Phytochemistry, 1977, Vol. 16, pp. 651–654. Pergamon Press Printed in England.

CATABOLISM OF SULPHOQUINOVOSYL DIACYLGLYCEROL BY AN ENZYME PREPARATION FROM *PHASEOLUS MULTIFLORUS*

D. DOUGAL BURNS, TERENCE GALLIARD* and JOHN L. HARWOOD
Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, U.K.;
*ARC Food Research Institute, Colney Lane, Norwich, U.K.

(*Received* 26 *October* 1976)

Key Word Index—*Phaseolus multiflorus*; Leguminosae; runner bean; sulphoquinovosyl diacylglycerol; acyl hydrolase; enzyme purification and properties.

Abstract—An enzyme which will deacylate sulphoquinovosyl diacylglycerol (SQDG) has been partially purified from the leaves of runner bean (*Phaseolus multiflorus*). No monoacyl intermediate was observed and the acyl hydrolase was more active towards unsaturated molecular species of SQDG than towards saturated species. The major peak of activity of SQDG acyl hydrolase, separated on both DEAE-cellulose and Sephadex columns, also contained galactolipid acyl hydrolase activity. The distribution of these activities together with substrate competition and inhibitor experiments indicated that at least part of the SQDG acyl hydrolase activity was due to an enzyme that also hydrolysed galactolipids.

INTRODUCTION

Sulphoquinovosyl diacylglycerol (SQDG, plant sulpholipid, sulphonolipid) was first observed by Benson and co-workers in a variety of photosynthetic microorganisms and higher plants [1–5]. Subsequently it has been found in all green plants examined [6, 7]. In contrast to the other glycolipids, monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG), it contains a high amount of palmitic as well as linolenic acid [8]. SQDG is localized in the chloroplast lamellae and it has been suggested that it may play a role in photosynthesis [9, 10]. Furthermore, during greening SQDG is formed prior to the appearance of chlorophyll with which it rapidly achieves a constant ratio [11].

Although the biosynthesis of SQDG has been investigated by several workers [12–15] its catabolism has received little attention. Extracts from *Scenedesmus obliquus* catalysed the breakdown of SQDG via a monoacyl intermediate to sulphoquinovosyl glycerol [16]. This enzyme, however, was not specific for SQDG but also hydrolysed phospholipids. Wolfersberger and Pieringer [17] showed that cell-free preparations of the alga *Chlorella pyrenoidosa* could also deacylate SQDG in a step-wise manner.

Extracts from the leaves of *Phaseolus* contain very active galactolipid acyl hydrolase activity. Sastry and Kates [18] suggested that enzymes specific for MGDG and DGDG were present but Helmsing [19] considered that the same enzyme was involved in hydrolysis of both lipids. No examination of SQDG as a substrate was made, however, nor is there any report of SQDG hydrolysis by an enzyme preparation from plant leaves, in spite of the high amounts of endogenous sulpholipid. This paper describes the partial purification and properties of such an enzyme which has been isolated from leaves of *Phaseolus multiflorus*.

RESULTS AND DISCUSSION

Preliminary experiments were carried out using crude enzyme preparations to determine the most suitable method of assay. Disappearance of substrate, measured by ester [20] or sugar determination [21] or appearance of fatty acid, estimated by complex formation [22], or GLC of methyl esters were compared. Of these, the ester determination and the GLC method were the most reliable. Comparable results were always obtained but the GLC technique was selected because of its sensitivity as well as the additional information it yielded concerning the release of different fatty acids.

Enzyme isolation and partial purification.

The procedure used was based on that of Helmsing [19] except that the ion-exchange step preceeded the gel filtration stage. This had the advantage of allowing a less concentrated protein solution to be loaded onto the ion-exchange column, which removed much of the pigments that interfered with gel filtration. There was no tendency for the protein to precipitate after concentration prior to gel filtration when treated as such. Elution was carried out using a salt gradient in 0.01 M phosphate buffer, pH 7 (Fig. 1) and the fractions assayed for activity towards MGDG, DGDG and SQDG. Although the major peak of activity was in the same fractions for all 3 substrates, they were not exactly superimposable. Fractions 28–48 (Fig. 1) from the ion-exchange column were bulked and the protein concentrated with ammonium sulphate (75 %) as described in the Experimental section. The concentrated protein solution was applied to a Sephadex G-200 column and eluted with 0.01 M phosphate buffer, pH 7 (Fig. 2). Fractions 24–28 were bulked and this partially purified preparation was used in all the subsequent experiments. This preparation had acyl hydrolase activity towards SQDG, DGDG and MGDG in the ratio of 1:4.4:7.6. This agrees very

