CATALASE STIMULATES LINOLEATE DESATURASE ACTIVITY IN MICROSOMES FROM DEVELOPING LINSEED COTYLEDONS

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1. Introduction

Evidence from in vivo [1–6] and in vitro [7,8] studies indicates that α-linolenate is synthesised in plants by sequential desaturation of oleate and linoleate. While oleate desaturases have been described in microsomal preparations from leaves, oil seeds and microorganisms [9–14], attempts to demonstrate linoleate desaturation in cell-free systems have been less successful [8,15]. The most clearly defined linoleate desaturase so far described is that from spinach chloroplasts which has 1,2-diacyl-3-galactosylglycerol as its substrate [7].

Cell free homogenates of soya bean (*Glycine max*) were shown to be capable of synthesising linolenate from [^{14}C]oleate of [^{14}C]linoleate; this activity was lost during subcellular fractionation [8,16]. Here, we report the stimulation by catalase (EC 1.11.1.6) of linoleate desaturation in microsomal preparations from developing linseed (*Linum usitatissimum*) cotyledons.

2. Experimental

Linseed plants (*Linum usitatissimum* cultivar Punjab) were grown in pumice peat in a heated glasshouse and watered daily with nutrient solution [17]. Sodium methoxide was prepared as in [12] and diazomethane was generated by the method in [18]. [1-^{14}C]Linoleic acid (56 Ci/mol) was from The Radiochemical Centre (Amersham), fatty acid methyl ester standards from Nu Check Prep (Elysian MN) and other biochemicals, including catalase (product no. C-30; 20 000 units/mg crystalline enzyme from beef liver suspended at 20 mg/ml in 0.1% aqueous thymol), were from Sigma (St Louis MO).

Developing linseed cotyledons were homogenised using a polytron apparatus in 50 mM Hepes/KOH buffer (pH 8.0) containing 0.33 M sorbitol, 0.2% defatted bovine serum albumin, 1 mM EDTA, 1 mM EGTA and 1 mM $MgCl_2$. Cell-free homogenates were obtained by filtration through two layers of miracloth. Microsomal preparations were derived from these crude homogenates either by the Mg^{2+} precipitation technique [19] or by centrifuging [14]. Results from these two methods were comparable.

Assays were performed at 25°C in screw-cap tubes (12 × 2 cm) shaken in a Warburg apparatus. Each reaction mixture (1.25 ml) contained enzyme equivalent to 0.1 or 0.2 g fresh wt of cotyledons, in the Hepes/KOH buffer above plus 0.25 mM CoA, 8 mM ATP, 2.7 mM NADH and 0.15 μCi NH_4[^{14}C]linoleate. Reactions were started by adding the labelled substrate and terminated after 45 min by adding 5 ml chloroform/methanol (1:2, v/v); 1.67 ml $CHCl_3$ and 1.75 ml water were then added and the tubes shaken. The chloroform phase was dried under N_2 and the lipids redissolved in 0.5 ml chloroform. A sample was transmethylated using sodium methoxide [12] and the fatty acid methyl esters separated by argentation TLC [20] or GLC [14] before scintillation counting of the linoleate and linolenate. Chloroform-soluble lipids were also separated by chromatography on silica gel G thin-layers using chloroform/methanol/15 M NH_3 (65:25:2, by vol.) as solvent. Individual lipid bands were scraped into tubes, transmethylated with sodium methoxide and the methyl esters separated for analysis as above.

The aqueous phase from the chloroform/methanol/water partition was saponified by adding 1.5 ml 40% KOH and heating to 80°C for 1 h, then 1.5 ml 7.2 M H_2SO_4 was added and the fatty acids extracted into

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5 ml light petroleum (b.p. 40–60°C). After evaporation of the solvent the fatty acids were methylated with diazomethane and the methyl esters separated and counted as described above.

