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Characterization of botryococcene synthase enzyme activity, a squalene synthase-like activity from the green microalga *Botryococcus braunii*, Race B

Shigeru Okada,[a] Timothy P. Devarenne,[b,1] Masahiro Murakami,[a] Hiroki Abe,[a] and Joseph Chappell[b,*]

[a] *Laboratory of Marine Biochemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan*
[b] *Plant Physiology/Biochemistry/Molecular Biology Program, Agronomy Department, University of Kentucky, Lexington, KY 40546-0091, USA*

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Abstract

The extracellular matrix of the alga *Botryococcus braunii*, Race B, consists mainly of botryococcenes, which have potential as a hydrocarbon fuel. Botryococcenes are structurally similar to squalene raising the possibility of a common enzyme for the biosynthesis of both. While *B. braunii* squalene synthase (SS) enzyme activity has been documented, botryococcene synthase (BS) enzyme activity has not been. In the current study, an assay for BS activity has been developed and used to show that many of the assay conditions for BS enzyme activity are similar to those of SS. However, SS enzyme activity is stimulated by Tween 80 while BS enzyme activity is inhibited. Moreover, BS enzyme activity was correlated with the accumulation of botryococcenes during a *B. braunii* culture growth cycle, which was distinctly different from the profile of SS enzyme activity. While the current results indicate a conservation of enzymological features amongst the BS and SS enzymes, raising the possibility of one enzyme capable of catalyzing both activities, they are also consistent with these two activities arising from separate and distinct enzymes.

Keywords: *Botryococcus braunii*; Botryococcenes; Botryococcene synthase; Squalene; Squalene synthase

Botryococcus braunii is a colony-forming green microalga with individual cells of the colony held together by an extracellular matrix consisting of liquid hydrocarbons embedded within a cross-linked hydrocarbon polymer core [1,2]. Previous studies have classified *B. braunii* into three races (A, B, and L) depending on the type of hydrocarbon associated with the extracellular matrix [3,4] (see Fig. 1). Triterpenoid hydrocarbons, referred to as botryococcenes, are the major matrix components associated with the B race, while the A race accumulates alkadienes and alkatrienes derived from fatty acids [3,5,6]. The L race accumulates yet another type of hydrocarbon, the tetraterpene lycopadiene [4]. The B race also accumulates other types of triterpenoids such as the squalene derivative tetramethylsqualene [7], its epoxide [8], botryoxanthins [9], and braunixanthins [10] as minor secondary metabolites in the extracellular matrix.

In the B race, C_{30} botryococcene is not the dominant botryococcene in the extracellular matrix. Feeding experiments using radiolabeled methionine or carbon dioxide have demonstrated that C_{30} botryococcene is the precursor of all botryococcenes and is rapidly converted into homologues of up to C_{34} by methylation with *S*-adenosylmethionine [11–13]. The composition of these various botryococcene homologues in the colony matrix depends on the algal strain, culture conditions, and growth stage [14,15]. The B race has attracted special attention for several reasons. One, this race can accumulate botryococcenes up to 30–40% of their dry weight

* Corresponding author. Fax: 1-859-257-7125.
E-mail address: chappell@uky.edu (J. Chappell).
[1] Present address: Boyce Thompson Institute for Plant Research, Tower Road, Ithaca, N.Y. 14853-1801, USA.

