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Solubilization and partial purification of squalene synthase from daffodil microsomal membranes

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A squalene synthase was solubilized from daffodil (*Narcissus pseudonarcissus L.*) microsomes with CHAPS, a zwitterionic non-denaturating detergent. By successive chromatography on DEAE Sephacel and APP Sepharose a fraction enriched in this enzyme (21-fold) was prepared.

Narcissus pseudonarcissus; Squalene synthase; CHAPS; Microsome

1. INTRODUCTION

Squalene is the first specific compound of the sterol branch of the isoprenoid pathway; its synthesis involves the 1'-2-3 condensation of two farnesyl diphosphates (FPP) to form pre-squalene diphosphate. The resulting diphosphate is reduced to squalene by NADPH [1]. The conversion of FPP to squalene is a two-step process which requires only one enzyme, squalene synthase [2].

A putative branch point for isoprenoid metabolism is situated at FPP. FPP can be diverted to sterol biosynthesis by squalene synthase or be utilized for sesquiterpenoid biosynthesis by specific enzymes known as sesquiterpene cyclases [3]. While squalene synthase is an intrinsic microsomal protein [4], it has been shown that sesquiterpene cyclases were weakly bound to endoplasmic reticulum membranes [5]. The two enzymes are on endoplasmic reticulum membranes and utilize FPP as a common substrate. A knowledge of the reaction mechanisms would be valuable for understanding of isoprenoid regulation in plants.

The major problem in studying of squalene synthase has been the difficulty of solubilizing this enzyme. Up to now, only squalene synthase from yeast has been purified to homogeneity [2]. In this paper we present the first solubilization of squalene synthase from higher plants and its partial purification.

Abbreviations: FPP, farnesyl diphosphate; APP, aminophenethyl diphosphate; TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; CHAPS, (3-[(3 cholamidopropyl)-dimethylammonio]-1-propanesulfonate).

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2. MATERIALS AND METHODS

Daffodil flowers were purchased from the local markets.

2.1. *Isolation procedure*

The coronae were homogenized in isolation medium consisting of phosphate buffer 67 mM, pH 7.5, 0.74 M sucrose, 5 mM $MgCl_2$, 0.2% (w/v) polyvinylpyrrolidone (PVP 10; Sigma) using a razor-blade equipped food mixer [6]. The homogenate was filtered through three layers of nylon cloth (40 μm mesh) and centrifuged 5 min at 1400 × *g*, 20 min at 16 500 × *g* and 90 min at 100 000 × *g*. The resulting pellet was washed in 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM dithioerythritol, and centrifuged at 100 000 × *g* for 90 min. The microsomal pellet obtained was resuspended in the same buffer and could be stored for several months at −20°C without loss of activity. All the above steps and all subsequent operations were carried out at 4°C.

2.2. *Enzyme solubilization*

CHAPS was used to solubilize membranes. It was synthesized and purified according to [7]. To the microsomal fraction was added a CHAPS stock aqueous solution to a final concentration of 5 mM CHAPS. The mixture was stirred gently with a magnetic stirrer for 45 min at 40°C and then centrifuged at 120 000 × *g* for 90 min. Glycerol (10%, w/v) was added to the obtained supernatant containing squalene synthase activity.

2.3. *Enzyme purification*

The supernatant was chromatographed on a DEAE Sephacel column (2 × 12 cm) which was previously equilibrated with buffer A (50 mM Tris-HCl buffer, pH 7.4, 2 mM dithioerythritol, glycerol 10%, (w/v) and 5 mM CHAPS. For removal of unadsorbed proteins the column was washed with buffer A. Bound proteins were eluted with a NaCl step gradient (36 ml each of 0.1, 0.2, 0.3 M in buffer A). Fractions (3 ml) were collected, and assayed for squalene synthase activity. The fractions containing squalene synthase activity were pooled and dialyzed against buffer A overnight. After addition of $MgCl_2$ (1 mM), the enzyme fraction was submitted to affinity chromatography on an aminophenethyl-diphosphate Sepharose column prepared according to [8]. The column (1.5 × 5 cm) was equilibrated with buffer B (buffer A + 1 mM $MgCl_2$), and elution was carried out by a step gradient of 0, 0.1, 0.3, 0.5, 0.8 M NaCl in buffer A. Fractions (5 ml) were collected and concentrated after dialysis against 20% polyethylene glycol 20 000 in buffer A.

