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SQUALENE SYNTHASE FROM CELL SUSPENSION CULTURES OF *TABERNAEMONTANA DIVARICATA*

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Key Word Index—*Tabernaemontana divaricata*; Apocynaceae; cell suspension culture; phytosterols; pentacyclic triterpenoid phytoalexins; squalene synthase; presqualene diphosphate synthase; purification; properties.

Abstract—Squalene synthase (SQS) was partially purified from a membrane-rich fraction obtained from cells of elicitor-treated *Tabernaemontana divaricata* cell suspension cultures. The enzyme was solubilised using a mixture of the non-ionic detergents *n*-octyl-β-D-glucopyranoside and Lubrol PX and then purified, always in the presence of the two detergents, by sequential anion-exchange, cation-exchange, and gel filtration chromatography. SDS-PAGE analysis of the partially pure enzyme gave one major band of M_r 64 000 and five minor bands with M_rs in the range 47–58 000. Gel filtration chromatography indicated a native M_r of 55–60 000, while isoelectric focusing of solubilised SQS (ex. microsomal fraction) gave a peak of activity corresponding to pI 7.3 and a minor peak. Throughout the purification procedure, when assayed in the presence of Mg^{2+} and NADPH, the enzyme catalysed both the condensation of two molecules of farnesyl diphosphate (FPP) to form presqualene diphosphate (PSPP) and the reduction of this intermediate to squalene, with a stoichiometry of close to 1.0. These results indicate that the two partial reactions of squalene synthesis by *T. divaricata* SQS are tightly coupled. Characterisation of SQS obtained from the cells of control and elicitor-treated *T. divaricata* cultures indicated differences in the apparent affinity (K_m) for FPP before solubilisation of the enzyme, while the pH optimum and profile were similar for enzyme obtained from both sources. These results suggest that more than one isoform of SQS may be present in cells of elicitor-treated *T. divaricata* suspension cultures.

INTRODUCTION

Squalene synthase (farnesyl diphosphate:farnesyl diphosphate farnesyl transferase, EC2.5.1.21; SQS) catalyses the NADPH- and Mg^{2+}-dependent formation of squalene from two molecules of farnesyl diphosphate (FPP) by way of the NADPH-independent formation of presqualene diphosphate. Since SQS is the first enzyme on the branch of the terpenoid biosynthetic pathway concerned with the biosynthesis of acyclic and cyclic triterpenoids, it might be expected to play a regulatory role in the production of these compounds. There is evidence that SQS regulates the accumulation of cholesterol and ergosterol in animal cells [1–4] and yeast cells [5], respectively, although it is generally concluded that in animals this level of regulation is of less importance than that provided by 3-hydroxy-3-methylglutaryl coenzyme A reductase.

Studies on the elicitor-mediated redirection of terpenoid biosynthesis away from phytosterol production and towards terpenoid phytoalexin accumulation in solanaceous plants and *Tabernaemontana divaricata* have revealed two features. Firstly, SQS activity and the *de novo* biosynthesis of phytosterols and sterol glycoalkaloids, when present, are rapidly and almost totally inhibited following the elicitor-induced accumulation of sesquiterpenoid phytoalexin accumulation in potato tuber discs [6–9] and cell suspension cultures of potato and tobacco [10–13]. Secondly, SQS activity is increased whereas phytosterol biosynthesis is inhibited following the induction of pentacyclic triterpenoid phytoalexin accumulation in cell suspension cultures of *T. divaricata* [14, 15]. These rapid changes in SQS activity point to a regulatory role for SQS in the biosynthesis of phytosterols, sterol glycoalkaloids and pentacyclic triterpenoids. However, it remains to be established whether the inhibition of SQS in elicitor-treated cultures and tissues of solanaceous plants is a means of channeling

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FPP away from phytosterol and sterol glycoalkaloid biosynthesis and towards sesquiterpenoid phytoalexin biosynthesis [6–15] or whether it is divorced from sesquiterpenoid phytoalexin biosynthesis as would be the case if the phytosterol and sesquiterpenoid phytoalexin biosynthetic pathways are in fact metabolically distinct, even though they are located in the same cellular compartment (i.e. metabolon concept) [16]. Nevertheless, whatever the precise regulatory function(s) of SQS, it is more than likely there will be a different isoform of this enzyme for each of the triterpenoid pathways which are operative in a cell.

