Pergamon

0031-9422(95)00061-5

Phytochemistry, Vol. 39, No. 5, pp. 1071–1075, 1995

0031-9422/95 $9.50 + 0.00

BIOSYNTHESIS OF SULPHOQUINOVOSYLDIACYLGLYCEROL BY CHLOROPLAST FRACTIONS FROM PEA AND LETTUCE

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(*Received in revised form* 7 *December* 1994)

Key Word Index—*Pisum sativum*; Leguminosae; *Lactuca sativa*; Compositae; sulpholipid; sulphoquinovosyldiacylglycerol; biosynthesis; isolated chloroplasts.

Abstract—Chloroplasts isolated from pea and lettuce leaves were found to synthesize sulphoquinovosyldiacylglycerol from [^{35}S]sulphate. Optimal conditions were defined for this synthesis. Rates of up to 1.3 and 0.8 nmol mg chlorophyll^{-1} hr^{-1} (at pH 7.9) were obtained for pea and lettuce chloroplasts, respectively. Synthesis by intact chloroplasts was stimulated by UTP (uridine triphosphate) and was light-dependent. The rates of synthesis achieved *in vitro* were sufficient to show that the chloroplast is autonomous with respect to synthesis of sulpholipid. The ready availability of pea leaves and the ease of isolation and activity of their chloroplasts make such preparations very suitable for detailed studies of sulpholipid synthesis.

INTRODUCTION

The photosynthetic membranes of all oxygen-evolving organisms contain three glycosylglycerides, rather than phosphoglycerides, as their major lipid components [1]. Of these, the plant sulpholipid, or sulphoquinovosyldiacylglycerol, contains a unique sulphonic acid group. Its structure of 1′,2′-di-*O*-acyl-3′-*O*-(6-deoxy-6-sulpho-α-D-glucopyranosyl)-*sn*-glycerol was originally elucidated by Benson [2]. In higher plants, sulpholipid represents *ca* 2–6 μmol g^{-1} dry weight [3] or *ca* 10% of the thylakoid lipids [1]. Some algae contain very large amounts, the highest being detected in *Padina paventia* [4], where 49% of the total lipids are sulpholipid. It is possible that these very large amounts in marine Phaeophyceae may be due to the low levels of phosphate in such environments, which limit the production of alternative polar membrane lipids, such as phosphoglycerides. In support of this idea, is the sensitivity of sulpholipid-deficient mutants of *Rhodobacter sphaeroides* to phosphate limitation [5].

Sulpholipid has been proposed as a prominent member of the global sulphur cycle [6]. Indeed, from an ecological point of view, knowledge of its biosynthesis and catabolism is important [3]. Furthermore, because of the ubiquitous occurrence of the plant sulpholipid in oxygenic photosynthetic membranes and its purported function there [7], elucidation of its biochemistry is needed.

Studies on the biosynthetic pathway to sulpholipid have been reviewed [3, 8, 9]. Despite recent progress, the pathway by which the sulphoquinovose head-group is formed is still unknown [3]. It is clear, however, that UDP-sulphoquinovose (UDP = uridine diphosphate) is used in the final step to form sulpholipid from diacylglycerol [10, 11]. In order to study the biosynthesis of sulpholipid in more detail, we needed a preparation that was active *in vitro*. Accordingly, we have examined a number of potential systems and report here the optimization of conditions for chloroplast fractions from pea leaves. These plants are easily grown in large quantities and can be simply processed to yield pure preparations of intact chloroplasts which show good activity with regard to sulphoquinovosyldiacylglycerol synthesis.

RESULTS AND DISCUSSION

Previous work with spinach chloroplasts [12, 13] has shown that these organelles are capable of incorporating $^{35}SO_4^{2-}$ into sulpholipid. Therefore, we isolated chloroplasts from pea leaves to test for biosynthetic capability. Because there was some doubt, following the low rate of incorporation of $^{35}SO_4^{2-}$ found in some laboratories [12], whether the chloroplast was autonomous for sulpholipid synthesis [14], we realized that it was very important to achieve good rates of formation. Under our original incubation conditions we achieved rates of *ca* 0.7 nmol mg chlorophyll^{-1} hr^{-1} which were comparable to those for spinach chloroplasts [13]. In isolated chloroplasts, the supply of diacylglycerol for use in the final step