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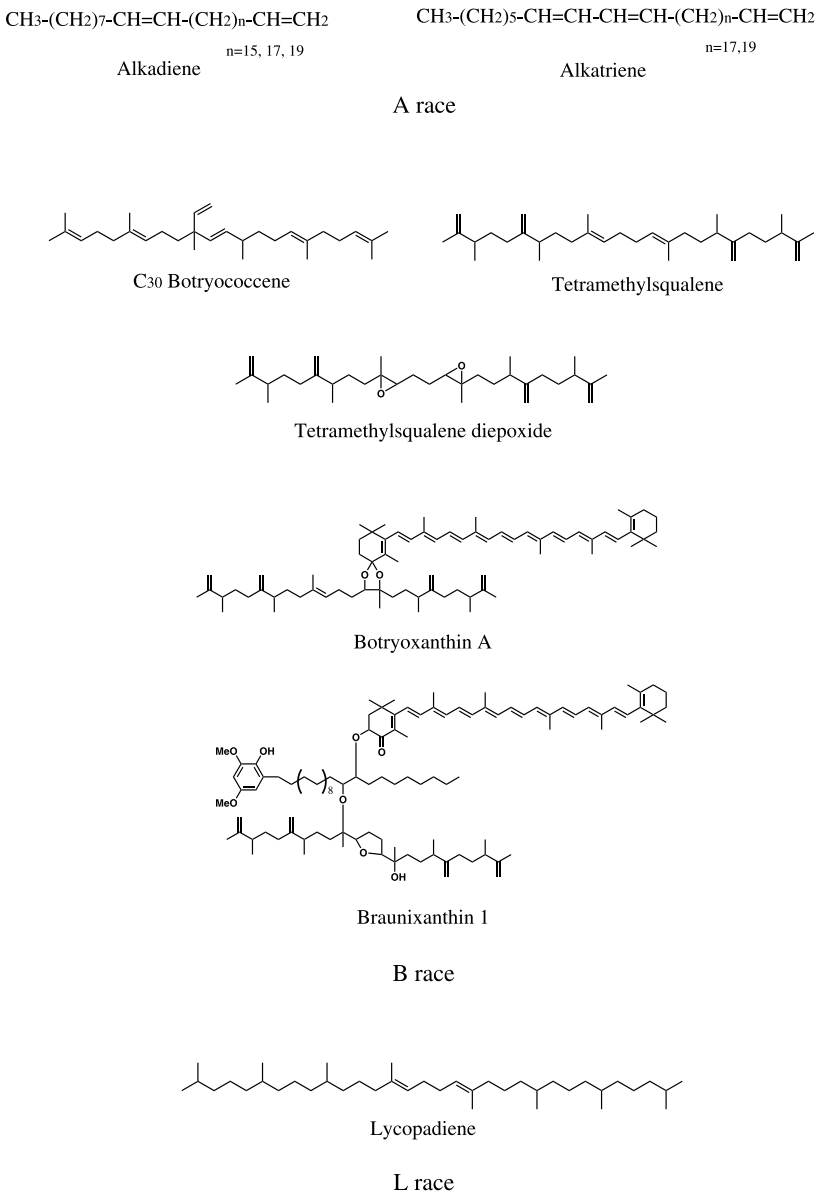
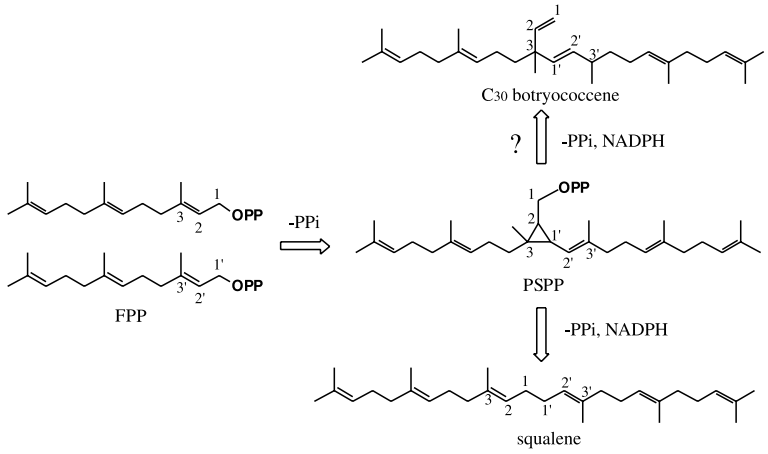


Fig. 1. Various lipids found in the colony matrix of different *B. braunii* races. Race B accumulates botryococcenes and methylated squalenes [3,7,8]. Race A produces alkadienes and alkatrienes while race L accumulates lycopadiene [3,4]. Race B also accumulates squalene derivatives, such as tetramethylsqualene epoxides [8], botryoxanthins [9], and braunixanthins [10] in the matrix.

[3,16]. Two, caustic hydrolysis of the highly branched botryococcene results in the generation of hydrocarbon fuels suitable for internal combustion engines [17]. This potential use of botryococcenes as a renewable energy source has led to many studies on the chemistry and biosynthesis of botryococcenes.

Because of the similarity between the structure of squalene and C_{30} botryococcene, studies on C_{30} botryococcene synthesis have begun with comparisons to squalene synthesis. Both botryococcene and squalene are C_{30} compounds with a common backbone of two C_{15} farnesyl residues. However, they differ in the linkage between the farnesyl residues; squalene has a 1′-1 linkage and botryococcene has a 1′-3 linkage (Scheme 1). Squalene is synthesized by the enzyme squalene synthase (SS)[2] in a two-step reaction in which the first step condenses two molecules of farnesyl diphosphate (FPP) to form presqualene diphosphate (PSPP) [18,19]. The second step cleaves the cyclopropane ring in PSPP to form the 1′-1 linkage followed by reduction with NADPH. The formation of the 1′-3 linkage found in C_{30} botryococcene can be rationalized by an similar two-step reaction carried out by a SS-like

[2] *Abbreviations used:* SS, squalene synthase; FPP, farnesyl diphosphate; BS, botryococcene synthase; PSPP, presqualene diphosphate; DTT, dithiothreitol; RuBisCO, ribulose bisphosphate carboxylase/oxygenase.



Scheme 1. The formation of squalene from FPP by SS and a proposed mechanism for the biosynthesis of C_{30} botryococcene from FPP by BS.

enzyme with a different mechanism for cleavage and rearrangement of the PSPP cyclopropane ring in the second step. Feeding experiments using stereospecifically deuterated farnesol are consistent with such a reaction [20,21].

