Solubilization, Partial Purification, and Immunodetection of Squalene Synthetase from Tobacco Cell Suspension Cultures

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ABSTRACT

Squalene synthetase, an integral membrane protein and the first committed enzyme for sterol biosynthesis, was solubilized and partially purified from tobacco (Nicotiana tabacum) cell suspension cultures. Tobacco microsomes were prepared and the enzyme was solubilized from the lipid bilayer using a two-step procedure. Microsomes were initially treated with concentrations of octyl-β-D-thioglucopyranoside and glycodeloxocholate below their critical micelle concentration, 4.5 and 1.1 millimolar, respectively, to remove loosely associated proteins. Complete solubilization of the squalene synthetase enzyme activity was achieved after a second treatment at detergent concentrations above or at their critical micelle concentration, 18 and 2.2 millimolar, respectively. The detergent-solubilized enzyme was further purified by a combination of ultrafiltration, gel permeation, and Fast Protein Liquid Chromatography anion exchange. A 60-fold purification and 20% recovery of the enzyme activity was achieved. The partially purified squalene synthetase protein was used to generate polyclonal antibodies from mice that efficiently inhibited synthetase activity in an in vitro assay. The apparent molecular mass of the squalene synthetase protein as determined by immunoblot analysis of the partially purified squalene synthetase protein separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 47 kilodaltons. The partially purified squalene synthetase activity was optimal at pH 6.0, exhibited a $K_m$ for farnesyl diphosphate of 9.5 micromolar, and preferred NADPH as a reductant rather than NADH.

Squalene synthetase is an integral membrane protein of the endoplasmic reticulum that catalyses the condensation of two FPP molecules to form squalene, an isoprenoid intermediate dedicated solely to sterol biosynthesis (11). Squalene synthetase also resides at a branch point in the isoprenoid biosynthetic pathway, representing a potentially important control point for sterol biosynthesis and other branch pathways competing for the available FPP (5). However, examples of squalene synthetase regulation are limited. Faust et al. (4) and James and Kandutsch (8) reported a suppression of squalene synthetase enzyme activity in mammalian cells grown in sterol-supplemented media, but concluded such regulation was of secondary importance relative to the control provided by 3-hydroxy-3-methylglutaryl coenzyme A reductase. The regulation of squalene synthetase enzyme activity in tobacco cell suspension cultures has also been described (18). Upon addition of fungal elicitors to tobacco cell cultures, sterol accumulation and biosynthesis cease, and the cells start synthesizing and secreting sesquiterpenes (3, 18). The induction of sesquiterpene biosynthesis has been correlated with the induction of a sesquiterpene cyclase enzyme, which is regulated by the transcription rate of the cyclase gene (20). The mechanism controlling the suppression of squalene synthetase enzyme activity in elicitor-treated tobacco cells is currently unknown.

To further investigate the mechanism(s) controlling the suppression of squalene synthetase enzyme activity in tobacco cells, we are intent on developing antibody probes to the purified protein to determine if the level of squalene synthetase protein is correlated with the respective enzyme activity. But because this enzyme is integrally associated with the membrane lipid bilayer, it was first necessary to develop a solubilization procedure that would result in continued catalytic activity and maintenance of stability for subsequent purification steps. The anionic detergent deoxycholate was used successfully to solubilize the yeast squalene synthetase protein, but recoverable activity was very low (1). The detergents octylglucopyranoside and Lubrol PX were also used to solubilize squalene synthetase activity from yeast microsomes, but large micelles were formed, hindering further purification steps (9). Sasiak and Rilling (13) were successful in purifying the yeast squalene synthetase enzyme to homogeneity when Lubrol PX was removed during the latter stages of the purification and octylglucopyranoside detergent was used alone, but the enzyme activity was very unstable. Similar attempts to solubilize and purify squalene synthetase activity from plant tissue have either failed (our laboratory) or not been reported.

In the present investigation, we describe the solubilization of squalene synthetase from tobacco microsomes by a two-step protocol using a combination of anionic and nonionic detergents. Additionally, we report on the partial purification...
and generation of polyclonal antibodies for the immunodetection of a putative squalene synthetase enzyme.