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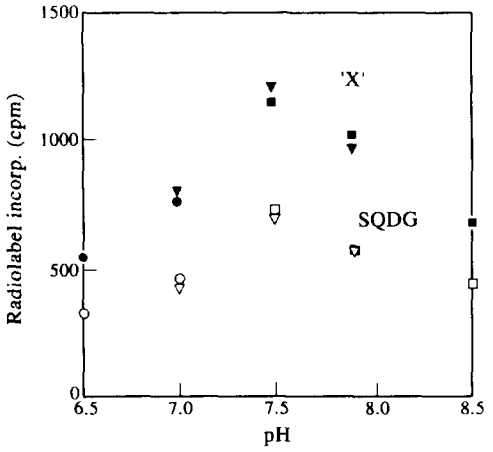

Fig. 1. Incorporation of radioactivity from $^{35}SO_4^{2-}$ into lipids by isolated pea chloroplasts as a function of incubation pH. Closed symbols show labelling of lipid 'X'; open symbols show sulphoquinovosyldiacylglycerol; (Circles), PIPEP buffer; (squares), TES buffer; (triangles), Tricine buffer; all at 22 mM. Results are means of duplicate assays.

is not limiting [13]; the role of molecular species of this substrate in sulpholipid has also been studied [15].

The pH requirement for optimal activity is shown in Fig. 1. Over the range tested, the best incorporation of $^{35}SO_4^{2-}$ into sulpholipid was achieved with a pH of 7.5. This compares with data for spinach chloroplasts [13], where optimal synthesis was found between pH 6.5 and 7.0. With spinach chloroplasts [13] and in other plant preparations [15], other ^{35}S-labelled compounds were found in the lipid extracts. For pea (and lettuce) chloroplasts a major component (containing comparable amounts of radioactivity to sulpholipid) had an R_f of *ca* 0.4 in the TLC system employed (see Experimental) in comparison to an R_f of 0.35 for sulpholipid. The relative labelling of sulpholipid and this unknown lipid remained approximately constant at the different pHs tested for pea chloroplasts, in contrast to the data for the chloroform-soluble lipids formed in spinach [13]. Although the highest rates of sulpholipid synthesis were achieved at pH 7.5 (Fig. 1), we wanted to study the process under conditions resembling those *in vivo* and, therefore, carried out our usual incubations at pH 7.9 (thought to be the stromal pH), where synthesis was still good.

We tested pea chloroplasts for nucelotide stimulation of sulpholipid synthesis (Table 1). Neither CTP nor GTP had any effect. ATP caused a small but significant increase in incorporation but UTP raised labelling rates by *ca* 110%. In four separate experiments, addition of 2 mM UTP approximately doubled incorporation of $^{35}SO_4^{2-}$ into sulpholipid. In contrast to the stimulation of sulpholipid labelling (Table 1), there was no effect of UTP addition on $^{35}SO_4^{2-}$ incorporation into other lipids. This result agrees generally with data for spinach, although stimulation in our pea chloroplast preparations is much more marked. This may reflect the high purity of pea chloroplasts [14] or their low endogenous levels of UTP.

Table 1. The effect of nucleotides on the incorporation of $^{35}SO_4^{2-}$ into sulphur-containing lipids by pea chloroplasts

	Total labelling (d.p.m. × 10^{-3})	
	SQDG	Lipid 'X'
No addition	15.3 ± 0.9	29.1 ± 2.0
ATP (2mM)	17.0 ± 0.7	30.4 ± 0.3
CTP (2mM)	14.4 ± 1.2	34.3 ± 1.6
GTP (2mM)	14.8 ± 1.5	33.1 ± 1.6
UTP (2mM)	32.0 ± 1.1	31.5 ± 1.7

Abbreviation: SQDG = sulphoquinovosyldiacylglycerol.

For discussion of lipid 'X', see text. Standard chloroplast incubation conditons were used (see Experimental). Results as means ± s.d. (n = 3).

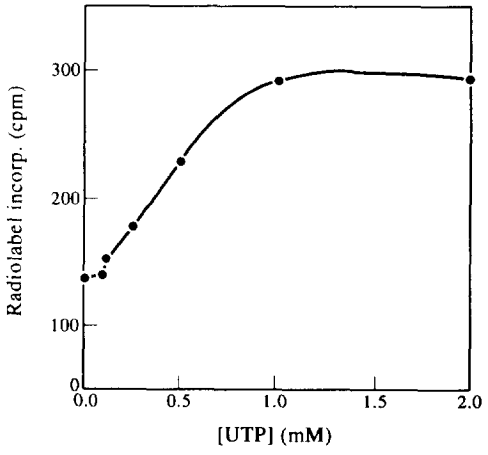

Fig. 2. Effect of UTP concentration on sulpholipid labelling from $^{35}SO_4^{2-}$ by isolated pea chloroplasts.