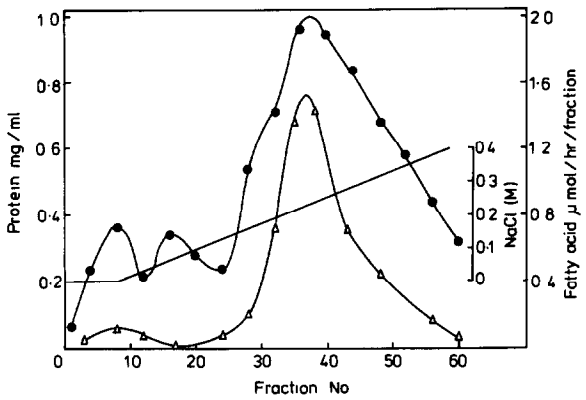

Fig. 1. Elution pattern of a DEAE-cellulose column. ●——● protein (mg/ml); △——△ SQDG deacylation (μmol/hr/fraction) For column details see text. Fractions of 8.4 ml were collected.

well with a ratio of 1.50:2.68 for DGDG and MGDG hydrolysis obtained by Helmsing [19] who concluded that the same enzyme catalysed deacylation of both lipids, but he did not test SQDG.

Determination of pH optima

The pH curve for SQDG hydrolysis showed a broad optimum around pH 5. Activity at pH 7.6 was 50% of that at pH 5. Similarly broad pH curves were obtained for MGDG and DGDG by Sastry and Kates [18]. No effect of different buffers was observed and subsequent incubations used succinate buffer at pH 5.

Determination of K_m

Varying concentrations of SQDG were incubated at 30° in a succinate buffer, pH 5. When plotted according to Lineweaver and Burk a linear relationship between 1/V and 1/S was obtained, which gave an 'apparent' K_m of 0.15 mM. This value compares favourably with 0.65 mM and 0.31 mM that Helmsing [19] obtained for MGDG and DGDG respectively. Sastry and Kates [18] obtained values of 7.8 mM and 1.5 mM for these two substrates.

Substrate competition experiments

Since Helmsing [19] had implicated a single enzyme which would act on both MGDG and DGDG, we were

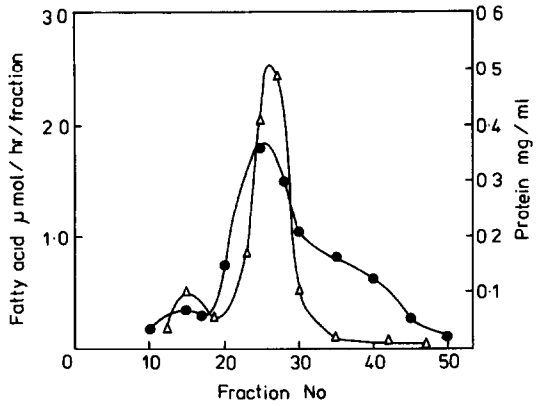

Fig. 2. Gel filtration on a Sephadex-G200 column. ●——● protein (mg/ml); △——△SQDG deacylation (μmol/hr/fraction) Fraction volumes were 11.2 ml. For column details see text.

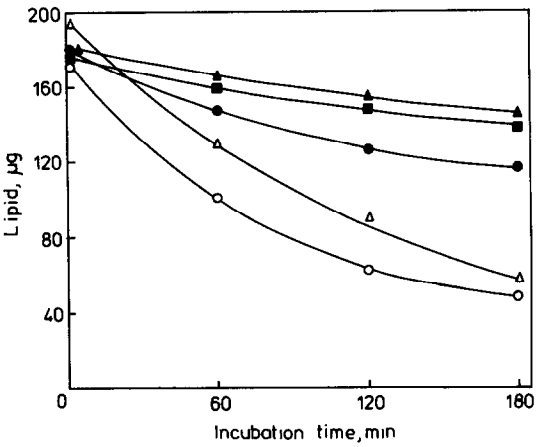

Fig. 3. Effect of competing substrates on SQDG deacylation. The reaction was followed by estimating the amount of substrate remaining at different time intervals (see Experimental). ●——● SQDG deacylation, ▲——▲ SQDG deacylation in presence of MGDG, ■——■ SQDG deacylation in presence of DGDG, △——△ DGDG deacylation in presence of SQDG, ○——○ MGDG deacylation in presence of SQDG. Equal weights of all lipids were incubated.

interested in whether there was any evidence that SQDG could be deacylated by the enzyme hydrolysing the galactolipids. Accordingly, a substrate competition experiment was set up and the results are shown in Fig. 3. The breakdown of SQDG was decreased in the presence of either MGDG or DGDG. These results can be interpreted as providing evidence for a single enzyne which can hydrolyse all 3 substrates. Since inhibition was not complete, the data does not preclude the possibility that another enzyme(s) may specifically deacylate SQDG without hydrolysing MGDG and DGDG.