MATERIALS AND METHODS

Cell Cultures

Tobacco (Nicotiana tabacum) cell suspension cultures were maintained in Murashige-Skoog medium as previously described (3). Cells were harvested during their rapid growth phase (3 d after subculturing) and kept frozen at −80°C until used for enzyme isolation.

Microsome Preparation

Microsomes were prepared in batches of 600 g frozen tobacco cells. Cells were mixed with 800 mL of 100 mM potassium phosphate buffer, pH 7.0, 4 mM MgCl₂, 25 mM sucrose, and 5 mM β-mercaptoethanol (buffer A), and kept at room temperature for 15 min. Cells were then homogenized twice (once with 800 mL buffer A, once with 400 mL buffer A) using a Polytron (30 s, medium speed). Homogenates were collected by filtration through two layers of nylon mesh and filtrates were centrifuged at 1000g for 10 min at 4°C. The supernatant was adjusted to 25% saturation with solid ammonium sulfate, allowed to equilibrate for 20 min at 4°C, and centrifuged at 10,000g for 50 min at 4°C to precipitate the microsomal fraction (14). The supernatant was discarded and the pellet was resuspended in 30 mL of 50 mM Tris buffer, pH 7.5, 2 mM MgCl₂, and 2 mM β-mercaptoethanol (buffer B), centrifuged at 10,000g for 20 min, and the pellet again resuspended in buffer B. The microsomes were kept frozen at −20°C and enzyme activity remained stable for several weeks.

Solubilization Procedure

Microsomal preparations were assayed for total protein and adjusted to a final concentration of 2.0 mg/mL with buffer B prior to detergent solubilization. The microsomal fraction was first treated with detergents at concentrations below their CMC to remove loosely associated proteins, and then at concentrations above their CMC to complete solubilization of squalene synthetase. Initially, the diluted microsomes were made to 4.5 mM OTGP (Ultral grade, Calbiochem) and 1.1 mM GDC (Calbiochem), kept on ice 15 min, and centrifuged at 100,000g for 1 hr at 4°C. The supernatant (SN1) was decanted and the pellet resuspended with buffer B to the original starting volumes. The resuspended pellet was adjusted to 18 mM OTGP (2 × CMC) and 2.2 mM GDC (1 × CMC) and treated as above. After ultracentrifugation, the supernatant (SN2) contained greater than 80% of the squalene synthetase activity and was used for further purification steps.

Enzyme Purification Procedures

All of the purification steps were performed without interruption at 4°C or on ice. The detergent-solubilized enzyme extract was concentrated to approximately one-third of the original volume by ultrafiltration (YM 30 Diaflo membrane, Amicon). The concentrated enzyme preparation was loaded onto an HR S-300 Sephacyr (Pharmacia) gel filtration column (1.5 × 60 cm) equilibrated in 20 mM Tris, pH 7.5, 9 mM OTGP, 1.1 mM GDC, 2 mM MgCl₂, and 5 mM β-mercaptoethanol (buffer C). Fractions containing squalene synthetase enzyme activity were pooled and loaded onto a 1.0 mL FPLC Mono Q anion exchange column (Pharmacia) equilibrated in buffer C minus the GDC detergent. Bound proteins were eluted with a 22 mL two-step buffer C-KCl salt gradient, 0 to 0.3 M KCl in 16 mL, then 0.3 to 1.0 M KCl in the remaining 6 mL.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was according to Laemmli (10) in an 11.5% (w/v) acrylamide/bis gel. Proteins were detected after staining with Coomassie brilliant blue. Immunoblot analysis of squalene synthetase protein was performed as previously described (19).

Polyclonal Antibody Production and Precipitation of an Enriched Antibody Fraction

Three mice were immunized with a primary intraperitoneal injection of 50 μg partially purified squalene synthetase protein in complete Freund's adjuvant. After 12 d, a secondary injection of 50 μg squalene synthetase protein in incomplete Freund's adjuvant was administered. Twelve to 14 d after the secondary injection, the mice were bled and serum collected. The antibody fraction was enriched from immune serum using ammonium sulfate fractionation (14). A 100 μL aliquot of antiserum or preimmune serum was adjusted to 33% saturation with ammonium sulfate, kept on ice 15 min, spun at 14,000g for 10 min, and the supernatant was discarded. The pellet was resuspended in Tris-buffered saline (20 mM Tris, pH 7.5, 500 mM NaCl) solution and ammonium sulfate was removed using a centricon-10 microconcentrator (Amicon). The final volume of the enriched antibody fraction was adjusted to 100 μL with Tris-buffered saline. The isolated antibody fraction was tested for antisqualene synthetase activity in an in vitro assay and used for immunodetection on a western blot.