SQS is an intrinsic, microsomal protein and has proved to be particularly resistant to solubilisation and isolation, thus limiting studies of the enzymes mechanism and its potential for regulating the flux of metabolites through the polyisoprenoid pathways. The enzyme was first solubilised from yeast with deoxycholate [17, 18] but the detergent significantly inhibited enzyme activity. Rilling and co-workers [19, 20] used a mixture of the non-ionic detergents Lubrol PX and *n*-octyl-β-D-glucopyranoside (OGP) to solubilise a M_r 47 000 polypeptide from yeast which was able to synthesise both PSPP and squalene from FPP, and which was subsequently purified. A truncated rat SQS has also been isolated [21]. However, SQS has yet to be isolated from plants although limited purifications have been described for daffodil [22], tobacco [23] and dandelion [24].

In this paper, we report on our attempts to purify SQS from cells of elicitor-treated cultures of *T. divaricata* via solubilisation with OGP and Lubrol PX. Elicited cells were chosen for this study because they contain a five-fold higher level of SQS than cells from untreated cultures, and, furthermore, the specific activity of the enzyme (122.2 pkat mg protein^{-1} [14]) in crude cell-free homogenates of such cells is comparable to that observed for rat liver microsomes [21]. In addition, we also report on some of the properties of the microsomal and solubilised forms of this enzyme obtained from control and elicited cells.

RESULTS AND DISCUSSION

Solubilisation of SQS from membrane-rich fractions

Cells from 5-day-old cultures which had been treated with the *Candida albicans* elicitor preparation 16 hr before harvest were used as the source of the SQS-containing preparations used in the experiments described in this and, except were otherwise stated, the next section.

Our original method for preparing microsomes contained an intermediate centrifugation step (16 000 *g* for 20 min) to remove organelles such as plastids and mitochondria. However, as 64% of the SQS activity was sedimented in this step it was subsequently omitted from the membrane-precipitation protocol, and a membrane-rich fraction (microsomal fraction) prepared by centrifugation of the cell-free extract at 180 000 *g* for 60 min. This gave *ca* two-fold increase in the specific activity and a yield of almost 80% of SQS activity, with 95% of the retained activity present in the pelleted fraction. Our results are similar to those obtained by Sasiak and Rilling [20], who found SQS activity was distributed throughout the particulate fractions in yeast. Microsomal fractions could be stored at −80° with only small losses in activity (5–10%) after 3 months, whereas about 50% of the activity was lost after 24 hr at 4°.

SQS was successfully solubilised from yeast membranes using a mixture of the non-ionic detergents Lubrol PX and OGP [19, 20] and we chose these detergents to solubilise SQS from *T. divaricata* membranes. Typically, treatment of resuspended *T. divaricata* microsomes with Lubrol PX (0.5% v/v) and OGP (5 mM) resulted in retention of 65–70% of SQS activity in the supernatant (termed 'solubilised microsomal fraction') following centrifugation (180 000*g* for 60 min), although there was no increase in specific activity. The degree of solubilisation could not be increased by increasing the detergent concentration, changing the mixture ratio, or increasing the time of exposure. These results suggest there was differential solubilisation of the *T. divaricata* membranes by mixtures of these detergents, with portions of the total cell SQS too tightly bound or otherwise unsuitable for solubilisation.

After SQS had been solubilised from the membranes with a mixture of these two detergents, their continued presence was essential for retention of SQS activity. Furthermore, solubilised SQS activity was not stabilised by methanol (10%) as observed for the yeast enzyme [20].

