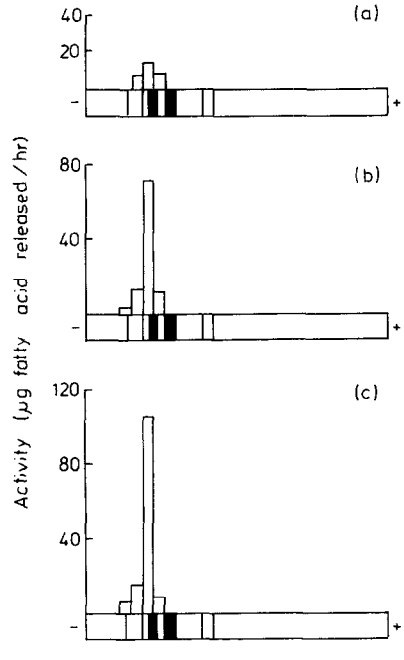

Fig. 1. Distribution of acyl hydrolase activity on polyacrylamide gels. For estimation of activity see Experimental. (a) SQDG substrate; (b) DGDG substrate; (c) MGDG substrate. Control gels were also stained with Coomassie blue. The histograms represent the average of duplicate determinations. The enzyme sample had been purified as far as the hexyl agarose-column stage.

phosphatidylcholine and a second which hydrolyses glycosylglycerides and oleoylglycerol.

RESULTS AND DISCUSSION

The initial purification procedure [13] was based on that of Helmsing [12]. Competition experiments [13] and the similar heat-lability of activity towards a number of lipid substrates [14] had indicated that the enzyme was a non-specific acyl hydrolase. Accordingly polyacrylamide gel electrophoresis was performed and the preparation was resolved into two major and 4 minor protein bands on 7.5% gels (Fig. 1). Enzyme activity estimated in slices from unstained gels was located in the same region of the gels for all 3 glycosylglycerides, again providing further evidence that the same enzyme was involved in their deacylation.

Because of the likely hydrophobic nature of the acyl hydrolase, hydrophobic chromatography was utilized for further purification. The final purification procedure is described in Experimental. Under such conditions acyl hydrolase activity was successfully separated from esterase activity (using naphthyl acetate) [15]. Such esterase activity was associated with the same region of polyacrylamide gels as acyl hydrolase and could have been responsible, in part, for oleoylglycerol hydrolysis at previous stages of the purification.

Since the partially purified enzyme had always shown acyl hydrolytic activity towards a number of lipids [14], the final purification procedure was followed by monitoring all stages with a number of substrates. The lipids used were the 3 leaf glycosylglycerides (MGDG, DGDG, SQDG), phosphatidylcholine and oleoylglycerol. If the same enzyme was responsible for the hydrolysis of all these substrates, then, not only should the respective activities be eluted at the same positions from different columns, but the ratio of their hydrolysis rates should also remain constant. With DEAE-chromatography (cf. [13]) all hydrolase activities were maximal with the first protein peak. Sephadex G-100 chromatography also gave only one peak of activity for each substrate, in every case at *ca* 3 times the void volume. Thus, all the steps in the purification up to this stage indicated that a single acyl hydrolase was present.

Hexyl agarose chromatography of the concentrated fractions from the Sephadex G-100 column used a salt