Enzyme Assay and Protein Determination

The squalene synthetase enzyme assay was slightly modified from that previously reported (18). Briefly, a 1 to 2 μL (0.1–5.0 μg protein) aliquot of sample was incubated in 500 mM potassium phosphate buffer, pH 6.0, 40 mM MgCl₂, 25 mM β-mercaptoethanol, 5 mM NADPH, and 40 μM trans-[1-3H]FPP (36 μCi/μmol) (18) in a final assay volume of 50 μL for 20 to 30 min at 35°C. The reaction was stopped by the addition of 100 μL of petroleum ether containing 0.4% squalene as carrier, vortexed for 10 s, and then spun in a microcentrifuge for 10 s. A 20 μL aliquot of the organic phase was separated on TLC silica gel plates (EM Merck) developed in cyclohexane:ethyl acetate (85:15). The squalene product was visualized by iodine vapor and the spot scraped from the plate for counting of radioactivity. Proteins were determined after TCA precipitation and assayed using a modified Lowry reaction (2).
RESULTS AND DISCUSSION

Solubilization and Purification

Squalene synthetase is a low abundant, integral membrane protein of the endoplasmic reticulum (11). The low abundance of this enzyme made it initially necessary for us to develop a procedure to isolate large quantities of microsomes. Because conventional preparations of microsomes by ultracentrifugation results in relatively small sample sizes, an alternative method of precipitating membranous material with a low concentration of ammonium sulfate (14) was evaluated. By comparison, the recovery of microsomal squalene synthetase activity for the equivalent of 1 g frozen tobacco cells was 0.5 nmol/min with a specific activity of 3.2 nmol/min·mg protein for the ammonium sulfate precipitation method versus 0.47 nmol/min and specific activity of 3.0 nmol/min·mg protein prepared by centrifuging the cell-free homogenate at 100,000g for 1 h. In addition, preparation of microsomes results in a 5 to 10-fold enrichment of squalene synthetase from the initial cell-free homogenate.

Membrane proteins can be dissociated from the lipid bilayer by various methods (17). Typically, non-denaturing detergents are used when it is necessary to maintain catalytic activity after solubilization. The choice of detergent(s) and concentrations needed must be determined empirically for each individual membrane protein (6). Initially, the detergents Triton X-100, Tween 20, Brij 96, and CHAPS were tested at 5 and 50 mM for inhibition of squalene synthetase activity (data not shown). Although both OTGP and GDC did not inhibit squalene synthetase activity, neither alone solubilized significant amounts of enzyme activity. However, in combination, OTGP and GDC solubilized greater than 80% of the microsomal enzyme activity (Fig. 1) and the solubilized activity remained stable for further purification steps.

Detergent solubilization, by definition, results in the disintegration of the membrane lipid bilayer (17). This can be achieved by determining the correct ratio of detergent molecules to cell membrane lipids. Because it is generally more cumbersome to assay for total membrane lipid content than total membrane proteins, the latter can be quantified and the detergent:protein ratio can be assessed for solubilization (6). The optimal ratio for detergent solubilization of tobacco squalene synthetase was determined empirically by varying the total membrane protein concentration. Crude tobacco microsomal proteins were solubilized using a two-step solubilization procedure as described in "Materials and Methods." This two-step protocol initially removed the loosely associated membrane proteins, followed by the solubilization of integral membrane proteins, including squalene synthetase, during the second detergent treatment. When crude extracts having a total microsomal protein concentration of 3.5 mg/mL were detergent-treated, 55, 40, and 5% of the starting microsomal squalene synthetase activity was recovered in the supernatants, SN2 and SN1, and pellet fractions, respectively (Fig. 1). When solubilization was attempted on microsomes having less than 1.75 mg/mL total protein, not more than 30% of the original microsomal enzyme activity was recovered in any of the fractions, with total combined activity not exceeding about 50% (Fig. 1). Optimal recovery of squalene synthetase activity in the SN2 fraction was achieved at a protein concentration of approximately 1.75 to 2.5 mg/mL (Fig. 1). This step also resulted in a twofold purification of the enzyme (Table I). Care was taken that all subsequent solubilizations for protein purification were done at approximately 2.0 mg/mL.