Purification of SQS from solubilized microsomal fraction

Various fractionation techniques were tested for their effectiveness in separating solubilised SQS from contaminating proteins. In addition to the four techniques eventually chosen to form the purification protocol (see below), the following were evaluated: ammonium sulphate precipitation, ion exchange chromatography on DEAE cellulose (DEAE-5PW High Performance, LKB); chromatography on hydroxy apatite (HA-Ultrogel, IBF Biotechnology); affinity chromatography on 2′,5′-ADP Sepharose 4B (Pharmacia). The conditions (pH, ionic strength, etc.) were altered to achieve the best increases in specific activity and/or greatest yields in activity. The purification procedure adopted involved anion-exchange chromatography on Q-Sepharose Fast Flow (Pharmacia), cation exchange chromatography on Cellulose Phosphate P11 (Whatman), gel filtration chromatography on LKB Ultropac TSK 3000 SW high performance (LKB) and chromatofocusing on Mono P 5/20 (Pharmacia). The whole procedure being completed in one go over a 24 hr period.

Chromatography of the solubilised microsomal

Table 1. Summary of the partial purification of SQS from cells of elicited *T. divaricata* cultures. Squalene synthesis was not detected in the fractions following chromatofocusing on a FPLC Mono P column and therefore the results are not shown

Step	Vol (ml)	Total protein (mg)	SQS recovery (%)	Sp. activity (pkat mg^{-1})	Purification (fold)
Crude cell-free preparation	280	624.8	100	21.3	1.0
Microsomes	35	260	76.0	40.6	1.9
Solubilised microsomes*	38	165.5	50.2	40.3	1.9
Q-Sepharose†	3	5.78	3.7	85.4	4.0
Phosphocellulose†	0.2	1.78	0.26	19.6	0.9
Gel filtration‡	3	0.224	0.07	40.9	1.9

* Sample was buffer-exchanged before squalene synthesis and total protein assays were performed

† Pooled active fractions were concentrated and desalted prior to assay for SQS and total protein.

‡ Pooled active fractions were buffer-exchanged prior to assay for SQS and total protein.

fraction on Q-Sepharose gave a single peak of SQS activity which eluted at *ca* 400 mM NaCl with a 2.1-fold increase in specific activity. SDS-PAGE analysis of the active fractions indicated there was enrichment for several proteins and especially for some in the size range M_r 50–65 000. However, the yield from this step was only 7.4%. Further purification on cation-exchange and gel filtration columns gave rise to a purified sample (224 μg total protein) with a specific activity of 40.9 pkat mg protein^{-1}, and which gave three major bands on SDS-PAGE with estimated M_rs of 64 000, 58 000 and 50 000. There was a particularly pronounced enrichment of the M_r 64 000 polypeptide in comparison to that for the other two major bands, this band accounting for some 75% of the total protein in the sample (based on integration analysis on a gel scanner). The overall yield and degree of purification were 0.07% and 1.9-fold, respectively, reflecting the labile nature of the solubilised enzyme, especially in chromatographic separations. Indeed, we were unable to detect SQS activity in any of the fractions obtained from a subsequent chromatofocusing step, although separation of solubilised microsomes on the same column gave clear peaks of SQS activity (Fig. 1).

The low overall yield reported here (0.07%) compares with yields of 0.39% reported for SQS purified from yeast microsomes solubilised with the same mixture of detergents [19], 3.1% for the partial purification of SQS from daffodil coronae microsomes solubilised with CHAPS [22], 21% reported for SQS partially purified from tobacco membranes using a mixture of glycodeoxycholate and octylthioglucopyranoside (OTGP) [23], and 0.043% for the SQS purified from dandelion cells using a mixture of glyoxydecholate and OTGP [24]. In contrast to the yield of activity, the A_{280}-profiles (not presented) and the protein yields (Table 1) at each stage of the purification suggested a greater purification. This was also demonstrated in the results for electrophoresis of the samples under denaturing conditions where the removal of several prominent bands was illustrated for each chromatographic stage of the purification. In

