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PROPERTIES AND PARTIAL PURIFICATION OF SQUALENE SYNTHASE FROM CULTURED CELLS OF DANDELION

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Key Word Index—*Taraxacum officinale*; Compositae; squalene synthase; biosynthesis; triterpenoids.

Abstract—Squalene synthase (SQS) activity was detected in the microsomal fractions of suspension-cultured cells of dandelion (*Taraxacum officinale*), which produce cycloartane (involved in phytosterol biosynthesis) and other classes (e.g. oleanane and ursane) of triterpenoids. High SQS activity was detected at the late logarithmic growth stage of the culture. The activity was solubilized from the microsomes by the use of detergents (glycodeoxycholate and octylthioglucopyranoside), and chromatographed on triple-joint-columns composed of cation-exchange, anion-exchange and hydroxylapatite resins. Active fractions were further subjected to chromatography on hydroxylapatite and anion-exchange reins, followed by fast protein liquid chromatography (FPLC) on a gel-filtration column. About a 50-fold purification compared with the microsomal activity was achieved at the hydroxylapatite stage, and, although the recovery was poor, the activity was retained in the final FPLC fractions.

INTRODUCTION

Squalene synthase (SQS; EC 2.5.1.21) catalyses the formation of squalene from two molecules of farnesyl diphosphate (FPP) in two sequential steps: in the first step an intermediate with a cyclopropane ring, presqualene diphosphate, is produced, and the subsequent reductive rearrangement gives squalene. Squalene is the first C_{30} intermediate in the biosynthesis of sterols and, especially in higher plant cells, numerous cyclic triterpenes. SQS is an intrinsic endoplasmic reticulum membrane enzyme, and its purification is difficult. It was first solubilized and purified to homogeneity from yeast [1], and then a truncated, soluble active fragment was purified and characterized from rat [2]. SQS genes from yeast [3, 4], rat [5], human [6] and mouse [7] have been cloned. From higher plant sources, however, only partial purification from cultured tobacco cells [8] and daffodil coronae [9] has been reported, and no complete purification has been accomplished. Very recently, the molecular cloning of SQS cDNA from *Arabidopsis thaliana* was reported [10].

Dandelion (*Taraxacum officinale* Weber) accumulates unique triterpenoids, especially in its latex. Tissue culture lines of dandelion and their constituents have been reported [11] and regeneration of complete plants from these cultures has been achieved [12–14]. Previous incorporation studies using [2-^{14}C]mevalonic acid with the cultured cells and regenerated and wild plant organs of dandelion have revealed different biosynthetic capabilities between the cells and organs [15]. Undifferentiated callus and suspension-cultured cells biosynthesize mainly oleanane- and ursane-type triterpene acids and also cycloartenol, whereas organogenesis favours the synthesis of taraxastreol which is primarily, if not exclusively, accumulated in latex. Cultured dandelion cells and tissues thus offer good systems in which to study changes of triterpenoid metabolism and the molecular mechanisms underlying them during organ differentiation.

We report the detection, characterization and partial purification of SQS from cultured dandelion cells.

RESULTS AND DISCUSSION

Detection and characteristics of SQS activity in microsomes

As expected, the microsomal fractions prepared by ultracentrifugation (160 000 *g*) from suspension-cultured dandelion cells displayed SQS activity, which was demonstrated by radioactive squalene formation (shown by reverse-phase TLC) from [1–^{3}H]FPP. Because partially purified SQS from dandelion cells was very labile and was obtained only in a low yield (see below), some of the characteristics of the enzyme were determined in the washed microsomes. As shown in Table 1 (Experiment 1), NADPH or NADH was

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required as a cosubstrate and Mg^{2+} as a cofactor. In the absence of NADPH or NADH, radioactive products other than squalene were present on the TLC plates. Presqualene diphosphate was one of the expected products but this was not confirmed. The requirement for Mg^{2+} was very strict, and with other divalent ions (Mn^{2+} and Ca^{2+}) no radioactive product was formed. Ca^{2+} inhibited the activity when present together with Mg^{2+} (Table 1, Experiment 2); this might be potentially important in post-translational regulation of SQS activity in the cells. The optimum pH for the activity was around 7.5 (data not shown).