Detergent solubilization of microsomal proteins results in a mixture of protein-detergent aggregates, protein-detergent micelles, and detergent-protein-lipid mixed micelles (7, 16). Because the diverse composition of the micelles interferes with the separation of individual proteins by conventional biochemical methods, the protein-detergent aggregates were separated from the large molecular mass micelles by gel

![Figure 1. Distribution of squalene synthetase enzyme activity recovered after detergent treatment of tobacco microsomes. Microsomes were prepared and protein was adjusted to 0.5 to 3.5 mg/mL and used in a two-step detergent-solubilization procedure as described in "Materials and Methods." The supernatants (SN1 and SN2) and remaining pellet were each assayed for squalene synthetase activity and plotted as a percentage of the initial microsomal squalene synthetase activity. Each value is the mean of two separate experiments.](http://www.plantphysiol.org/fig1.jpg)
were collected and assayed for onto loaded membrane proteins molecular mass were of total micrograms gel SN2; 3, partially purified squalene synthetase separated in 10 kD using molecular mass standards. Figure 2. Separation of squalene synthetase activity from solubilized membrane proteins by gel filtration chromatography. Tobacco microsomes were detergent-solubilized, concentrated by ultrafiltration, and loaded onto an HR S-300 Sephacryl column. Two milliliter fractions were collected and assayed for enzyme activity and total protein. The molecular mass of the detergent-enzyme complex was calculated as 110 kD using molecular mass standards.

Figure 3. SDS-PAGE of proteins associated with microsomal and partially purified squalene synthetase active fractions. One hundred micrograms of total protein from each of the different steps in the purification protocol were separated on an SDS-polyacrylamide/bis gel and proteins stained with Coomassie brilliant blue. Fractions separated in each lane: 1, crude microsomes; 2, detergent-solubilized SN2; 3, concentrated SN2; and active fractions after gel filtration (4) and Mono Q chromatography (5).

permeation. To improve resolution of the solubilized protein mixture on a gel filtration column, it was first necessary to reduce the volume of our sample. When the SN2 fraction was concentrated by ultrafiltration using a membrane with a molecular mass cut-off of 30 kD, a significant purification with very little loss of enzyme activity was achieved (Table I). The concentrated sample was separated by gel filtration and the large molecular mass micelles (600–200 kD) appeared as cloudy fractions just after the void volume. All of the recoverable detergent-solubilized squalene synthetase activity was detected in fractions 9 through 13 (Fig. 2), corresponding to a molecular mass of 110 kD. A broad range of different sized proteins were recovered from the active fraction, as detected after separation by SDS-PAGE (Fig. 3). This possibly results from different sized proteins complexed with one or more detergent or membrane molecules or mixed micelles, thus increasing the apparent molecular mass anticipated for the protein alone (16). Fractions 10 through 13 were pooled and used for further purification.

Separation of enzyme proteins by charge or hydrophobicity may be particularly difficult when trying to resolve detergent-solubilized proteins. Detergent moieties can interfere with the specificity of the protein binding, or elution from the column material may destabilize the protein-detergent complex and result in loss of catalytic activity (7). Preliminary experiments indicated that detergent-solubilized squalene synthetase bound to DEAE anion exchange resins, but enzyme activity eluted with a very broad elution pattern (data not shown). To increase resolution, the pooled gel filtration fraction was loaded onto an FPLC Mono-Q column and proteins separated with a two-step KCl gradient (Fig. 4). Squalene synthetase activity was eluted in two fractions at approximately 550 mM KCl. Approximately 20% of the starting enzyme activity was recovered with a 60-fold purification (Table I).
A modest increase of specific activity from 2.8 to 15.4 nmol/min·mg protein was observed after detergent solubilization and gel permeation (Table I). This specific activity was increased further to 164 nmol/min·mg protein after the separation on a Mono Q column. Numerous unsuccessful attempts were made to further purify the tobacco squalene synthetase protein, including separation by chromatofocusing, hydrophobic interaction, dye ligands, and hydroxyapatite. Aliquots of proteins isolated from each step in the purification protocol were separated by SDS-PAGE and visualized after Coomassie blue staining. We detected a modest enrichment of several protein bands (Fig. 3), but with no clear candidate for the squalene synthetase protein. The enriched protein band detected at about 100 kD is most likely not the squalene synthetase protein. Even though squalene synthetase eluted from the gel filtration column with an apparent molecular mass of approximately 100 kD, this fraction represents a protein-detergent aggregate that inflates the true molecular mass of the protein component (16).