The identity of the [^{14}C]linolenate product was confirmed by co-chromatography of the methyl ester with genuine methyl linolenate during GLC and argentation TLC and by the quantitative recovery of [^{14}C]-stearate following hydrogenation [21] of the [^{14}C]-linolenate.

3. Results

After 45 min incubation <10% of the [^{14}C]linoleate supplied to crude homogenates of linseed cotyledons remained as unesterified fatty acid. Considerable desaturation of glycerolipid label to [^{14}C]-linolenate occurred in freshly prepared homogenates without added catalase, but the activity declined when the homogenate was stored at 0°C prior to assay (table 1). Passage of the homogenate through a Sephadex G-25 column, the addition of polyvinyl-polypyrrolidone or ascorbate and the use of nitrogen purged buffers during isolation failed to stimulate or stabilise the desaturase activity (not shown). Microsomal preparations from homogenates without added catalase contained considerable label in glycerolipids, but such preparations desaturated [^{14}C]linoleate to only a very slight extent. Addition of catalase to the homogenisation buffer stimulated the crude homogenate activity only slightly, but the microsomal preparations from such homogenates when resuspended in catalase-containing buffer exhibited higher levels of desaturation than the crude homogenates and 10–15-times the activity of microsomes prepared in the absence of catalase (table 1). Linolenate synthesis by microsomal preparations also declined with storage time at 0–4°C, but was largely retained in samples stored at −18°C (table 1). Incorporation of

Table 1
The effects of catalase and storage conditions on [1-^{14}C]linoleate desaturation by cell free homogenates and microsomal preparations from linseed cotyledons

Experiment	Percentage desaturation of [1-^{14}C]linoleate incorporated into glycerolipids				
	Storage time at 0°C (min)				
	0	60	100	200	24 h
1. Crude homogenate					
− catalase	26.7	17.9		11.1	
+ catalase	31.0	23.0		14.5	
2. Crude homogenate					
+ catalase	19.2				
Microsomes					
+ catalase	24.2				
3. Crude homogenate					
+ catalase	11.2		6.2	6.3	
Microsomes					
− catalase	2.3		1.2	0.6	
+ catalase	35.5		15.2	11.5	
+ boiled catalase	2.4				
Microsomes					
+ catalase stored at					
4°C					7.0
−18°C					24.2

Homogenates were prepared as in section 2 with or without the addition of 2000 units catalase/ml. Microsomes prepared by Mg^{2+} precipitation were resuspended in the same homogenisation buffer. Assays contained the equivalent to 0.2 g fresh wt cotyledons

Table 2
The effects of cytoplasmic supernatant and catalase on desaturation by linseed microsomes

Prepared and assayed	
+ 2000 units catalase/ml	18.5
Prepared without catalase	
assayed without additions	5.4
+ 0.2 ml supernatant	8.5
+ 0.8 ml supernatant	9.3
+ 2000 units catalase/ml	17.0
+ 6000 units catalase/ml	19.0

Microsomes were prepared by differential centrifuging in the presence or absence of catalase. All assays contained microsomes equivalent to 0.1 g fresh wt cotyledons. The 105 000 × *g* supernatant from the preparation without catalase added back to assays 3 and 4 contained to equivalent of 0.1 g fresh wt cotyledons/ml

label into lipids was dependent on added CoA and ATP, while conversion of [^{14}C]linoleate to linolenate required in addition both NADH and oxygen (not shown).

In cellular fractions separated by differential centrifugation most of the desaturase activity was confined to the 105 000 × *g* pellet, while the 15 000 × *g* pellet also showed some activity (not shown). Addition of catalase to microsomes immediately prior to assay stimulated the desaturase to levels equivalent to those found in microsomes which had been protected by catalase during preparation, but addition of cytoplasmic supernatant had a far smaller effect on linolenate synthesis (table 2).