The rather selective effect of UTP, of the nucleotides tested, was not unexpected because of the demonstrated involvement of UDP-sulphoquinovose in sulpholipid synthesis [10, 11]. Optimal stimulation for UTP addition was found at 1 mM or above (Fig. 2). In the plant cell UTP, or derivatives thereof, may have to be transported into the chloroplast, although such a process is not defined [cf. 16]. This possibility is raised by experiments with spinach which located the UDP-sulphoquinose–diacylglycerolsulphoquinovosyltransferase on the envelope [11] with its active site inaccessible for exogenously added UDP-sulphoquinovose [3]. Such a result could be explained by the biosynthesis of UDP-sulphoquinovose in the stroma.

Because a significant amount of radioactivity was incorporated into chloroform-soluble components other than sulpholipid, we carried out some experiments to characterize these compounds. In agreement with data from Joyard *et al.* [15], a fast-moving band at the solvent

front, which contained *ca* 10% of the incorporated radioactivity under standard incubation conditions, had properties consistent with it being elemental sulphur.

The sulphoquinovosyldiacylglycerol band (co-migrating with leaf sulpholipid) and another lipid 'X', which migrated with an R_f of 0.4 in the standard chromatography system (see Experimental), were subjected to mild alkaline, acid and enzymatic hydrolysis. Both radiolabelled lipids were stable to acid hydrolysis (Fig. 3A) under conditions where complete hydrolysis of control sulphate esters (for example, glucose 6-sulphate) was observed. At the same time, sulpholipid standard, isolated from leaves, was shown to be stable. These results imply that the ^{35}S-labelling is contained in a sulphonate (or similarly stable) moiety for both lipids. By contrast, mild alkaline hydrolysis caused complete degradation of the sulphoquinovosyldiacylglycerol to fatty acids and sulphoquinovosylglycerol, while compound 'X' was stable (Fig. 3B). Deacylation of [^{35}S]sulphoquinovosyldiacylglycerol to yield the water-soluble [^{35}S]sulphoquinovosylglycerol in this experiment served to confirm further the nature of the sulpholipid labelled in our *in vitro* experiments as sulphoquinovosyldiacylglycerol. The lack of degradation of compound 'X' showed that acyl groups were not present and that the hydrophobic moieties occurred in an alkali-stable linkage, such as an ether bond.

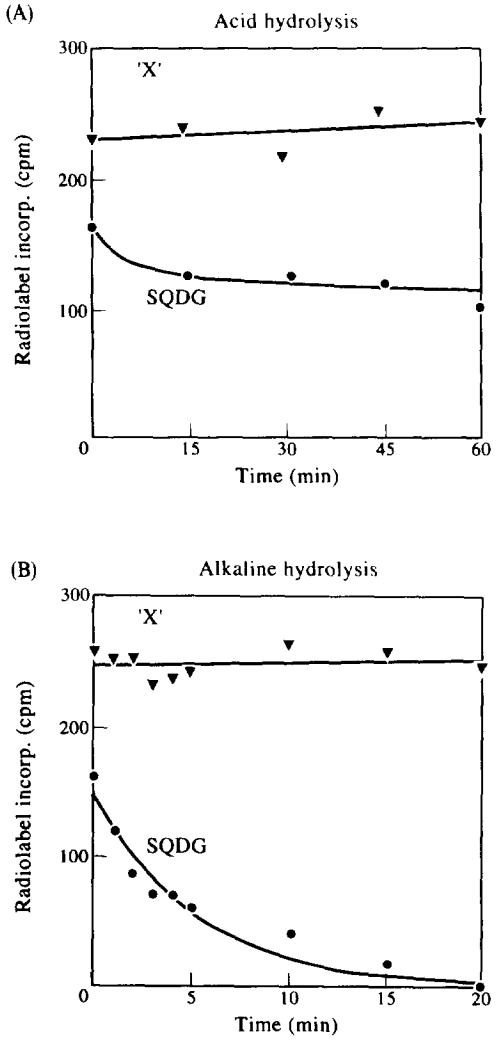

Fig. 3. Stability of sulphoquinovosyldiacylglycerol and lipid 'X' to acid (A) or alkaline (B) hydrolysis. Acid hydrolysis was carried out with 0.7 M H_2SO_4 in 90% MeOH at 90°. Alkaline hydrolysis was carried out with 0.3 N NaOH in 90% MeOH at 90°. Results are means of duplicates.