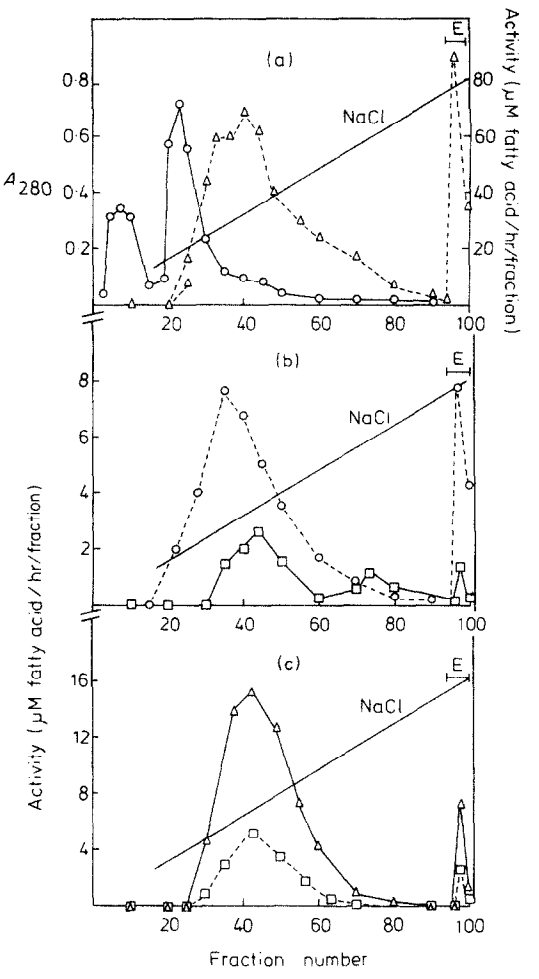

Fig. 2. Elution profile of hexyl agarose column. The column used was 20 × 1 cm and was loaded with 14 mg protein. Elution procedure was described under Experimental. (a) O——O, Protein (A_{280}), Δ---Δ, acyl hydrolase activity with 1-oleoylglycerol substrate; (b) □——□, acyl hydrolase with SQDG (Swiss chard) substrate, O---O, acyl hydrolase with phosphatidylcholine (soya bean) substrate; (c) Δ——Δ, acyl hydrolase with MGDG (Swiss chard) substrate, □---□, acyl hydrolase with DGDG (Swiss chard) substrate.

Table 1. Activity of fractions prepared at different stages of the purification procedure towards various substrates

Substrate	Fraction used (a)	(b)	(c)	(d)	(e)	(f)
SQDG	23	21	16	20	14	46
DGDG	46	48	43	51	30	132
MGDG	132	117	135	125	58	259
Phosphatidylcholine	100	100	100	100	100	100
1-Oleoylglycerol	632	720	770	613	400	878

Results are expressed as % of the activity towards phosphatidylcholine. Identity of fractions: (a) 104000 *g* supernatant; (b) after heat treatment; (c) after DEAE-cellulose chromatography; (d) after Sephadex G-100 gel filtration; (e) hexyl agarose column fractions 28–35 (see Fig. 2); (f) hexyl agarose column fractions 41–51 (see Fig. 2).

gradient which was sufficiently long to elute more than 90% of the acyl hydrolase activities before the ethanolic elution. Absorbance at 280 nm showed two major peaks and acyl hydrolytic activity was eluted after these (Fig. 2). With oleoylglycerol substrate the elution profile showed a shoulder at fraction 34 and a peak at fraction 41. In contrast, the two galactosylglycerides showed a single peak of activity which was maximal at fraction 42. SQDG activity was maximal at fraction 44 and is probably associated with the same peak as the galactosylglyceride activity. However, it also showed a second smaller peak in later fractions. In marked contrast to the glycosylglycerides, activity against phosphatidylcholine peaked at fraction 35 where it coincided with the shoulder of the oleoylglycerol peak. This result indicated that two acyl hydrolases may have been partially resolved. Both enzymes hydrolyse oleoylglycerol but one was active with phosphatidylcholine while the other cleaved glycosylglycerides. To gain further information about these activities, fractions 28–35 and 41–51 were combined and examined further.

The activity of preparations at different stages of purification is shown for the 5 substrates in Table 1. Whereas the ratio of activities was constant for heat-treatment, DEAE-cellulose chromatography and Sephadex G-100 filtration, a significant change was seen for the two combined fractions from hexyl agarose chromatography. This confirmed the elution profile data and suggested the presence of two different acyl hydrolases. Thus, for example, in fractions 28–35, MGDG was hydrolysed at half the rate of phosphatidylcholine whereas in fractions 41–51 it was hydrolysed at 2.5 times the rate. Although the results shown in Fig. 2 and Table 1 indicated that both activities will hydrolyse oleoylglycerol, it was possible that the activity towards oleoylglycerol in fractions 28–35 was caused by poor separation of the enzyme found mainly in fractions 41–51.

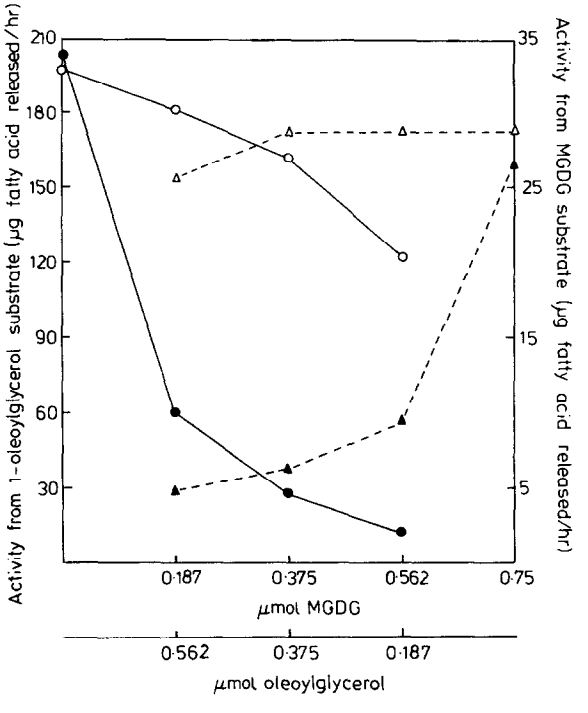

Fig. 3. Competition between diacylgalactosylglycerol and 1-oleoylglycerol substrates for acyl hydrolase. The enzyme preparation had been purified as far as the hexyl agarose-column stage. Substrates were incubated with the enzyme separately or in mixed solution. The final amount of lipid for mixed substrates was always 0.75 μmol. Activity was measured by loss of substrate [13]. ○——○, Hydrolysis of 1-oleoylglycerol; ●——●, hydrolysis of 1-oleoylglycerol in the presence of MGDG; △---△, hydrolysis of MGDG; ▲---▲, hydrolysis of MGDG in the presence of 1-oleoylglycerol.