Enzyme specificity

The fatty acid patterns obtained for the product of hydrolysis of both Swiss chard and Spring cabbage SQDG were not the same as the substrates (Table 1). Nor was there any good agreement with the fatty acids present in the 2-position of SQDG (Table 1). These data provide further evidence that deacylation of SQDG occurs at both positions and explains the absence of a lyso-intermediate, assuming that the second deacylation was the more rapid. Previous results with deacylation of

Table 1. Distribution of fatty acids in SQDG compared with those released by enzymic hydrolysis

		% Total fatty acids					
Lipid	Position	16:0	16:1	18:0	18:1	18:2	18:3
Swiss chard SQDG	Both	38.0	0.4	1.2	2.3	6.8	51.3
	1–	46.2	0.4	2.6	3.9	7.3	39.0
	2–	25.1	2.8	2.0	2.3	2.8	65.0
Fatty acid products		32.5	0.5	0.5	0.2	5.6	60.7
Spring cabbage SQDG	Both	30.5	1.9	1.7	1.0	7.6	57.3
Fatty acid products		22.8	0.8	1.0	1.4	7.0	67.0

Table 2. Fatty acid analysis of molecular species of Swiss chard SQDG

Species	% Total fatty acids					
	16:0	16:1	18:0	18:1	18:2	18:3
'Saturated' SQDG	59.8	1.4	2.0	9.3	22.7	4.8
'Trienoic' SQDG	41.3	0.6	1.7	1.2	2.1	53.0
'Hexaenoic' SQDG	10.5	0.2	1.1	0.7	1.5	86.0

MGDG and DGDG by higher plants also failed to reveal a lyso-intermediate [18, 19]. Algal preparations, on the other hand, deacylated SQDG in a step-wise manner with the lyso-intermediate clearly isolatable [16, 17].

From Table 1 it can be seen that linolenic acid is released in preference to palmitic acid. One could, therefore, predict that the enzyme preparations would be more active towards molecular species containing a high proportion of linolenic acid. Accordingly, $AgNO_3$-TLC was conducted which resulted in the separation of 3 molecular species of SQDG (Table 2). These were, predominantly, saturated, palmityl-linolenyl and dilinolenyl, representing 13%, 71% and 16% respectively of the original Swiss chard SQDG.

Incubation of these molecular species with the enzyme preparation (Fig. 4) showed quite clearly that the dilinolenyl species was hydrolysed preferentially. The relative rates of hydrolysis of the molecular species, together with their relative abundance, fully explain the pattern of fatty acids released during hydrolysis.

Effect of cysteine

Helmsing [19] had noted complete inhibition of his enzyme preparation by 1 mM cysteine but, in contrast, a galactolipid acyl hydrolase from *Phaseolus vulgaris* [23] was unaffected. We found no effect of cysteine up to a level of 2 mM on hydrolysis of SQDG, MGDG or DGDG by our preparation. It is, perhaps, pertinent to note that the cysteine solutions must be carefully buffered, otherwise the consequent change in pH of the incubation medium naturally renders the enzymes inactive.

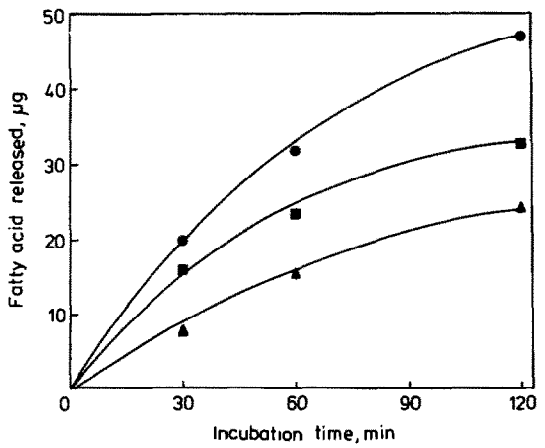

Fig. 4. Enzyme release of fatty acids from molecular species of SQDG ▲—▲ 'saturated' SQDG, ■—■ 'trienoic' SQDG, ●—● 'hexaenoic' SQDG.