To confirm that the ^{35}S-labelled lipid was sulphoquinovosyldiacylglycerol, we used *Rhizopus arrhizus* lipase (Fig. 4). This enzyme has been shown to be specific for the *sn*-1 position of sulpholipid [17], as well as of other phospholipids and glycolipids. Exposure of the ^{35}S-sulpholipid to the *Rhizopus* lipase resulted in the progressive degradation of the lipid with time (Fig. 4). Analysis of the products showed the simultaneous appearance of a new ^{35}S-lipid with an R_f of 0.1, which was the same as that of 2-acylsulphoquinovosylglycerol [18]. Thus, one of the two major lipids labelled by [^{35}S]sulphate in pea chloroplasts was confirmed as sulphoquinovosyldiacylglycerol. On the other hand, compound 'X' was resistant to *Rhizopus* lipase attack (Fig. 4), as it had been to both acid and alkaline treatment (Fig. 3). This confirmed that it did not contain an acyl group in the required position. The properties that compound 'X' showed seemed to be rather similar to a ^{35}S-labelled compound in spinach chloroplasts [13], although details of the latter are lacking. We also detected a similar compound in lettuce chloroplast incubations. However, compound 'X' was not found in incubations using intact pea or lettuce leaves, although small amounts were detected following germination of *Vicia faba* or *Pisum sativum* seeds in [^{35}S]sulphate (data not shown). Compound 'X' showed similar staining with α-naphthol as sulphoquinovosyldiacylglycerol, suggesting that it could be a glycolipid. Alternatively, other sulphur-containing

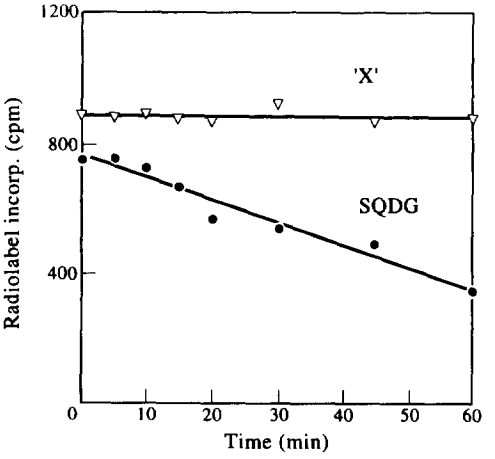

Fig. 4. Time-course of degradation of sulphoquinovosyldiacylglycerol or lipid 'X' when incubated with *Rhizopus arrhizus* lipase. For incubation conditions see Experimental.

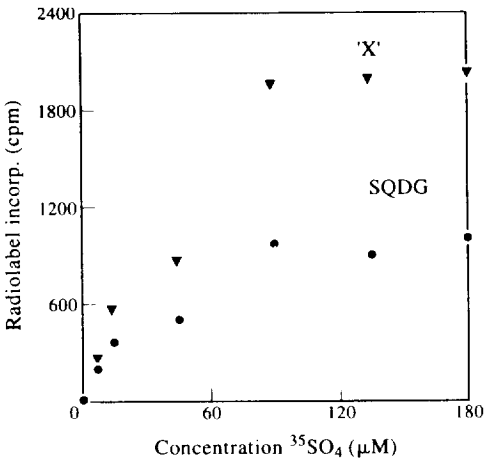

Fig. 5. Effect of sulphate concentration on labelling of lipids from $^{35}SO_4^{2-}$ by isolated pea chloroplasts. Triangles show labelling of lipid 'X', circles that of sulphoquinovosyldiacylglycerol, as analysed by TLC.

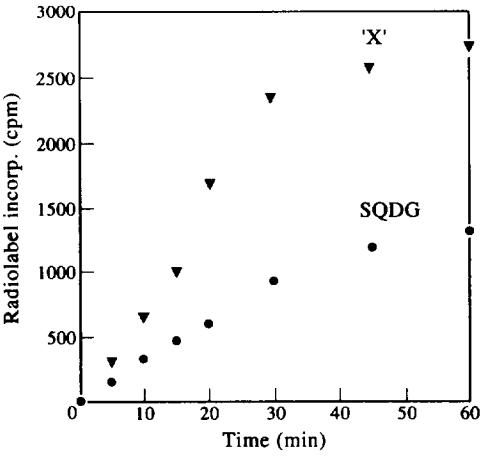

Fig. 6. Time-course of lipid labelling from $^{35}SO_4^{2-}$ by isolated pea chloroplasts. For key see legend of Fig. 5.

lipids, such as those from amino acids, have been detected in various organisms [19], some of which could have similar properties to compound 'X'. However, because it seemed that 'X' was only formed *in vitro* to any significant extent, we did not pursue its identification any further.