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2.4. *Assay procedure*

Assays for squalene biosynthesis were carried out at 28°C for 1 h. The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.4, 10 mM $MgCl_2$, 1 mM NADPH, [1-^{3}H]FPP (77 MBq/mmol) and enzyme fractions in a final volume of 1 ml. The reaction was started with addition of FPP, and was stopped with 500 μl of 40% KOH and 500 μl of 95% ethanol. Squalene was added as carrier and each reaction mixture was saponified for 30 min at 55°C. The unsaponified lipids are extracted three times from the cooled samples with 1.5 ml of light petroleum ether. The pooled petroleum ether fractions were evaporated to dryness and 500 μl of chloroform was added. An aliquot was counted by liquid scintillation spectrometry.

TLC was carried out on silica gel plates from Merck with the solvent system petroleum ether/ether/acetone (40:10:5, v/v/v). In this system, squalene moves in the solvent front and was visualized with iodine vapor.

Radioactive zones were localized using a Berthold TC scanner.

Boiled enzyme was used as a control to confirm the enzyme activity of squalene synthase.

Protein was determined by a modified Lowry method [11].

2.5. *Substrate*

[1-^{3}H]Farnesyl diphosphate was prepared from *trans-trans* farnesol by the method of Davisson et al. [12]. FPP was purified by chromatography on a Kieselegel (Merck) column (20 × 1.5 cm) eluted with *n*-propanol/ammonium hydroxide/water (60:30:10, v/v/v). Fractions of 1 ml were collected and tested on silica gel 60 plates developed with the above solvent mixture. The spots were detected after drying the plates and spraying of Zindzadge reagent [13]. Activity of FPP was dissolved in Tris-HCl 50 mM, pH 7.6 and stored at −20°C.

2.6. *Analysis of polypeptides*

Separation of proteins by SDS-PAGE electrophoresis was done essentially according to [9]. Silver staining of the gels was carried out by the method of Henkershoven and Dernich [10].

3. RESULTS AND DISCUSSION

3.1. *Solubilization of squalene synthase*

The amounts of protein recovered in the supernatant after centrifugation were enhanced with increasing CHAPS concentrations in the solubilization mixture as shown in Fig. 1. Analysis of squalene synthase activity in the fractions shows an increase in the supernatant above 5 mM CHAPS. At 4–6 mM CHAPS the activity was almost equal whereas above 6 mM CHAPS a progressive decrease of activity was observed, and a partial inhibition of the enzyme occurred at higher concentration of CHAPS.

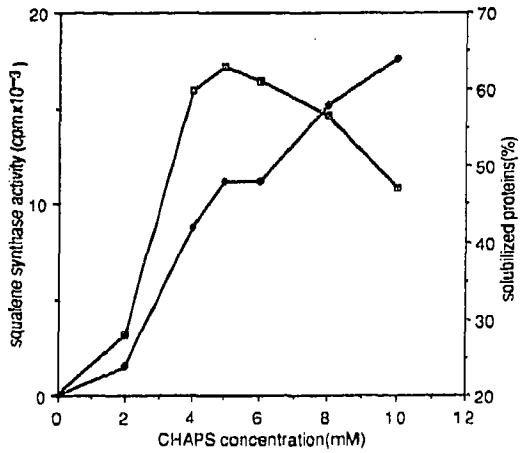

Fig. 1. Effect of CHAPS concentration on the solubility of protein from the microsomal pellet (■) and on squalene synthase activity (□).

We have attempted the solubilization of squalene synthase with a mixture of the detergents CHAPS and lubrol PX. Slightly better results were obtained with 5 mM CHAPS and 1 mM Lubrol PX (not shown). However, Lubrol PX has been discarded because the addition of this detergent makes large micelles containing different proteins and the subsequent chromatographic methods separate class of micelles rather than individual proteins.

Up to now solubilization of squalene synthase has only been achieved on yeast using a mixture of *N*-octyl-β-D-glucopyranoside (OGP) and Lubrol PX [2,4] and can with deoxycholate [14], but it appeared that the enzyme solubilized by deoxycholate was unstable.

3.2. *Stability of activity*

Squalene synthase microsomal activity is stable and can be stored for months at −20°C. However, when solubilized, this enzyme becomes labile (Table I). At 4°C, squalene synthase was stabilized in 10% glycerol (v/v). Methanol (10% v/v) and sucrose (10% w/v) were good stabilizing agents for the solubilized activity from yeast [4] but not for activity from daffodil.