SQS activity changes in the culture cycle

Over the 3-week culture period, the cells proliferated to *ca* eightfold in *ca* 2 weeks, and then entered the stationary phase (Fig. 1). The SQS activity in the early stages of growth was somewhat variable, but usually increased between 5 and 10 days of culture. The highest activity was attained in the late logarithmic growth phase, and the activity rapidly decreased in the stationary phase.

The high SQS activity in rapidly-growing cells has been reported in tobacco suspension cultures [16], in which the activity was correlated with sterol biosynthesis. In dandelion cells also, the SQS activity in proliferating cells is assumed to be involved primarily in phytosterol synthesis. In contrast, in our previous feeding experiments with dandelion callus [15], [2-^{14}C]mevalonate was roughly equally incorporated into phytosterols, oleanolic/ursolic acids, and triterpenols (among which α- and β-amyrins were predominantly labelled), indicating that dandelion cells possess strong biosynthetic activities toward triterpene skeletons, as distinct from sterols. Whether these triterpenes are synthesized from a common squalene pool which is produced by a single (or a small number of) SQS or whether separately-regulated SQS isozymes are involved in the synthesis of different classes of triterpenes (cf. 'metabolic channels' hypothesis [17]) needs to be examined.

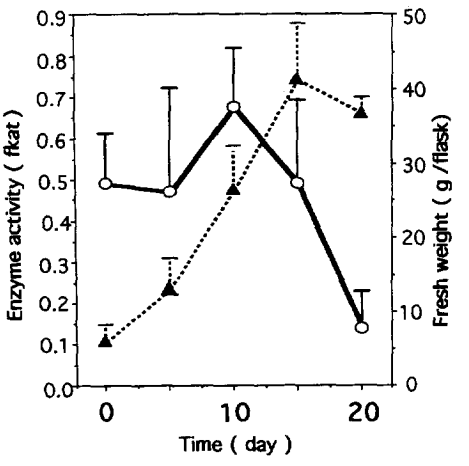

Fig. 1. Growth of suspension-cultured dandelion cells (▲) and squalene synthase activity (○) in their microsomes. Data are average value±standard deviation from three independent experiments.

Table 1. Squalene synthase activity in washed microsome fractions from suspension-cultured dandelion cells in the presence or absence of cosubstrates (hybride-donor) and divalent ion cofactors

Experiment number	Assay condition	Relative activity (%)
1	Complete system*	100†
	−NADPH	3
	−NADPH + 5 mM NADH	175
	$-MgCl_2$	1
	$-MgCl_2$ + 25 mg $MnCl_2$	2
	$-MgCl_2$ + 25 mM $CaCl_2$	1
2	Complete system*	100†
	+5 mM $CaCl_2$	97
	+10 mM $CaCl_2$	4
	+20 mM $CaCl_2$	3
	+50 mM $CaCl_2$	3

*The complete system consisted of 4.4 pmol [1-^{3}H]FPP (3.7 kBq) and microsomal protein (1.3 mg ml^{-1} for Exp. 1 and 0.48 mg ml^{-1} for Exp. 2) in 50 μl 50 mM K-Pi buffer containing 5 mM NADPH, 25 mM $MgCl_2$ and 5 mM 2-mercaptoethanol.

†Actual enzyme activity was 3.1 fkat mg^{-1} protein in Experiment 1 and 2.3 fkat mg^{-1} protein in Experiment 2, respectively.

Solubilization and partial purification of dandelion squalene synthase

Microsomes were prepared from 10- to 15-day-old suspension-cultured cells. After treatment with the solubilization buffer containing glycodeoxycholate (GDC) and octylthioglucopyranoside (OTGP), SQS was recovered in the 160 000 *g* supernatant fraction. The composition of the detergents was the same as that used for solubilization of tobacco SQS [8]. The following chromatography steps were carried out in a short period (within 48 hr) because the SQS activity was unstable. Figure 2A–D shows the elution profiles of the activity on successive column chromatographies, and Table 2 summarizes the results of each step of purification. The triple-joint-columns (composed of three columns packed with SP-Sepharose, Q-Sepharose and Bio-Gel HTP) were designed for the preliminary removal of unnecessary proteins by the top two columns and a rough separation of proteins with the hydroxylapatite Bio-Gel HTP (Fig. 2A). The subsequent Bio-Gel HTP (Fig. 2B) column effectively concentrated the activity. After this stage, a 55-fold purification compared with the microsomal activity was achieved (Table 2), which is comparable with the purification level (59-fold) of SQS from tobacco cells using FPLC [8] and better than the 20-fold purification from daffodil [9]. Although a sharp activity peak was observed in the elution profile