It is unknown whether a single enzyme, subjected to environmental and/or cellular signals, is responsible for the biosynthesis of both squalene and C_{30} botryococcene, or whether separate enzymes dedicated to the biosynthesis of squalene and botryococcene exist. Current data support both contentions. For example, recombinant yeast SS incubated in the absence of NADPH can synthesize the 1′-3 linked (10*S*, 13*S*)-10-hydroxybotryococcene in addition to the two 1′-1 linked squalene derivatives (*Z*)-dehydrosqualene and (*R*)-12-hydroxysqualene [22]. This may indicate that under the proper conditions SS is capable of producing C_{30} botryococcene. Alternatively, it may indicate that SS is not capable of C_{30} botryococcene biosynthesis and that a separate botryococcene synthase (BS) enzyme must exist.

Studies to determine the true nature of the C_{30} botryococcene reaction mechanism have been limited by the inability to determine the true nature of the C_{30} botryococcene precursor. In earlier studies, feeding of [^{14}C]farnesol or [^{14}C]farnesal to living alga resulted in the formation of ^{14}C-labeled botryococcenes [23,24]. Moreover, protoplasts of *B. braunii* converted [1-^{14}C]farnesal to [1-^{14}C]3-hydroxy-2, 3-dihydrofarnesal which, when fed to a culture of *B. braunii*, was incorporated into botryococcenes [23]. However, incubation of a cell-free extract of *B. braunii* with [^{3}H]FPP showed incorporation of radiolabel into squalene but not botryococcenes [24]. This led to the hypothesis that 3-hydroxy-2,3-dihydrofarnesal may be the direct precursor of botryococcene. In an attempt to better understand the reaction mechanism for and physiology of botryococcene biosynthesis, an enzyme assay for the detection of BS enzyme activity in algal homogenates has been developed and characterized. The BS assay has also been used to demonstrate that FPP is directly incorporated into botryococcenes and to distinguish BS activity from SS activity.

Materials and methods

Culture of the B. braunii Berkeley strain and hydrocarbon analysis

Botryococcus braunii Berkeley (Showa) strain [25] was cultured in modified Chu13 medium [26], grown under illumination of 150 μE/m^2/s on a 12 h light:12 h dark cycle at 20 °C, and aerated with filter-sterilized air containing 2% CO_2. Subculturing was carried out by transferring 300 mL of the 30-day-old culture to 900 mL of newly prepared medium. Aliquots (10 mL) of the culture were collected every 3 days by vacuum filtration onto preweighed Whatman GF/C filters (Whatman, Madison, England). The filters with algal cells were freeze-dried and then weighed. Hydrocarbons were extracted from the algal cells on the glass filters with acetone. A known amount of squalene was added as an internal standard. Solvent was removed from the extracts by a rotary evaporation and the residue was dissolved in a small amount of *n*-hexane. The hydrocarbon fraction was obtained by passing the sample over a silica gel column (Wakogel C-300, Wako Pure Chemicals, Osaka, Japan) and eluting with *n*-hexane. Components of the hydrocarbon fraction were analyzed by GC with a fused silica capillary column (DB-1, 60 m × 0.25 mm). The GC conditions were as follows: column temperature was held at 200 °C for 1 min after injection and raised to 250 °C at a rate of 8 °C/min, and then to 280 °C at a rate of 0.6 °C/min. The total amount of hydrocarbon was determined by comparing the total peak areas of the

hydrocarbon components to that of the internal standard, squalene. To measure BS and SS enzyme activities at different stages of the culture period, multiple flasks containing 1200 mL algal culture were prepared and 500 mL aliquots of the algal culture were removed from separate flasks on days 0, 1, 3, 6, 12, 18, and 24. The algal cells were harvested by filtration with a 20 μm mesh nylon cloth, frozen with liquid nitrogen, and stored at −80 °C for later use.

Assays for SS and BS enzyme activities

Characterization of SS and BS assays was carried out by TLC analysis using a 2000*g* supernatant of algal cell homogenates. Actively growing algal cells (after 5–7 days of cultivation) were harvested by filtration with a 20 μm mesh nylon cloth and stored at −80 °C for future use. The algal cells were initially ground in liquid nitrogen and then homogenized in extraction buffer containing 100 mM sodium phosphate (pH 7.0), 250 mM sucrose, 4 mM $MgCl_2$, and 5 mM β-mercaptoethanol. A superior homogenization buffer was subsequently developed (50 mM Mops (pH 7.3), 20 mM $MgCl_2$, 5 mM DTT, 5 mM EGTA, and 20% (v/v) glycerol) and used in preparing extracts for the enzyme kinetic and squalestatin sensitivity studies. The algal homogenate was centrifuged at 2000*g* for 10 min at 4 °C and the supernatant was filtered through a cotton plug before use.