Immunological Studies

The partially purified squalene synthetase protein recovered from the Mono Q column was used to generate polyclonal antibodies from mice. Three mice were immunized with the partially purified squalene synthetase protein. Because serum samples interfered with the in vitro enzyme assay (data not shown), the antibodies were enriched from both the preimmune and immune sera using ammonium sulfate fractionation (14). Enriched antibodies from preimmune serum did not inhibit the in vitro squalene synthetase enzyme assay (Fig. 5). We did not detect any cross-reactivity between either the preimmune serum or enriched antibody fraction and the partially purified squalene synthetase protein in a western blot (data not shown). When the enriched antibodies from mouse-1, -2, and -3 immune sera were tested for the ability to inhibit the squalene synthetase enzyme activity, only mouse-1 efficiently inhibited the in vitro assay (Fig. 5). Increasing amounts of mouse-1 antibodies resulted in a proportional decrease of enzyme activity (Fig. 5). When the polyclonal antibody sera from all three mice were used separately to probe western blots of partially purified squalene synthetase protein, a prominent band located just below the 68 kD molecular mass marker was detected in all three immunoblots (Fig. 6). This band was less intense in mouse-1 as compared with -2 and -3. Another prominent band located at about 47 kD was also detected after probing with mouse-1 serum. The 47 kD band was significantly less visible in the western blot probed with mouse-2 serum, and was almost undetectable with mouse-3 serum (Fig. 6). An enrichment of the 47 kD band in both mouse-1 and -2 was detected with increasing purification of the squalene synthetase protein (Fig. 6). The enhanced appearance of the 47 kD band from mouse-1 and the decreased intensity detected using the other two sera correlates well with the inhibition of enzyme activity (Fig. 5). We tentatively identify this protein as tobacco squalene synthetase.

Biochemical Comparison

Squalene synthetase has previously been isolated and characterized only from yeast (1, 9, 12, 13, 15). We have observed some similarities between the yeast and plant enzymes. Both the tobacco and the yeast (1, 9, 12, 13) enzyme have an absolute requirement for a divalent cation in the reaction buffer for catalytic activity. Similar to the yeast protein (1,
tobacco squalene synthetase can utilize either NADH or NADPH as a reductant, but preferred NADPH (data not shown). Kinetic studies were conducted using both the crude tobacco microsomes and the partially purified gel filtration fraction. The microsomal squalene synthetase enzyme had an apparent $K_m$ for FPP of 26 $\mu$M as determined by the double reciprocal plot of enzyme velocity versus substrate concentration (Fig. 7A). After partial purification of the squalene synthetase enzyme, the apparent $K_m$ for FPP was 9.5 $\mu$M (Fig. 7B). Affinity for the substrate is in close agreement with previous kinetic studies reported in yeast (1, 13).

Squalene synthetase in yeast has been reported from two separate laboratories as having an apparent molecular mass of 47 (13) and 55 kD (1). The correlation between staining intensity on a western blot and inhibition of enzyme activity also suggests that the tobacco squalene synthetase is of a similar molecular mass. Currently, attempts are underway to further characterize this putative tobacco squalene synthetase protein.

**LITERATURE CITED**


**Figure 7.** $K_m$ determination of squalene synthetase for FPP. Double reciprocal plots of the enzyme velocity versus FPP concentration for microsomal (A) and partially purified (through gel filtration step) (B) squalene synthetase were used to determine the apparent $K_m$ for FPP. Each value represents the mean of two replications.