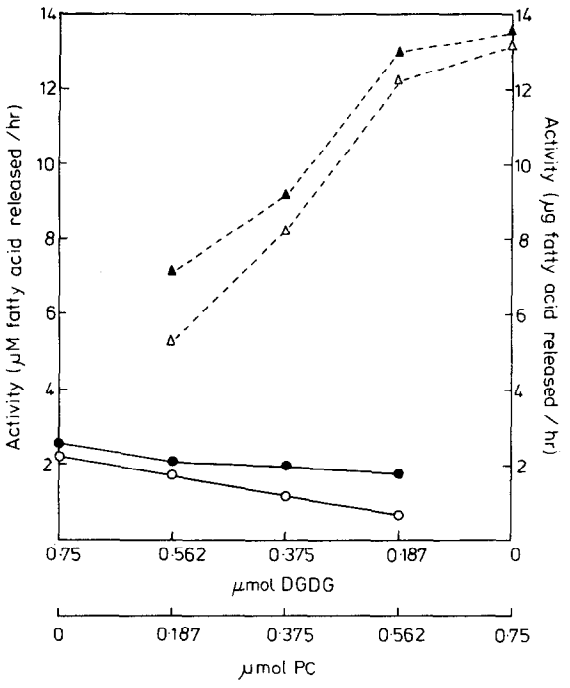

Fig. 4. Competition between phosphatidylcholine and diacylgalabiosylglycerol as substrates for an acyl hydrolase preparation. The enzyme preparation used was the combined fractions 28–35 from a hexyl agarose column (Fig. 2). Substrates were incubated with the enzyme either separately or together. The total lipid for mixed substrates was always 0.75 μmol. PC = Phosphatidylcholine (soya bean). Activity was measured by loss of substrate [13]. ●——●, Hydrolysis of DGDG; ○——○, hydrolysis of DGDG in the presence of phosphatidylcholine; ▲——▲, hydrolysis of phosphatidylcholine; △——△, hydrolysis of phosphatidylcholine in the presence of DGDG.

To obtain further evidence for the two different acyl hydrolases, competition experiments with various substrates were undertaken. Previous work [13] had already shown that the hydrolysis of each of the 3 glycosylglycerides was reduced in the presence of one of the others. This agreed with data from Fig. 2 and Table 1 that a single acyl hydrolase would deacylate MGDG, DGDG and SQDG. In Fig. 3 the competition between oleoylglycerol and MGDG as substrates is shown. The hydrolysis of either lipid is severely reduced in the presence of the other. The data would suggest that both substrates are competing for the same active site on the enzyme. However, the result does not eliminate the possibility that the lipids may form mixed micelles which then affect the rate of hydrolysis due to the non-availability of substrate.

A similar competition experiment was set up between phosphatidylcholine and DGDG and the result is shown in Fig. 4. In marked contrast to the previous experiment,

Table 2. Overall purification of acyl hydrolase activity

Fraction	1-Oleoylglycerol substrate Protein (mg)	Activity (μM/hr)	Purification	SQDG substrate Protein (mg)	Activity (μM/hr)	Purification
104000 *g* Supernatant	580	746	—	340	28.8	—
Heat treatment	130	715	4.3	225	24.2	1.3
DEAE-cellulose	37	613	12.8	90	18.0	2.4
Sephadex G-100	16.2	502	23.9	14	14.5	12.2
Hexyl agarose	3.5	302	66.4	—	—	—
Hexyl agarose (fractions 41–51, cf. Fig. 2)		—	—	1.06	6.8	73.2

Two separate purifications are shown.

there was very little evidence for competition in this case. The small amount of hydrolysis of DGDG confirmed the data in Table 1 and probably arises from contamination by the glycosylglyceride-hydrolysing enzyme. These results confirm Fig. 2 and Table 1 in that two different acyl hydrolases were separated by hexyl agarose chromatography.