Enzyme assays were typically initiated by the addition of 2 μL of the 2000*g* supernatant (corresponding to 3 μg protein) to 48 μL reaction buffer (50 mM Mop, pH 7.3, 10 mM NADPH, 2.5 mM β-mercaptoethanol, 20 mM $MgCl_2$, 20 μM [1-^{3}H]FPP (86.9 μCi/mmol)) at 37 °C and incubated for 30 min. Reactions were terminated by adding 100 μL of 1 M KOH, and 100 μL *n*-hexane followed by mixing and centrifugation. Twenty microliters of the *n*-hexane fraction was applied to TLC plates (PE SIL G, Whatman) with authentic standards of squalene ($R_f = 0.14$) and purified C_{30} botryococcene ($R_f = 0.2$) [27] and developed with *n*-hexane. The spots of authentic standards were visualized with iodine vapor, scraped from the TLC plate, and placed into a scintillation vial to measure radioactivity. The lower detection limit for both enzymes under these conditions was 0.1 nmol of product/h/mg protein, which corresponds to approximately 10 dpm above background.

The enzyme reaction products were also analyzed by reverse phase HPLC (RP-HPLC). The reactions were carried out as described above, except the *n*-hexane extract of the reaction was evaporated to dryness using a rotary evaporator, resuspended in MeOH, and then analyzed by RP-HPLC with a Cosmosil ODS AR (4.6 mm × 250 mm) column (Nacalai tesque, Kyoto, Japan) using MeOH as a mobile phase at a flow rate of 1 mL/min and detection at 210 nm.

Distribution of SS and BS enzyme activities in algal cells

An algal 2000*g* supernatant was prepared as mentioned above except that sucrose was omitted from the homogenization buffer to facilitate rupture of organelles. The 2000*g* supernatant was further centrifuged at 4 °C for 30 min at 100,000*g* to obtain a soluble protein fraction (100,000*g* supernatant) and a crude microsomal pellet. The pellet was washed twice with homogenization buffer and resuspended to the original volume with homogenization buffer. SS and BS activities in the 2000*g* supernatant, 100,000*g* supernatant and 100,000*g* pellet were measured using the TLC assay. The activity of ribulose-bisphosphate carboxylase (RuBisCO) in these fractions was also measured to evaluate the efficiency of the cell fractionation and plastid rupture. Fifty microliters of each fraction was incubated with 199 μL of 125 mM Bicine buffer (pH 8.2) containing 20 mM $MgCl_2$, 15 mM $NaH^{14}CO_3$ (380 pCi/nmol) in a screw-capped vial at 30 °C for 10 min. The reaction was initiated by adding 1 μL of 3 mM ribulose 1,5-bisphosphate into the vial and terminated by adding 100 μL of 2 N HCl after incubation at 30 °C for 1 min. The contents in the vials were completely dried and dissolved in 500 μl of H_2O, and the incorporation of radioactivity was measured with a scintillation counter.

Results

Previous in vivo precursor feeding studies on the synthesis of botryococcene have suggested that FPP may not be a substrate for a putative BS enzyme [23,24]. To evaluate this possibility, an aliquot of a 2000*g* supernatant prepared from a *B. braunii* cell homogenate was incubated at 37 °C for 30 min in the presence of [^{3}H]FPP, $MgCl_2$, and NADPH. Analysis of the reaction products by TLC revealed two spots of radioactivity with identical R_f values to C_{30} botryococcene ($R_f = 0.2$) and squalene ($R_f = 0.14$), respectively. Additional analysis of the reaction products by reverse phase HPLC also indicated incorporation of radioactivity into squalene and C_{30} botryococcene (Fig. 2). This assay system was hence used to compare properties of SS and BS enzyme activities in *Botryococcus* cell homogenates.

SS enzyme activity is known to be stimulated by some detergents [18,28]. The effects of the detergent Tween 80 on *B. braunii* SS and BS activities were examined (Fig. 3). Increasing concentrations of Tween 80 up to 2% enhanced SS activity approximately 2.5-fold. In contrast, BS activity was progressively inhibited by increasing amounts of Tween 80. In fact, BS activity was completely abolished by 0.5% detergent. The detergent Triton X-100 was also tested and shown to stimulate SS activity but inhibit BS activity (data not shown).