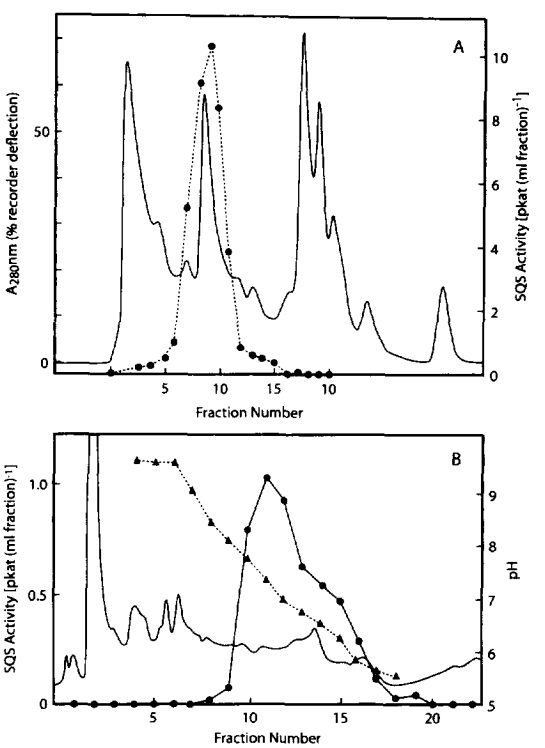

Fig. 1. Gel filtration (A) and chromatofocussing (B) column chromatography of solubilised microsomal fractions. —, A; ● — ●, SQS activity; ▲ — ▲, pH.

each of the three other purifications of SQS from plant sources [22–24], SDS-PAGE separation of the most pure preparation gave rise to several (six or more) stained bands, indicating the difficulties associated with isolation of membrane-bound plant proteins.

Separation of solubilised SQS on a gel filtration column in the presence of detergents and 400 mM NaCl indicated a native M_r of 55–60 000 [Fig. 1(A)]. Taken in conjunction with the results from SDS-PAGE, *T. divaricata* SQS is a monomer of about M_r 64 000. This is higher than values reported previously for cultured yeast SQS (47 000 [20]) and during this

study for rat liver SQS (45–47 000 [21]) and tobacco SQS (47 000 [23]). The M_r is closer to the 55 000 value reported for SQS from cultured yeast microsomes [18].

Elution of solubilised microsomal fraction from a FPLC chromatofocussing column gave a major peak of SQS activity corresponding to a pI of 7.3, with an additional shoulder of activity corresponding to a pI of 6.95 (Fig. 1B). The SQS activity corresponding to the lower pI was several-fold lower than that for the higher pI. In keeping with the changes observed in total SQS activity in the cells of elicitor-treated *T. divaricata* cultures, it is possible the minor peak of activity was due to the enzyme present constitutively, while the major peak of activity was due to an elicitor-induced SQS. The p*I* values for both forms are significantly higher than the value of 6.3 obtained previously for SQS purified from cultured yeast [20], this being the other p*I* for SQS reported to date.

Enzymology of T. divaricata *SQS*

Cell-free homogenates prepared from the cells of both control and elicited (*C. albicans* preparation, 18 hr) cultures of *T. divaricata* were stripped of endogenous cofactors by passage through a pre-packed column containing Sephadex G-25 (PD-10, Pharmacia) and subsequently assayed for the synthesis from [1-$^3H_2$]FPP of both PSPP and squalene. The results (Table 2) showed that, as expected, the synthesis of squalene is both Mg^{2+}- and NADPH-dependent, whereas the synthesis of PSPP required only Mg^{2+}. The two component reactions were very tightly coupled (i.e. PSPP–squalene, 1:1) in the presence of NADPH and this was reflected in the very small amounts of PSPP produced in the absence of this cofactor. Although the specific activity for PSPP and squalene synthesis in the presence of NADPH and Mg^{2+} by SQS obtained from cells of elicited cultures was some four to five-fold higher than for enzyme from cells of control cultures, the relative rates of the two partial reactions remained constant (PSPP–squalene, 1:1) for the enzyme from both sources.