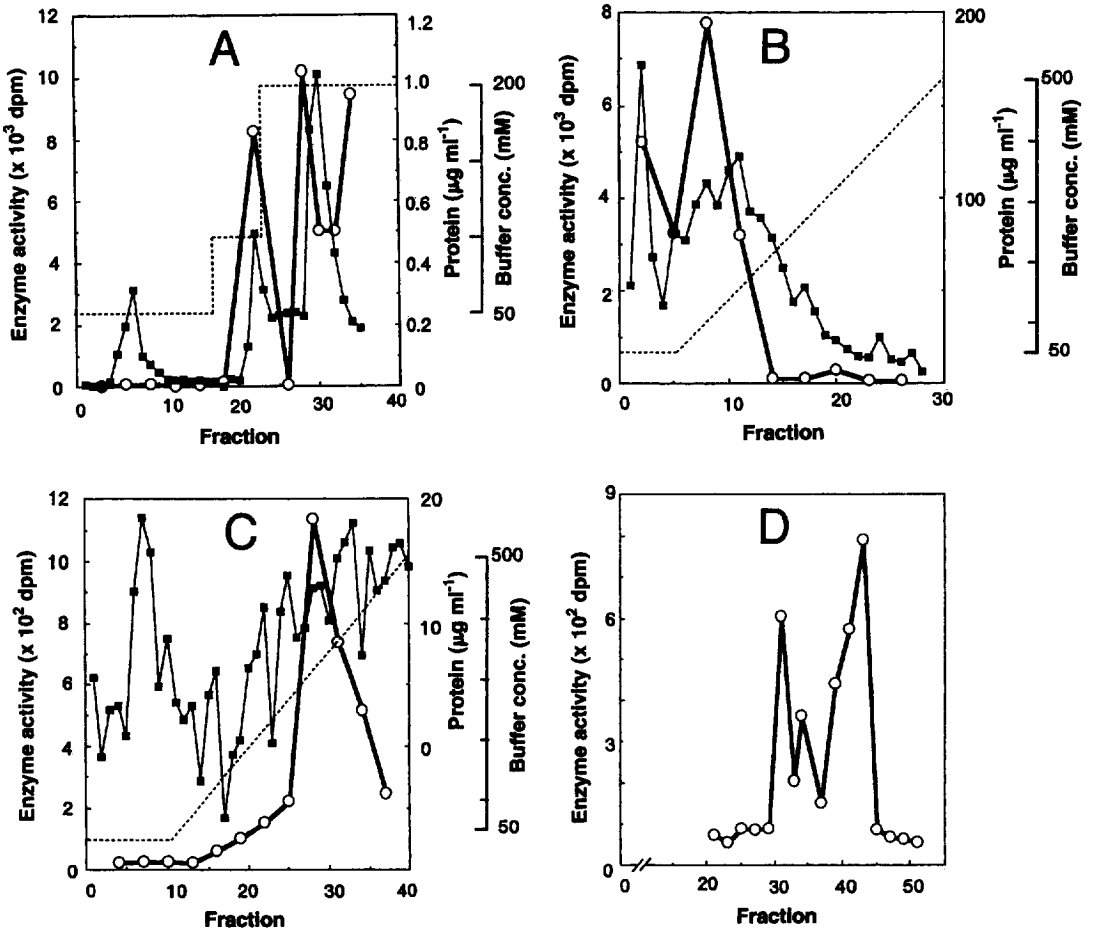

Fig. 2. Elution profiles of squalene synthase activity (○) and protein (■) on column chromatography of solubilized microsomes from suspension-cultured dandelion cells. Dashed lines represent buffer concentrations. A, Triple-joint-columns (see the footnote to table 2); B, Bio-Gel HTP; C, Q-Sepharose; D, FPLC Superdex.