Since Agnew and Popjak [14] observed that enzyme activity was increased by phospholipids, we tested the effect of phosphatidylcholine on solubilized enzyme. The addition of liposomes of phosphatidylcholine (2 mg/ml) to the incubation mixture did not activate squalene synthase immediately after the solubilization of microsomes. But after the first step of purification (chromatography on DEAE Sephacel), squalene synthase was stimulated by the presence of phosphatidylcholine (Table II). One can suggest that lipid compo-

Table I

Stability of squalene synthase activity after solubilization of microsomal pellet

Addition	Temperature (°C)	Days	Initital activity (%)
none	4	1	81
none	4	3	34
10% glycerol (w/v)	4	1	98
10% glycerol (w/v)	4	3	92
10% methanol (w/v)	4	1	53
10% methanol (w/v)	4	3	18
10% methanol (w/v) + 10% sucrose (w/v)	4	1	84
10% methanol (w/v) + 10% sucrose (w/v)	4	3	38

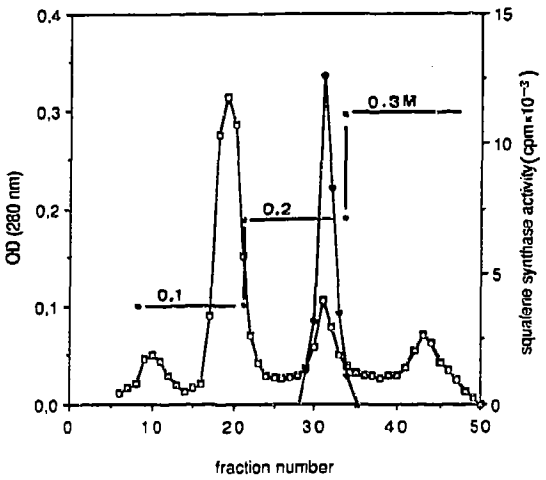

Fig. 2. Chromatography on DEAE Sephacel column of squalene synthase extracted from microsomal pellet with 5 mM CHAPS. Elution was carried out by an NaCl step gradient (36 ml each of 0.1, 0.2, 0.3 M). Protein (□) was measured by absorbance at 280 nm. Squalene synthase (◆) was measured as described in section 2.

nents present in solubilized extract were eliminated after the ion exchange chromatography and the added PC liposomes recreate the lipophilic environment necessary for good enzymatic activity.

3.3. *Partial purification of squalene synthase*

As a first step towards purification of the squalene synthase, DEAE Sephacel chromatography was used. Solubilized proteins in 5 mM CHAPS were loaded on the column. After elution of unbounded proteins, a NaCl step gradient (36 ml each of 0.1, 0.2, 0.3 M in buffer A) was applied to the column (Fig. 2). Squalene synthase was eluted with 0.2 M NaCl. The active fractions were pooled and dialysed overnight against buffer A. Since affinity chromatography was efficient in the purification of other enzymes involved in prenyl-lipid metabolism [8], the fraction containing squalene synthase was chromatographed on a Sepharose aminophenetyl-diphosphate column in the presence of CHAPS. A NaCl step gradient was used and squalene synthase activity was recovered with 0.5 M NaCl. As shown in Table III, in which are summarized the steps of purification, this procedure resulted in a 21-fold purification of squalene synthase. Squalene synthase has been purified to homogeneity from yeast [2], but because the low amount of squalene synthase in plant tissue, the ultimate method used for yeast (isoelectric focusing) was not possible with the enzyme from daffodil.

Table II

Stimulation by phosphatidylcholine (PC) of squalene synthase activity after chromatography on DEAE Sephacel

PC/protein (w/w)	Initial activity (%)
-	100
0.4	148
0.86	155
1.72	52

Table III

Partial purification of squalene synthase

Fraction	Protein (mg)	Specific activity (nmol FPP incorporated·h^{-1}·mg^{-1} protein)	Purification factor
Solubilized microsomes	52.6	8.12	1
DEAE-Sephacel	4.4	38.89	4.79
Sepharose-APP	0.076	173.76	21.40

SDS electrophoresis carried out after affinity chromatography revealed the presence of 2 major and 4 minor polypeptides (not shown). Unfortunately the identification of squalene synthase was not possible on the electrophoresis gels. This is mainly due to the low quantity of this enzyme recovered from daffodil coronae. Nevertheless this is the first successful attempt at purification of the squalene synthase from higher plants reported and it is reasonable to think that this determination could be obtained using antibodies raised against yeast squalene synthase enzyme which has been purified to homogeneity [2]. These experiments are in progress in our laboratory.

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