The two activities of SQS (condensation to form PSPP and reduction of PSPP to squalene) co-purified upon anion-exchange chromatography. Further, the ratio of PSPP: squalene synthesis remained constant and close to 1.0 upon solubilisation of the enzyme from the membranes and through the chromatographic separations. A single polypeptide is therefore responsible for both activities of SQS in *T. divaricata* (reported here) and in yeast [20]. However, the observed ratio of PSPP–squalene synthesis for the yeast enzyme was *ca* six, whether the enzyme was enzyme-bound or solubilised. The yeast and *T. divaricata* enzymes therefore differ significantly in the way they mediate the formation of squalene from two molecules of FPP. For assays *in vitro* with the yeast enzyme, the product of the first reaction, PSPP, is relatively free to leave the enzyme active site. In contrast, the two partial reactions are tightly coupled for the *T. divaricata* enzyme assayed *in vitro*, with the product of the first reaction unable to leave the enzyme-intermediate complex until it has been reduced to squalene.

Solubilised SQS was active over a wide pH range for the three buffers tested (HEPES, phosphate and Tris–HCl) with the optimum activity occurring between pH 7–8. The highest activities were recorded in phosphate buffer which also acted as an effective inhibitor of phosphatase activity. While yeast [30] and pig liver [25] also possess pH optima in the range 7–8, partially purified tobacco SQS [23] was most active

Table 2. Requirements and properties of bound and solubilized forms of SQS from control and elicitor-treated cultures

Requirements for activity and properties	Sp. activity [pkat (mg protein)$^{-1}$] Control cells PSPPS	SQS	Elicited cells PSPSS	SQS
(a) Requirements of cell-free homogenates				
Additions:				
None	0	0	0	0
$MgCl_2$ (10 mM)	0.14	0	0.59	0
NADPH (0.24 mM)	0	0	0	0
$MgCl_2$ and NADPH	7.08	6.83	32.41	30.82
PSPSS: SQS	0.96		0.95	
(b) Properties of solubilized SQS				
M_r (gel filtration)	ND		55–60 000	
pH optimum	7.5		7.5	
p*I*	ND		7.3 + 6.95 (minor)	
Optimal [Mg^{2+}]	*ca* 10 mM		*ca* 10 mM	
K_m (NADPH)	ND		45 μM	
K_m (FPP)	4.25 ± 0.63 μM (micro)*		7.76 ± 0.64 μM (micro)*	
K_m (FPP)	7–8 μM		7–8 μM	

* Values obtained with microsome preparations.

at pH 6.0. The ratio of PSPP to squalene synthesis was pH independent and remained *ca* 1.

SQS activity was dependent on the presence of Mg^{2+}. The solubilized enzyme reached a maximum at *ca* 10 mM $MgCl_2$ and was inhibited by concentrations above 35 mM $MgCl_2$. Optimal concentrations for squalene synthesis of about 10 mM $MgCl_2$ have been observed for mammalian [25, 26], yeast [27, 28] and higher plant [22] SQS. The rate of synthesis of PSPP was also optimal at about 10 mM $MgCl_2$ but showed less sensitivity to deviations in $MgCl_2$ concentration from the optimum. As a result, the ratio of PSPP to squalene rose above 1.0 in assays of SQS containing $MgCl_2$ concentrations below (e.g. 1.7:1 at 1.7 mM) and above (e.g. 1.3:1 at 71.3 mM) the optimum. This was one of only two instances where the stoichiometry of the two partial reactions catalysed by *T. divaricata* SQS in the presence of NADPH deviated appreciably from 1.0, the other being under conditions of low or high substrate concentration (see below).