from the anion exchange Q-Sepharose chromatography (Fig. 2C), the recovery of 0.32% at this stage (Table 2) is much lower than the 21% final recovery from tobacco [8], presumably owing to the inactivation of the enzyme. In the final step of the attempted purification, a FPLC Superdex 200 HR gel-filtration column was employed, and at least two activity peaks were observed (Fig. 2D). Unfortunately, the amount of the protein eluted from the FPLC column was too small to evaluate the specific enzyme activity (Table 2). Also, Q-Sepharose and FPLC fractions gave no clear band on SDS–PAGE even by silver-staining.

Table 2. Purification of squalene synthase from suspension-cultured dandelion cells

Preparation	Total protein (mg)	Enzyme activity (fkat)	Specific activity (fkat mg^{-1})	Recovery (%)	Purification factor (fold)
Microsome	95	1976	21	100	1
Solubilized microsome	35	1599	45	81	2.2
Triple-joint-columns*	3.6	1947	535	99	26
Bio-Gel HTP	0.43	495	1151	25	55
Q-Sepharose	0.012	6.3	527	0.32	25
FPLC Fraction 31	ND	0.8	ND	0.043	ND
Fraction 43	ND	1.1	ND	0.056	ND

*System consisted of SP-Sepharose, Q-Sepharose and Bio-Gel HTP (for details, see Experimental).
ND, not detected.

Concluding remarks

Further purification of SQS from dandelion cells requires more effective and stable solubilization methods as also fewer chromatography steps. Our recent reinvestigation of the solubilization of SQS activity from dandelion microsomal membranes revealed more effective detergents (e.g. CHAPS, Tween 20) than OTGP plus GDC (unpublished results), although the stability after solubilization has not yet been determined. However, even using improved methods, the complete purification of SQS appears difficult. The cloning of SQS cDNA from *A. thaliana* [10] and successful heterologous expression of yeast and human SQS genes [18, 19] would make the molecular biological approach to dandelion SQS purification feasible. We are currently pursuing the possible detection of the SQS transcripts by Northern analysis and also by the reverse-transcription polymerase chain reaction.

SQS activities from higher plant cells are either induced (*Tabernaemontana divaricata* [20]) or suppressed (tobacco [21, 22], potato [23]) by treatment with elicitor: the suppression parallels the induction of the synthesis of sesquiterpenoid phytoalexins. As dandelion has also been shown to produce a fungitoxic sesquiterpenoid (lettucenin A) in response to heavy-metal stress [24], the regulation of FPP metabolism in response to environmental stimuli, possibly involving SQS activity, must be important.

EXPERIMENTAL

Plant materials. Callus cultures of *T. officinale* were derived from seedlings as previously described [13], and maintained at 25° in the dark on Murashige–Skoog's agar (7%) medium containing 1 ppm 2,4-D, 0.1 ppm kinetin and 7% coconut water with a 3-week subculture cycle. Suspension cultures were initiated by inoculating 5–10 g of 15-day-old callus cells into freshly prepared liquid media (250 ml) of the same composition except for the omission of agar.

Preparation of microsomes. All the procedures were carried out at 0–4°. Cells freshly collected by suction filtration were mixed with the same vol. (v/w) of 0.1 M K-Pi buffer (pH 7.0) containing 5 mM $MgCl_2$, 50 mM sucrose and 10 mM 2-mercaptoethanol (extraction buffer) and 3% Polyclar SB 100, and homogenized in a Waring blender. The homogenate was filtered through eight layers of cheesecloth, and the filtrate was centrifuged at 10 000 ***g*** for 15 min. The supernatant soln was then centrifuged at 160 000 ***g*** for 60 min. The pellet was recovered (and designated crude microsomes), resuspended in the extraction buffer, and centrifuged as before to yield washed microsomes. The crude microsomes were flushed with a N_2 stream and stored at −20°.