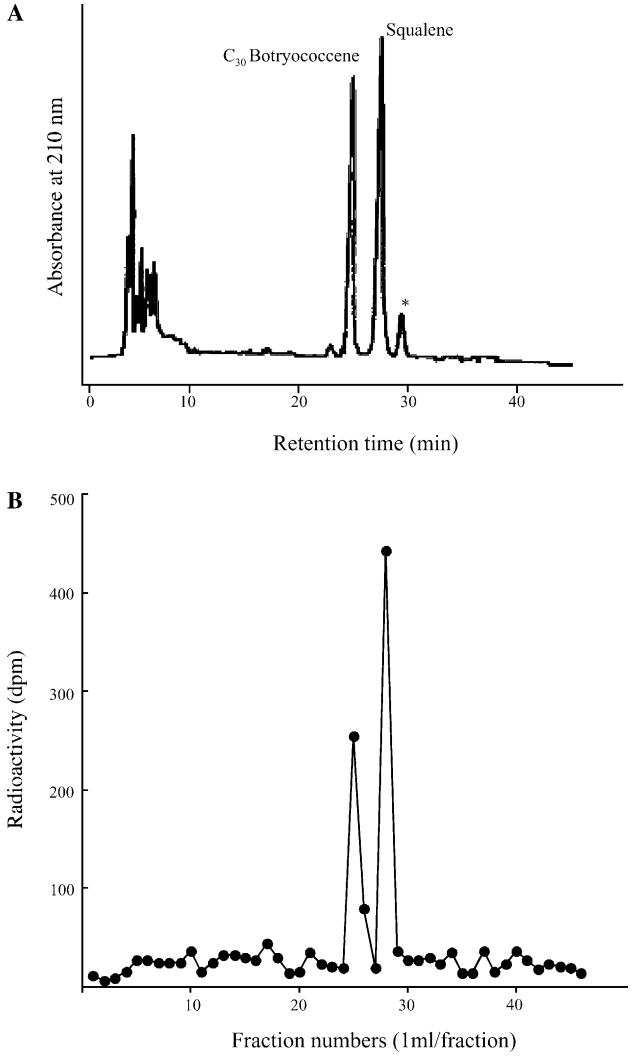


Fig. 2. HPLC analysis of the reaction products derived from incubation of the algal cell homogenate with ^{3}H-FPP. The HPLC system was calibrated by the addition of authentic standards of C_{30} botryococcene and squalene to the samples analyzed. Botryococcene and squalene migrated with retention times of 24.8 and 27.6 min, respectively (A). A minor peak indicated by the asterisk at 29.8 min corresponds to endogenous C_{34} botryococcene extracted from the algal supernatant used in the reaction assay. Aliquots of fractions were also monitored for incorporated radioactivity (B). The two peaks of radioactivity correspond to C_{30} botryococcene and squalene. The slight discordance between the absorbance and radioactivity traces arises from a 0.5 min delay between absorbance readings and fractions collected.

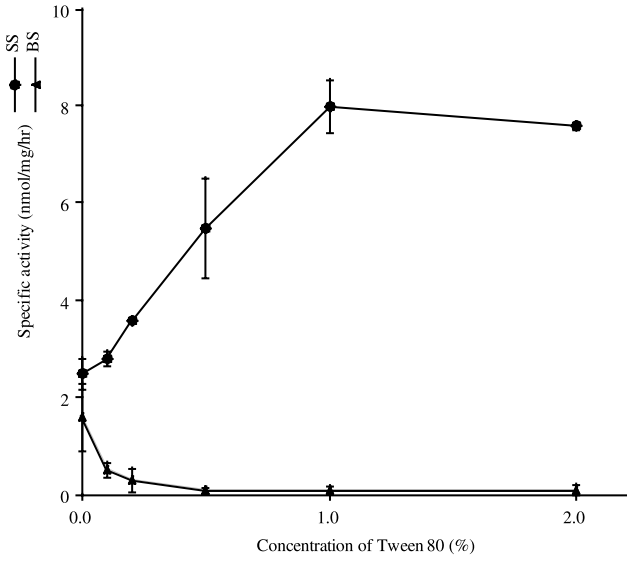


Fig. 3. Tween 80 stimulates SS enzyme activity, but inhibits BS enzyme activity. A 2000*g* supernatant of a *B. braunii* cell homogenate was used as the source for both enzymes, and the activities were determined using the TLC assay method. Tween 80 was added prior to substrate to the final concentrations indicated.

Table 1
Optimal conditions for botryococcene synthase and squalene synthase activities in the algal homogenate

	BS	SS
pH optimum	7.3 (Mops)	7.3 (Mops)
Temperature optimum	37 °C	37 °C
Divalent metals	Mg^{2+}	$Mg^{2+} > Mn^{2+} > Co^{2+}$
Coenzyme dependence	NADPH, NADH	NADPH, NADH
K_m (FPP) (μM)	5.7	3.2[a]
V_{max} (FPP) (pmol/h)	66	81[a]

[a] Enzyme assays were carried out at 2% Tween 80.

A list of biochemical properties determined simultaneously for SS and BS enzyme activities is presented in Table 1. The optimum temperature and pH for both SS and BS enzyme activities were identical, 37 °C and 7.3, respectively. A Mops-based buffer system also provided higher activities than Tris even at pH 7.3. Both enzymes require a divalent cation for activity. Much lower SS enzyme activity was detected when 20 mM $MgCl_2$ was replaced with 20 mM $CoCl_2$, and activity with 2 mM $MnCl_2$ was almost half compared to that of 2 mM $MgCl_2$. Manganese concentrations higher than 20 mM completely inhibited SS enzyme activity. In contrast, no BS enzyme activity was evident with cobalt or manganese as a cofactor. Both SS and BS enzymes required reducing equivalents for activity, with NADH or NADPH serving equally well. The apparent K_m for FPP and apparent V_{max} for SS activity were 3.2 μM and 81 pmol/h, compared to 5.7 μM and 66 pmol/h for BS enzyme activity. Neither [1-^{3}H]farnesol nor [1-^{3}H] farnesal, prepared from [1-^{3}H]farnesol by oxidation with MnO_2, was incorporated into either squalene or C_{30} botryococcene under the conditions tested here (data not shown).