Squalene synthesis by solubilized SQS reached a maximum at about 0.5 mM NADPH and no inhibition occurred at the maximum concentration (2.0 mM) of NADPH used. Squalene synthesis reached about 90% of its maximum value at the NADPH concentration utilised in standard assay conditions (0.24 mM). The value of K_m (NADPH) was tentatively determined from the intercept of the abscissa through a plot of the reciprocal of the initial velocity at pH 7.5 and 30°, against the reciprocal of the NADPH concentration according to the method of Lineweaver and Burk. A K_m value of 45 μM was obtained, which is in close agreement with value (40 μM) obtained for rat liver [21] and for yeast (61 μM) [29]. However, a K_m value for NADPH of only 4 μM was obtained for partially purified SQS from yeast microsomes [20]. The stoichiometry of protons released to squalene synthesised was between 1.0 and 1.1 for all concentrations of NADPH tested. Thus, in the presence of NADPH all the PSPP synthesized in the condensation reaction was efficiently converted to squalene regardless of the actual concentration of NADPH in the incubation. This supports the findings of Shechter and Bloch [17], who reported that in incubations containing partially purified yeast SQS, PSPP is converted to squalene three times faster than FPP is converted to PSPP.

Squalene synthesis by microsomes in the absence of the non-ionic detergents used in this study was maximal at substrate concentrations between 25 and 50 μM for microsomes from both control and elicited cells, with inhibition occurring at concentrations above 50 μM. Similar substrate concentrations have been shown to be inhibitory to pig liver [25, 30] and yeast [29, 31] SQS. Maximal squalene synthesis by microsomes in the presence of detergents at solubilizing concentrations (5 mM OGP and 0.5% Lubrol) was achieved at 100 μM (compared to 50 μM in the absence of detergents) for SQS obtained from cells of both control and elicited suspension cultures. In addition, no inhibition of squalene synthesis was observed at the maximum concentration (250 μM) of FPP used when detergents were present in the assay medium. These findings cannot be explained solely by the apparent stimulation of *T. divaricata* SQS activity by the mixture of detergents used in this study since this only accounts for an increase of some 15% (results not presented).

The K_m (FPP) value was calculated for microsomal SQS obtained from cells of control and elicited suspension cultures, both in the presence and absence of detergents, by the method of Wilkinson [32] which also provides an estimate of the standard error. K_m values (μM) of 4.25 ± 0.63, and 7.76 ± 0.64, were calculated for FPP for incubations containing elicited and control microsomal SQS, respectively. There was, therefore, a significant difference ($p = < 0.05$) in substrate specificity between the membrane-bound enzyme obtained from cells of control cultures and that obtained from cells of elicited cultures, with the enzyme obtained from elicited cells exhibiting a lower affinity for FPP than the enzyme from untreated cells. However, in the presence of detergents, the difference in K_m (FPP) values was no longer significantly different, the value for SQS from both sources being about 7–8 μM. These results suggest the differences observed in the K_m (FPP) between SQS obtained from cells of control and elicitor-based cultures could be due to the differences in the position of the enzyme molecules in the membrane rather than a true difference in substrate affinity. However, the results of chromatofocussing have indicated that an inducible form of SQS may predominate in elicitor-treated *T. divaricata* cells (see above).

To summarise, we have solubilised and partially purified SQS from elicitor-treated cells of suspension-cultured *T. divaricata*, and characterised the enzyme obtained from both control and elicitor-treated cells. The stoichiometry of the two partial reactions of squalene synthesis remained tightly coupled even after solubilisation of the enzyme. There were differences in the K_m (FPP) for the remaining membrane bound form of the enzyme obtained from control and elicited cells, while enzyme solubilised from microsomal fractions derived from elicitor-treated cultures gave two distinct peaks of SQS activity upon chromatofocussing. These results indicate that more than one SQS isoform may be present in cells of elicitor-treated *T. divaricata*.