Incubation conditions for sulpholipid labelling were further optimized with regard to sulphate concentration (Fig. 5) and time (Fig. 6). Optimal labelling occurred at 90 μM SO_4^{2-} or above, as did that of compound 'X'. This was somewhat higher than the value of 50 μM obtained for spinach chloroplasts [13] but, as in that preparation, sulpholipid labelling represented a larger proportion of the total ^{35}S-labelled chloroform-soluble lipids (65%) at 10 μM SO_4^{2-} than at maximal labelling (32% total). Labelling of sulpholipid was linear for at least 30 min (Fig. 6), a time which was used as the standard incubation condition (see Experimental). The synthesis was also found to be light dependent, with only 18% of standard rates achieved in the dark (data not shown).

A major consideration in the use of chloroplasts in order to study sulpholipid synthesis is whether they are autonomous and, if so, whether they can catalyse rates comparable to values *in vivo*. For spinach chloroplasts, values of up to 0.7 nmol mg chlorophyll^{-1} hr^{-1} were reported [13]. From our experiments, we calculate that pea and lettuce chloroplasts can synthesize sulpholipid at rates of 1.3 and 0.8 nmol mg chlorophyll^{-1} hr^{-1}, respectively. These are good rates and enough to maintain the requirements of these species for sulpholipid synthesis. Clearly, pea chloroplasts contain all the necessary enzymes for sulpholipid formation and are autonomous in this respect. The convenience of preparation and good activity of these chloroplasts make them very suitable for further investigations of the sulpholipid biosynthetic pathway.

EXPERIMENTAL

Materials. Pea (*Pisum sativum* cv. Feltham First) seeds were obtained from Nutting (Leicester, U.K.). They were germinated at 20° and grown in a 12-hr light/dark cycle in universal compost for 10–12 days. Lettuce (*Lactuca sativa*) of the Cos-type were obtained from a local market. Chloroplasts were isolated from de-veined leaves by a Percoll-gradient method [14]. Intactness was estimated by microscopy as being *ca* 90% for pea and 85% for lettuce chloroplasts, respectively. Sulphoquinovosyldiacylglycerol was isolated from pea leaves as described previously [20].

Incubations. Standard incubation conditions were as follows: 100 μM [^{35}S]sulphate (39 MBq mmol^{-1}), 2 mM UTP, 330 mM sorbitol, 2 mM $MgCl_2$, 0.5 mM DTT, 2 mM NaH_2PO_4, 33 mM Tricine buffer, pH 7.9 and chloroplasts, *ca* 120 μg chlorophyll ml^{-1}. Incubations were at 25° for 30 min with illumination (650 μE m^{-2} sec^{-1}). Reactions were terminated by addition of 3 vols of $CHCl_3$–MeOH (1:2).

Lipid analysis. Lipids were extracted [21] and sepd by TLC on silica gel G plates (Merck) using $CHCl_3$–MeCOMe–MeOH–HOAc–H_2O (10:4:2:3:1). Lipids were visualized routinely using the non-destructive 1-anilino-8-naphthalenesulphonic acid (0.5% in MeOH) and viewing under UV light. Radioactively labelled bands were detected with a spark chamber autoradiograph and by autoradiography with X-ray film. Labelled lipid bands were removed and counted for radioactivity by liquid scintillation counting using quench-correction by the external standard channel ratios method.

Degradation studies. Acid hydrolysis of lipid bands was carried out in 0.7 M H_2SO_4 in 90% MeOH at 90° for various times in sealed tubes. Alkaline hydrolysis was carried out using 0.3 N NaOH in 90% MeOH at 90° for various times in sealed tubes [21]. Incubations with 1000 U *Rhizopus arrhizus* lipase (Boehringer) were carried out as described previously [17]. Degradation products were extracted [20] and analysed by TLC as described above.

Acknowledgements—We are grateful to the SERC and AFRC (now BBSRC) and to Zeneca Agrochemicals for financial support and to Dr A. B. Roy (Cardiff) for helpful discussions.

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