The compounds into which the [^{14}C]linoleate was incorporated by homogenates and microsomal preparations differed (table 2). However, the patterns of ^{14}C-incorporation were not influenced by the presence of catalase and did not change with time of storage prior to assay, indicating that the desaturase rather than acyl-CoA synthetase or acyl transferase was the labile enzyme in the assay system. Between 15–25% of the total label in the crude homogenates was present in a non-saponifiable water-soluble breakdown product which was almost completely absent from the microsomal preparations. In both the homogenates and the microsomal preparations 3-*sn*-phosphatidylcholine was the major labelled lipid, while other phospholipids and neutral lipids (largely diacylglycerol) contained smaller amounts of radioactivity. In addition, the microsomal preparations contained nearly 20% of the incorporated label as long chain fatty acids partitioning into the aqueous phase of the Bligh and Dyer extraction – presumably as CoA esters. In the presence of catalase linolenate was found in all the compounds formed (table 2).

4. Discussion

Whereas linolenate synthesis was shown from added [^{14}C]oleoyl- and [^{14}C]linoleoyl-CoA by soya bean homogenates [8], this activity was lost during cell fractionation [16]. Similar inactivation of several desaturases has been reported for microsomal preparations from rat liver and in those systems cytoplasmic

Table 3
Products of [1-^{14}C]linoleate incorporation by cell-free homogenates and microsomal preparations from linseed cotyledons

^{14}C-Labelled compound	Cell-free homogenate		Microsomal preparation	
	Activity incorp. (dpm × 10^{-3})	Percent desaturation	Activity incorp. (dpm × 10^{-3})	Percent desaturation
3-*sn*-Phosphatidylcholine	126.1	20.9	181.5	24.4
Other polar lipids	49.0	19.7	18.2	18.1
Neutral lipids	82.8	5.3	24.6	24.6
Saponifiable lipids of the aqueous methanol phase	8.8	6.2	53.1	14.2
Water soluble breakdown product	51.0	–	<10.0	–
Unmetabolised [^{14}C]linoleate	6.7	–	45.6	–

Cell-free homogenates and Mg^{2+}-precipitated microsomes equivalent to 0.2 g fresh wt cotyledons were prepared and assayed in the presence of 2000 units catalase/ml as in section 2

proteins or catalase preserve or restore the activity [22–24]. Here, we have shown that high rates of linoleate desaturation by linseed microsomal preparations are similarly dependent on catalase. Although it is assumed that catalase enhances desaturase activities by removing H_2O_2, the mechanism by which H_2O_2 inhibits desaturation is not known. Oxidative changes in the state of cytochrome b_5 or the desaturase enzyme have been suggested and a stimulation of lipid peroxidation by H_2O_2 may also be involved [23,24].

Microsomal preparations from several plants have been shown to contain oleate desaturase activity without supplementary catalase or other additions [10,12–14,16]. No results of the effect of catalase on plant oleate desaturases have been reported, but it is noteworthy that in [24] the stearate, α-linolenate and eicosatrienoate desaturases of rat liver microsomes responded differently to additions of catalase.

The appearance of [^{14}C]linolenoyl phosphatidylcholine as the major product of the desaturase in both crude homogenates and microsomal preparations (table 2) as well as the association of the desaturase with the 105 000 × g pellet after differential centrifugation suggest that in developing linseed cotyledons the endoplasmic reticulum is the major site of linoleate desaturation and not the chloroplasts as appears to be the case in leaves [3,7]. This suggestion is consistent with evidence from labelling studies in vivo (C. R. S., L. C. Campbell, unpublished) which indicates that diacylgalactosylglyceride is not involved in the synthesis of linolenate by developing linseed cotyledons. The endoplasmic reticulum is the probable site of the oleoyl-phosphatidylcholine desaturase in developing oilseeds [13,14] and the fact that linolenoylphosphatidylcholine is the major lipid species produced by the microsomal linoleate desaturase strongly suggests that both enzymes may have this phospholipid as their substrate. However, the appearance of [^{14}C]linolenate in the aqueous/methanol phase of the extracted assay mixtures indicates that further work is required to clarify the role of acyl-CoA species [25].

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