EXPERIMENTAL

Radiochemicals and chemicals. (2*E*,6*E*)-[1-$^3H_2$] Farnesyl diphosphate (32 mCi mmol^{-1}) was available from previous work in this laboratory [11]. (2*E*,6*E*)-Farnesol, squalene and *n*-octyl-β-D-glucopyranoside (OGP) were purchased from Sigma and Lubrol PX from ICN Biochemicals.

Biological materials. The growth and maintenance of the *T. divaricata* cell suspension cultures and the source of the *Candida albicans* elicitor prepn have been

described elsewhere [14]. For the prepn of membrane-rich cell-free systems for purification of enzyme, the contents of two 14-day-old cultures were transferred asceptically to 700 ml fresh medium in a 2 l. conical flask and grown in an illuminated orbital incubator (120 rpm, 25°, subdued light). Experimental cultures were elicited by addition of 2 ml (10 mg total dry wt; 250 ml conical flask) or 20 ml (100 mg total dry wt; 2 l. conical flask) of stock *C. albicans* elicitor after 4 or 5 days of growth. Cultures were harvested by filtration through Miracloth and the retained cells washed with distilled H_2O. The washed cells were either used immediately or snap-frozen in liquid N_2 and stored at −80° until required.

Preparation of cell-free systems. Cell-free homogenates were prepd from fresh or freshly thawed cells according to a previously published method [14]. Membrane-rich frs were prepared by centrifuging ($1.5 \times 10^4 g$ for 20 min) cell-free preps and resuspending the pellets in 100 mM KPi buffer, pH 7.5, containing 2 mM EDTA, 10 mM β-mercaptoethanol and 1 M sucrose.

Solubilisation and purification of SQS. The protocol for solubilisation of SQS used here was based on a method described previously for the yeast enzyme [20]. Lubrol PX and OGP were added to membrane-rich preps (*ca.* 5 mg protein mol^{-1}) to a final concn of 0.5% and 5 mM, respectively. PMSF (0.5 mM final concn) was added to inhibit the activity of proteases. After gentle agitation on ice (30 min), the soln was centrifuged (240 000 *g* for 60 min) and the supernatant (S_{240}) retained.

SQS was purified from membrane-rich frs prepd (see above) from about 2 l. (3 × 700 ml) of 5-day-old *T. divaricata* suspension culture elicited (*C. albicans*) 18 hr previously. Following solubilisation, all procedures were performed in a coldroom (4–5°). Firstly, the solubilised microsomal protein (27 ml) was applied to several disposable pre-packed columns packed with Sephadex G-25 (PD10, Pharmacia) previously equilibrated with 20 mM Tris–acetic acid, pH 9.0 (at 5°), containing 1 mM EDTA, 1 mM DTT, 10% sucrose, 1 mM OGP and 0.1% Lubrol PX (buffer 1) which was eluted with the same buffer. The eluents were pooled (total vol. 38 ml) and applied to a column of Q-sepharose Fast Flow (Pharmacia; column dimensions 5 cm i.d. × 12 cm length, vol. = 230 ml) and eluted at 1 ml min^{-1} with 70 ml buffer 1 followed by a 120 ml linear gradient up to 500 mM NaCl. Frs (5 ml) were collected and assayed for SQS activity. Frs containing highest SQS activity (295–360 mM NaCl) were pooled, concd to 3 ml (Amicon ultrafiltration device; 10 kDa cut-off) and passed through a PD10 column previously equilibrated with 50 mM K–Pi buffer, pH 7.5 (5°), containing 1 mM EDTA, 1 mM DTT, 10% sucrose, 1 mM OGP and 0.1% Lubrol PX (buffer 2) and eluted with the same buffer. The concd pooled active frs were applied to a glass column packed with phosphocellulose P11 (Whatman), and eluted at 1 ml min^{-1} with 65 ml buffer 2 followed by 50 ml buffer 2 plus NaCl (500 mM). Frs (5 ml) were collected and assayed for squalene synthesis. Frs containing the peak of SQS activity (80–115 ml) were pooled and concd to 300 ml (Amicon ultrafiltration and microconcentrator devices). A TSK 3000SW gel filtration column (LKB-Bromma) was equilibrated with buffer 2 containing 400 mM NaCl (0.4 ml min^{-1}, 40 ml) and 200 ml of the concd active frs from the phosphocellulose column were applied to the column followed by elution at 0.4 ml min^{-1} over 32 ml. Frs (0.5 ml) were collected and assayed for squalene synthesis. Active frs (2 ml total vol) were pooled and eluted (2.8 ml buffer 3) from a PD10 column previously equilibrated with 25 mM Tris–acetic acid, pH 8.3 (5°), containing 1 mM EDTA, 1 mM DTT, 10% sucrose, 1 mM OGP and 0.1% Lubrol PX (buffer 3) before being applied to chromatofocusing column (Mono P HR 5/20, Pharmacia) previously equilibrated with buffer 3 (20 ml). The column was eluted at 0.5 ml min^{-1} with buffer 3 (4 ml) and then buffer 4 (20 ml; 2% polybuffer 94–5% polybuffer 74, pH 5.0, containing 1 mM EDTA, 1 mM DTT, 10% sucrose, 1 mM OGP and 0.1% Lubrol PX). Frs (2 ml) were collected and assayed for squalene synthesis and the pH of each fr. measured using a microprobe. The procedure from solubilisation of microsomes to collection of frs from the Mono P column is carried out without interruption and took about 28 hr.