CONCLUSION

The data presented in this paper show that *P. multiflorus* contains an active SQDG acyl hydrolase. The results provide an explanation for the rapid turnover of the acyl groups of SQDG in green leaves [6]. Furthermore, the specificity of the enzyme also enables the differential turnover of individual molecular species. At present we do not know how many enzymes are capable of deacylating SQDG. Certainly, an appreciable part of the lipolysis is due to an enzyme which also hydrolyses galactolipids. However, the lack of complete substrate competition and the non-superimposability of lipase activity towards the different substrates in the column fractions suggest that other enzymes may also be involved. These enzymes may be specific for SQDG or may be unspecific acyl hydrolases [*cf* 24, 25]. Further work is now continuing in our laboratories to answer this question.

Acknowledgements—This work was supported by a grant from the Agricultural Research Council to J.L.H.

EXPERIMENTAL

Runner bean seeds (*Phaseolus multiflorus* cv Scarlet Emperor) were obtained from Thompson and Morgan Ltd., Ipswich. Leaves were harvested from the plants between the second and fourth weeks of growth. Swiss chard (*Beta vulgaris* cv Cicla) was obtained from Samuel Dobie and Son Ltd., Llangollen and grown for 3 months before harvesting. Spring cabbage (*Brassica oleracea*) was purchased from the local market.

Substrates. SQDG, MGDG and DGDG were isolated from Swiss chard and Spring cabbage by the procedure of ref. [26]. MGDG and DGDG were further purified by preparative-Si gel TLC using $CHCl_3$–MeOH–HOAc–H_2O (170:30:20:7). Lipids were eluted with 6 changes of $CHCl_3$–MeOH–HOAc (200:100:1). SQDG was further purified on a second DEAE-cellulose column as previously described [26]. Purity of the substrates was checked by TLC.

Enzyme isolation. Enzyme was prepared from runner bean leaves (200–400 g) by a method based on ref. [18]. After storage at 4° for 1 hr in the dark, the leaves were homogenized with an equal wt of H_2O for 2 min. The homogenate was filtered through 2 layers of cheesecloth and the residue was further homogenized with H_2O and filtered. The pooled homogenates were centrifuged for 30 min at 12000 *g* and the resulting supernatant was further centrifuged for 1 hr at 104000 *g*. Solid $(NH_4)_2SO_4$ was added to the supernatant to 75% satn and the resulting ppt. was collected by centrifugation at 8000 *g* for 20 min. The ppt. was resuspended in 10 ml of 0.01 M Pi buffer, pH 7 and was dialysed against 5 l. of the same buffer for 24 hr. DEAE cellulose DE32 (microgranular anion exchanger, Whatman) was treated according to the manufacturers instructions. Ion exchanger (10 g) was resuspended in 0.01 M Pi buffer, pH 7. A column of 25 × 2 cm i.d. was used with a bed height of 10 cm. Elution was carried out using a gradient in the same buffer. Gel filtration was carried out on a 50 × 4 cm i.d. column of Sephadex G-200. Elution was carried out with 0.01 M Pi buffer, pH 7 with a bed height of 45 cm.

Enzyme assay. A modified procedure of ref. [18] was adopted. Succinate buffer, pH 5 was used for SQDG, maleate buffer pH 5.6 for DGDG and Pi biffer pH 7 for MGDG assays. Substrate (*ca* 0.25 μM) was resuspended in 15 μl of MeOH, buffer added and the substrate thoroughly dispersed. The reaction was initiated by enzyme addition and incubation carried out at 30°. The final buffer concn were 0.1 mM and the final vol. 0.5 ml. The reaction was stopped with 0.55 ml of 0.1 M HCl in 90% MeOH, $CHCl_3$ (0.625 ml) and MeOH (0.7 ml) were added and the mixture allowed to stand for 10 min.