The effect of squalestatin, a specific inhibitor of SS enzyme activity [29,30], on BS enzyme activity in the 2000*g* supernatant of a *B. braunii* cell homogenate was examined (Fig. 4). Both BS and SS enzyme activities were inhibited to a very similar extent at all concentrations of squalestatin with an apparent K_i of approximately 1 nM.

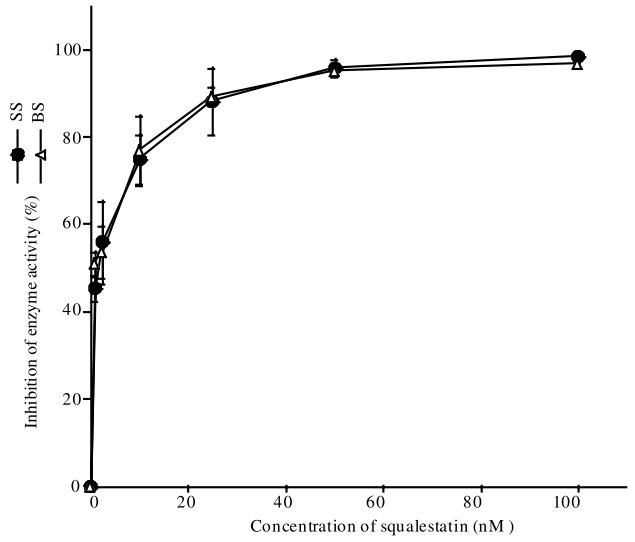


Fig. 4. Squalestatin inhibits both BS and SS enzyme activities. A 2000*g* supernatant of a *B. braunii* cell homogenate was used as the source for both enzymes, and the activities were determined using the TLC assay method.

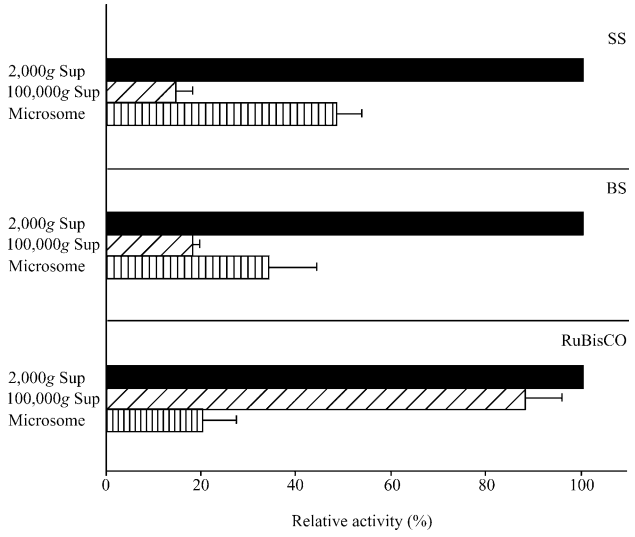


Fig. 5. Distribution of BS and SS activities in *B. braunii* cells. A 2000*g* supernatant of an algal homogenate was centrifuged at 100,000*g* for 30 min to generate a soluble protein fraction (100,000*g* supernatant) and a microsomal (membrane) fraction. Enzyme activities for SS, BS, and RuBisCO in the 100,000*g* supernatant and microsomal fractions are expressed as the percentage relative to the amount of that enzyme detected in the initial 2000*g* supernatant.

While SS enzyme activity is known to be membrane localized [18], it is not known whether BS enzyme activity is soluble or associated with a membrane fraction. The distribution of SS and BS enzyme activities in the algal cell was therefore studied by measuring enzyme activities in fractionated cell components. Botryococcus does not lend itself to traditional fractionation techniques of organelle isolation via density centrifugation