The two combined fractions were each run by 7.5% polyacrylamide gel electrophoresis and by SDS-10% polyacrylamide gel electrophoresis. Polyacrylamide gels revealed 4 protein bands for fractions 28–35 and 3 protein bands, all of similar mobility, from fractions 41–51. SDS-polyacrylamide gels of the latter gave 5 protein bands with MWs in the region 62000–79000. While the glycosylglyceride-hydrolysing enzyme had been purified to the greatest extent it was still not a homogeneous protein. It is likely that further purification of the enzyme would be rather difficult because the proteins in the fraction appear to have similar charges and MWs. The MW for the acyl hydrolase acting on glycosylglycerides would appear to be in the range 70–78000 from the results from SDS-polyacrylamide gels and because it was excluded from Sephadex G-75. From Sephadex G-100 filtration it had a MW of *ca* 70–90000. These results compare with the acyl hydrolase isolated from potato which was estimated to have a MW of 70000 [12].

The overall purification of enzyme activity is shown in Table 2 for two different preparations. With either oleoylglycerol or SQDG as substrate, a *ca* 70-fold purification was achieved. Recovery of total activity was particularly good for oleoylglycerol substrate.

Conclusions

The results show that enzymes present in runner bean leaves can deacylate a wide variety of acyl lipids. Purification of the activity through a number of intermediate steps produced fractions which conformed to the general characteristics of non-specific acyl hydrolases similar to those observed in potato [8, 12]. However, use of hexyl agarose columns and competition experiments indicated that two acyl hydrolases were present. The first could hydrolyse oleoylglycerol and phosphatidylcholine and the second would deacylate glycosylglycerides as well as oleoylglycerol. The presence of those potentially destructive enzymes within the leaves of *Phaseolus multiflorus* poses problems as to how they are kept inactive or, at least, relatively inactive *in vivo*. One possibility is that the acyl hydrolases may be present in a lysosome-like particle [16] although the chloroplast stroma has been suggested to be the location for a galactolipase in spinach [17]. In the latter case there may be an inhibitor present in order to prevent the rapid destruction of thylakoid lipids. The presence of two different hydrolases (cf. potato isoenzymes [18]) would allow more flexibility in the alteration of endogenous lipid metabolism with leaves.

EXPERIMENTAL

Substrates. DGDG, MGDG and SQDG were isolated from Swiss chard or Spring cabbage as previously described [13, 19]. Fatty acid standards and other lipids were obtained from Sigma. Purity of substrates was checked before use by TLC [13]. 2-Naphthyl agarose was from Miles Laboratories.

Enzyme assay. Acyl hydrolase activity was determined by using the CH_2N_2 procedure as described in ref. [13]. Quantitation was with an internal standard of pentadecanoic acid. Fatty acid Me esters were separated by GLC on 15% diethylene glycol succinate on Chromosorb W AW or on 15% EGSS-X on Supelcoport columns at 185°. Protein was assayed, after precipitation with 10% TCA and solubilization at 100° for 5 min with M NaOH, by the method of ref. [20]. Where the total protein in the sample was 10 mg, the method of ref. [21] was used. In both cases, BSA was used as standard.

Enzyme isolation. Runner bean seeds (*Phaseolus multiflorus* cv Scarlet Emperor) were obtained from Thompson and Morgan. Leaves were harvested from plants grown in John Innes No. 2 compost with normal daylight conditions between the second and fourth weeks of growth. Leaves (100–400 g) were stored at 4° for 1 hr in the dark. They were homogenized with an equal wt of H_2O, filtered, and the residue resuspended in an equal vol. of H_2O and treated as above. The pooled homogenates were centrifuged at 12000 *g* for 30 min and the supernatant spun at 104000 *g* for 1 hr. The supernatant was collected and stored at −20° until required. The soluble fraction was incubated with stirring at 50° for 20 min. Denatured protein was removed by centrifuging at 8000 *g* for 20 min. DEAE-cellulose (DE-32, Whatman) was equilibrated with 10 mM KPi buffer, pH 7 and elution of protein carried out in a linear NaCl gradient of 0–1 M in 10 mM KPi buffer, pH 7. Sephadex G-75 and G-100 columns were eluted with 10 mM KPi buffer, pH 7. Hexyl agarose (20 ml) (Miles Laboratories), washed according to the manufacturer's instructions, was used for hydrophobic chromatography. After loading the enzyme sample, the columns were washed with 10 mM KPi pH 7 (2–3 ml). Protein was then eluted with a 0–2 M NaCl gradient in the same buffer. Residual protein was eluted with EtOH/0.1 M $NaHCO_3$–0.5 M NaCl (1:1, v/v).

Electrophoresis. 7.5% polyacrylamide gels were run at pH 8.5 in a Tris–glycine buffer and 10% SDS-polyacrylamide gels were run at pH 7.2 [22]. Esterase activity on the gels was estimated by the method of ref. [15] and acyl hydrolase activity by incubating unstained gel slices under the usual assay conditions [13].

Acknowledgement—The authors are grateful to the Agricultural Research Council for financial support.

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