$CHCl_3$ (0.625 ml) and 0.625 ml of 2M KCl in 0.5M Pi, pH 7.4 were added and shaken vigorously. The extraction procedure is similar to that described in ref. [27] which has been shown to quantitatively extract acyl lipids [15]. The lower phase was removed and the upper phase was re-extracted with fresh lower phase. The combined lower phases were subjected to analysis by one of the following methods. (a) The products were separated by TLC on Si gel G in $CHCl_3$–MeOH–HOAc–H_2O (170:30:20:7) and visualized with 0.001% Rhodamine 6G. SQDG, DGDG and MGDG were methylated using M NaOMe in MeOH. MGDG, DGDG and free fatty acids were methylated using 14% BF_3 in MeOH. (b) Free fatty acids were methylated using CH_2N_2 Fatty acid Me esters were separated by GLC on 15% DEGS at 185°. The R_fs were compared to authentic standards and quantified by comparing with a Me pentadecanoate standard.

Isolation of molecular species. Swiss chard SQDG (up to 20 mg) was separated on a 0.75 mm $AgNO_3$-Si gel (1:4) TLC plate developed in $CHCl_3$–MeOH–H_2O (65:25:4). The bands were visualized using 0.001% Rhodamine 6G and were eluted with 6 changes of $CHCl_3$–MeOH (2:1). The Rhodamine was removed on a DE 32 column.

REFERENCES

1. Benson, A. A., Daniel, H. and Wiser, R. (1959) *Proc. Natl. Acad. Sci. (US)* **45**, 1582.
2. Lepage, M., Daniel, H. and Benson, A. A. (1961) *J. Amer. Chem. Soc.* **83**, 157.
3. Daniel, H., Miyano, M., Mumma, R. O., Yagi, T., Lepage, M., Shibuya, I. and Benson, A. A. (1961) *J. Am. Chem. Soc.* **83**, 1765.
4. Shibuya, I. and Benson, A. A. (1961) *Nature (London)* **192**, 1186.
5. Miyano, M. and Benson, A. A. (1962) *J. Am. Chem. Soc.* **84**, 57.
6. Hitchcock, C. and Nichols, B. W. (1971) *Plant Lipid Biochemistry*. Academic Press, New York.
7. Mudd, J. B. and Garcia, R. E. (1975) in *Recent Advances in the Chemistry and Biochemistry of Plant Lipids* (Galliard, T. and Mercer, E. I., eds.) pp 161–201. Academic Press, New York.
8. Tulloch, A. P., Heinz, E. and Fischer, W. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 879.
9. Miyachi, S., Miyachi, S. and Benson, A. A. (1966) *J. Protozool.* **13**, 76.
10. Klopfenstein, W. E. and Shigley, J. W. (1967) *J. Lipid Res.* **8**, 350.
11. Thomas, D. R. and Stobart, A. K. (1970) *J. Exp. Botany* **67**, 274.
12. Benson, A. A. and Shibuya, I. (1961) *Federation Proc.* **20**, 79.
13. Shibuya, I. and Hase, E. (1965) *Plant Cell Physiol.* **6**, 267.
14. Davies, W. H., Mercer, E. I. and Goodwin, T. W. (1966) *Biochem. J.* **98**, 369.
15. Harwood, J. L. (1975) *Biochim. Biophys. Acta* **398**, 224.
16. Yagi, T. and Benson, A. A. (1962) *Biochim. Biophys. Acta* **57**, 601.
17. Wolfersberger, M. G. and Pieringer, R. A. (1974) *J. Lipid Res.* **15**. 1.
18. Sastry, P. S. and Kates, M. (1964) *Biochemistry* **3**, 1280.
19. Helmsing, P. G. (1969) *Biochem. Biophys. Acta* **178**, 519.
20. Snyder, F. and Stephens, N. (1959) *Biochim. Biophys. Acta* **33**, 244.
21. Roughan, P. G. and Batt, R. D. (1968) *Anal. Biochem.* **22**, 74.
22. Anderson, M. M. and McCarty, R. E. (1972) *Anal. Biochem.* **45**, 260.
23. Anderson, M. M., McCarty, R. E. and Zimmer, E. A. (1974) *Plant Physiol.* **53**, 699.
24. Galliard, T. (1975) in *Recent Advances in the Chemistry and Biochemistry of Plant Lipids* (Galliard, T. and Mercer, E. I., eds.) pp 319–357. Academic Press, New York.
25. Galliard, T. and Dennis, S. (1974) *Phytochemistry* **13**, 1731.
26. O'Brien, J. A. and Benson, A. A. (1964) *J. Lipid Res.* **5**, 432.
27. Garbus, J., DeLuca, H. F., Loomans, M. E. and Strong, F. M. (1963) *J. Biol. Chem.* **238**, 59.