Samples were subjected to SDS-PAGE by the method of Laemmli [33]. Gels were stained with Coomassie Brilliant Blue and lanes analysed using a Chromoscan 3 electrophoresis gel scanner before silver staining to improve band visualisation. Sample protein M_rs were estimated from a plot of migration distance against $\log M_r$ for a range of proteins of known M_r. SQS activity was not detected in any of the frs from chromatofocussing so frs from this sepn were subjected individually to SDS-PAGE.

Assay of enzyme activities. The basic assay mixture (total vol. 0.5 ml) for measurement of squalene synthesis or presqualene pyrophosphate synthesis by SQS contained 20 μl of an appropriate enzyme prepn (containing 10–50 μg protein), 0.48 ml 50 mM K–Pi buffer (pH 7.5), containing 1 mM EDTA, 2 mM β-mercaptoethanol, 0.5 M sucrose, 10.7 mM $MgCl_2$ (added last to avoid pptn), 0.24 mM NADP, 3.22 mM glucose-6-phosphate, 0.8 IU G-6-P dehydrogenase and 12.5 nmol [1-$^3H_2$]FPP (32 μCi μmol^{-1}). The pH optima of the two activities were determined using HEPES (pH 6.5–8.5), KPi (5.5–8.0) and Tris–HCl (7–9) (ea. 50 mM). In experiments where both the SQS and PSPP synthetase activities of enzyme preps were to be measured, the total assay vol. and vol. of enzyme prepn used were doubled to 1 ml and 40 μl, respectively. At the end of the incubation period the assay was divided into 2 × 0.5 ml portions prior to termination with either 5 ml $CHCl_3$–MeOH (1:2, squalene synthesis) or 2.5 ml MeOH (PSPP synthesis). The workup for measurement of squalene synthesis was by a modification of the method described in ref. [11].

The washed and dried lipid-extract was dissolved in petrol (40–60°) and a portion applied to a glass column containing Bio-Sil A (200–400 mesh, BioRad; 0.5 × 3 cm) previously equilibrated with petrol. The column was eluted with petrol (3 ml; only squalene eluted) and then Et_2O (3 ml; ^{3}H-farnesol when present) before the two frs were dried (N_2 stream) and counted for radioactivity. PSPP was assayed by measuring the ^{3}H present as ^3H–H_2O in the reaction mixt. by the method of Rilling [34].

Protein estimation. Total protein was determined using a modified version [35] of the protein assay procedure in ref. [36] using BSA as a reference. When performing the assay, we used sodium tartrate in place of potassium tartrate.

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