Enzyme assay. (a) *Microsomal SQS assay.* Crude or washed microsomes and [1^{-3}H]FPP (New England Nuclear; 3.7 kBq, 4.4 pmol) were made up to a total vol. of 50 μl (0.5–1.5 mg protein ml^{-1}) in a buffer containing 25 mM $MgCl_2$, 25 mM 2-mercaptoethanol and 5 mM NADPH. 50 mM K-Pi (pH 7.0) was the basal buffer used in the experiments on cofactor and divalent ion effects. For the determination of pH-dependency, 500 mM Tris-MES (pH 6.0–8.5) was employed. (b) *SQS assay in solubilized microsomes and in chromatography fractions.* The reaction mixture contained appropriate vols of the test soln and other ingredients as above. After incubation at 35° for 30–60 min, 10 μl of the reaction mixture was separated and diluted with 10 μl 4% ethanolic soln of squalene (as a carrier), and subjected to TLC on RP-$18F_{254S}$ (Merck; developed with 95% Me_2CO). The squalene spot was visualized with I_2 vapour, scraped from the plate and placed into a vial, and mixed with scintillation cocktail (ACS II). The radioactivity in the spot was then measured in a liquid scintillation counter.

Solubilization of SQS activity from the microsomes. Microsomal pellets were suspended in 50 mM K-Pi buffer (pH 7.0) containing 5 mM 2-mercaptoethanol and 5 mM EGTA, and centrifuged at 160 000 ***g*** for 1 hr at 4°. The pellets (EGTA-treated microsomes) were resuspended in 50 mM K-Pi buffer (pH 7.0) containing 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 10% (v/v) glycerol, 9 mM OTGP and 1.1 mM GDC, and left to stand at 0° for 30 min. The supernatant fr. after the centrifugation (160 000 g, 1 hr, 4°) was designated the solubilized enzyme.

Column chromatography. The buffer used throughout this section was K-Pi (pH 7.0) containing 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 10% (v/v) glycerol, 9 mM OTGP and 1.1 mM GDC. All the procedures were carried out at 0–4° without interruption. (a) *Triple-joint-columns.* Three columns (SP-Sepharose (Pharmacia; 1.5 × 9.0 cm), Q-Sepharose (Pharmacia; 1.5 × 3.0 cm), Bio-Gel HTP (Bio-Rad; 1.5 × 3.0 cm)) were equilibrated with 50 mM buffer and connected in this order. Solubilized microsomal proteins from 1 kg (fr. wt) cells in 50 mM buffer (25 ml) were applied to the joint columns, and the columns eluted with the same buffer (15 ml). The top SP-Sepharose column was then removed, and a further 50 ml of the same buffer was applied to the remaining two columns. Finally, after the removal of Q-Sepharose column, the Bio-Gel column was eluted with a stepwise gradient using 100 mM and 200 mM buffers, then the SQS-active fractions in the 200 mM buffer (7 ml) were applied to a Sephadex G-25 column to replace the solvent with the 50 mM buffer. (b) *Bio-Gel HTP.* The SQS-containing soln was applied onto a Bio-Gel column (1.0 × 3.0 cm) equilibrated with the 50 mM buffer, and eluted with a linear gradient (total 40 ml) of 50–500 mM buffer (0.3 ml min^{-1}; 1-ml frs). The active fractions (9-13; 5 ml) were subjected to solvent substitution to 50 mM buffer by Sephadex G-25 CC as above. (c) *Q-Sepharose.* This column (1.0 × 3.0 cm) was also equilibrated with 50 mM buffer and, after loading of the sample, it was washed with a linear gradient of 50–500 mM

buffer (total 40 ml; 0.5 ml min^{-1}; 1-ml frs). (d) *Superdex 200HR.* A FPLC system (Pharmacia) connected to a Superdex 200HR column (1.0 × 30 cm) equilibrated with 50 mM buffer containing 150 mM NaCl in addition to the above constituents was used. After the application of the SQS-active fraction (fr. 28; 0.5 ml) from the Q-Sepharose column, the column was eluted with the same buffer as that used for the equilibration (0.25 ml min^{-1}; 0.5 ml frs).

Protein determination. A protein assay kit (Bio-Rad) was used for usually 0.1 ml samples according to the protocol of the supplier. Bovine serum albumin was used as the calibration standard.

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