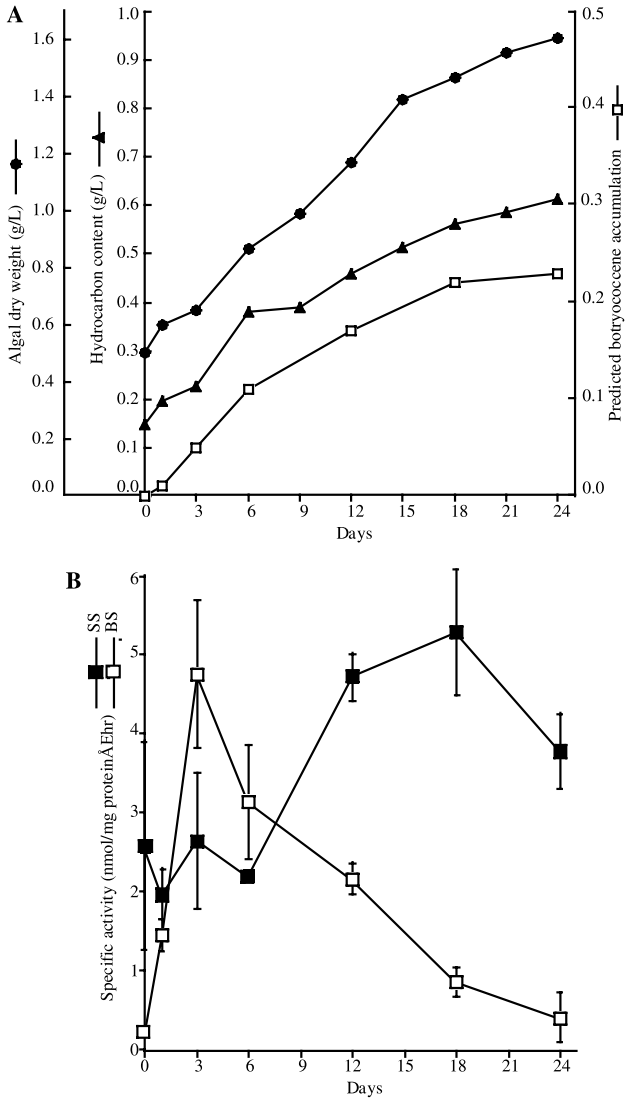


Fig. 6. Growth curve and hydrocarbon accumulation in *B. braunii*, Berkeley cell cultures over a 24-day culture period (A), and the corresponding changes in BS and SS enzyme activities over this same time period (B). SS enzyme activity was measured in the presence of 2% (w/v) Tween 80. The predicted pattern of botryococcene accumulation shown in (A) was calculated by integration (cut-n-weigh method) of the BS activity measured in (B).

because of its extensive extracellular matrix and its difficult to rupture cell walls. Instead, a 2000*g* supernatant of an algal homogenate that contained both SS and BS activities was centrifuged at 100,000*g* to separate functionally soluble proteins from those associated with the microsomal (membrane) fraction, and enzyme activities for SS and BS in these fractions were determined (Fig. 5). RuBisCO activity was used as a marker for the release of chloroplast stromal proteins in these fractions. Approximately 30–40% of the BS and SS enzyme activities were recovered in the crude microsome fraction with another 10–20% remaining in the 100,000*g* supernatant. In contrast, about 85% of the RuBisCO enzyme activity

was recovered in the 100,000*g* supernatant. Thus, like SS, BS enzyme activity appears to be associated with the membrane fraction.

The relationship between the enzyme activities of SS and BS and hydrocarbon production was investigated during a growth cycle of a *B. braunii* culture (Fig. 6). Algal dry weight increased rapidly after inoculation into new medium and corresponded to a similar rate of increase in the hydrocarbon content between day 3 and 6 (Fig. 6A). BS enzyme activity was initially low in the cells, but rose greater than 10-fold to a maximum by day 3, and then decreased gradually to almost undetectable levels by the end of the culture period (Fig. 6B). In contrast, significant levels of SS enzyme activity were detected throughout the whole culture period with a 2-fold increase at the later stages of culture growth (Fig. 6B).

Discussion

Many properties of the BS enzyme activity such as pH and temperature optimum, Mg^{2+} and NADPH requirements, and the Michaelis' constants for FPP are very similar to those of SS. These results suggest that the protein(s) responsible for BS enzyme activity may be very similar to the SS enzyme, or perhaps even one enzyme could be responsible for both activities. A single enzyme responsible for both activities was purported by the finding that, in the absence of NADPH, recombinant yeast SS is capable of producing the 1′-3 linked (10*S*, 13*S*)-10-hydroxybotryococcene [22], suggesting that under very specific conditions SS may produce C_{30} botryococcene. We previously isolated a cDNA corresponding to SS from the B race of *B. braunii* and have carefully characterized the encoded enzyme [31]. Using the *B. Braunii* SS enzyme obtained from bacteria expressing the corresponding cDNA and the optimal conditions for BS enzyme activity determined in this study, we were able to readily detect SS activity, but never have been able to document BS activity. Those observations plus the data presented in the current study are consistent with another SS-like enzyme in the B race of *B. braunii* responsible for BS enzyme activity.

Previous studies have shown detergent stimulation of enzyme activities similar to BS in which the substrates are water-soluble and the products are hydrophobic. Buffers containing detergents such as Tween 80 or Triton X-100 along with BSA are known to stimulate a soluble form of yeast squalene synthase [28], solanosyl diphosphate synthase [32], and dehydrodolichyl diphosphate synthase [33]. The detergent/BSA stimulation of these enzyme activities appears to be due to the hydrophobic nature of the products. Since the products may inhibit enzyme activity, the enzymes require a lipophilic reservoir to release the products. BSA acts as the reservoir and the detergent aids in the product/BSA association [28,32]. The proposed BS reaction is quite similar to that of SS and the other enzymes in terms of a very hydrophobic reaction product, C_{30} botryococcene, from a water-soluble substrate, FPP. However, the BS activity in the crude algal cell homogenate was strongly inhibited by several detergents while SS activity was stimulated. The finding of BS activity mainly in the microsomal fraction suggests that the association of the enzyme(s) responsible for BS activity with membranes may be a requirement for enzyme activity. The sensitivity of BS enzyme activity to detergents may suggest some sort of loose association with membranes that is necessary for full activity. This is in contrast to SS, which is tethered to the endoplasmic reticulum via a carboxy-terminal membrane spanning domain [18]. It is also formally possible that the localization of BS enzyme activity to the membrane fraction is mediated by an interaction with another integral membrane protein(s). Detergents in the reaction buffer may disrupt such interactions leading to a loss of enzyme activity.

Several lines of evidence in our study indicate that FPP is the true substrate for BS enzyme activity. The nature of the direct precursor for botryococcene biosynthesis has generated much speculation. Previous studies have shown incorporation of radiolabeled farnesol [23,24] and farnesal [23] as well as 3-hydroxy-2, 3-dihydrofarnesal [23] into botryococcenes in vivo. However, incorporation of FPP into botryococcenes in vitro was not observed, perhaps as a consequence of the Triton X-100 and BSA used in these assays [24]. To our knowledge, this is the first report of FPP incorporation into C_{30} botryococcene in a cell-free system. Neither ^{14}C farnesol nor ^{14}C farnesal was converted to C_{30} botryococcene using the assay conditions defined here. Additionally, our study shows that BS activity was completely inhibited by a specific inhibitor of SS, squalestatin, which mimics the SS reaction intermediate PSPP [29,30]. This would suggest the likelihood that C_{30} botryococcene is synthesized via Scheme 1 with FPP as a substrate and PSPP as an intermediate, and that the BS and SS reactions differ in the mechanism for opening the cyclopropane ring of PSPP. Consistent with this notion, two independent studies on the stereochemistry of botryococcenes have indicated that PSPP is also the likely intermediate in C_{30} botryococcene biosynthesis [20,21].

The in vivo incorporation of farnesol into botryococcenes likely proceeds through FPP. *B. braunii* cultures have been shown to convert farnesol to FPP by successive monophosphorylation events [24]. This mechanism for the conversion of farnesol to FPP has been shown to occur in mammalian cells [34,35] and other plant cells [36]. FPP produced from farnesol in this manner has been shown to be utilized for the production of sterols by SS in tobacco cells [36], making it likely that farnesol is used in a similar manner to produce

botryococcenes in *B. braunii*. Incorporation of farnesal and 3-hydroxy-2,3-dihydrofarnesal into botryococcenes is more difficult to explain. However, since the incorporation rate of these compounds into botryococcenes was only 2.3 and 2.4%, respectively, compared to the 16% incorporation of farnesol [24], it is unlikely that they are directly incorporated into botryoccenes by the *B. braunii* cells. Perhaps, farnesal and 3-hydroxy-2,3-dihydrofarnesal were incorporated into botryococcenes through a conversion first to farnesol and then to FPP. Regardless, the current results demonstrate that FPP is a direct precursor of botryococcene and that botryococcene is probably synthesized via a PSPP intermediate in a similar manner to squalene.

During the synthesis of botryococcenes, C_{30} botryococcene is rapidly converted to higher molecular weight (C_{31}–C_{34}) botryococcenes by successive methylation reactions [11–13]. The relative abundance of C_{30} botryococcene in the population of accumulated botryococcenes depends on the balance between the rate of biosynthesis of new molecules of C_{30} botryococcene and that of their methylation. As a culture of *B. braunii* ages and botryococcenes accumulate, the synthesis of botryococcenes is shifted toward C_{31}–C_{34} botryococcenes. Therefore, C_{30} botryococcene is usually a minor component in the population of accumulated botryococcenes in the later stages of a culture cycle. Shortly after inoculation into new culture media, the total culture content of C_{30} botryococcene becomes higher when cell division rapidly increases [14]. Since the BS enzyme activity assayed in this study measures only the synthesis of C_{30} botryococcene, the rapid rise of BS enzyme activity by day 3 of the culture cycle, and decrease in activity at the later stages of the culture period, are consistent with the change in botryococcene composition seen during the culture cycle [14]. This is further corroborated by a prediction of the botryococcene accumulation pattern derived from integration of the BS activity measured in Fig. 6B. The expected accumulation pattern directly parallels that observed (Fig. 6A) and is indicative of the important role BS likely plays in hydrocarbon accumulation.

The SS enzyme activity measured in the current study is in contrast to our earlier study [31], which observed a single peak of SS activity at an early stage of the culture cycle. We cannot fully explain the discrepancy between these two studies at this time, but note that two phases of SS enzyme activity during the culture cycle of *B. braunii* have been anticipated [31]. The first phase would consist of SS enzyme activity dedicated to sterol biosynthesis for membrane biogenesis, while the second phase would utilize squalene for extracellular biopolymer accumulation in which squalene derivatives are deposited. It is possible in our initial study [31] we detected SS enzyme activity corresponding to the first phase, while in the current study we were better able to detect SS enzyme activity due to optimization of assay conditions and have measured activities corresponding to both phases.

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