Phytochemistry, 1978, Vol. 17, pp 431–433 Pergamon Press Printed in England

SQUALENE BIOSYNTHESIS BY A CELL-FREE EXTRACT OF *RHIZOPUS ARRHIZUS*

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(*Revised received* 29 *July* 1977)

Key Word Index—*Rhizopus arrhizus*; squalene biosynthesis; cell free extract.

Abstract—Squalene was identified as the principal radioactive component (85%) of the hexane-soluble products formed from mevalonate-[2-^{14}C] by a cell-free preparation from *Rhizopus arrhizus*. The system required ATP, NADH and Mn^{2+} to obtain 20–40% incorporation of the substrate into squalene. Temperature and pH optima for the system were 20° and 7.0, respectively.

INTRODUCTION

Sterols are produced through a long series of reactions with mevalonate as the first committed intermediate [1–4] and an understanding of the reaction mechanisms was made possible with the availability of double and stereospecifically labeled mevalonic acid. However, little is known about the regulation of these reactions during the life cycle of sterol-producing microorganisms, including fungi. Studies of this type have been hindred by the unavailability of the required radiolabeled intermediates. Although [^{14}C] and [^{3}H] labeled mevalonate and lanosterol are available, radiolabeled sterol precursors such as *trans*-farnesol and squalene are not commercially available. This paper describes some properties of a cell-free preparation of *Rhizopus arrhizus* mycelia that can be used to efficiently produce radiolabeled squalene for use in metabolic studies.

RESULTS AND DISCUSSION

We reported previously that squalene was the major hydrocarbon component of *R. arrhizus* mycelia grown in liquid shake culture, but it did not accumulate when the fungus was grown in stationary cultures or on solid media [5]. Squalene accumulation was attributed to a lack of available oxygen due to the fungus growing submerged in the shake culture medium. In this study, a partially purified cell-free preparation of *R. arrhizus* mycelium grown under aerated submerged culture conditions readily incorporated mevalonate-[2-^{14}C] into three hexane-soluble substances. In numerous incubations between 20 and 40% of the [^{14}C] present was incorporated into these substances in 1 hr. The products could be separated into the three major radioactive components by TLC. The principal radioactive product of this system had chromatographic properties, on TLC (R_f 0.9) and GLC, very similar to authentic squalene. Column chromatography was used to purify squalene-[^{14}C] from the combined hexane fractions obtained from several incubations. After passing *n*-hexane through the Si gel column, squalene was the sole radioactive component of the 2% Et_2O in hexane fraction. Squalene was confirmed as the principal product of the *Rhizopus* cell-free preparation by GC–MS. Both the sample and squalene standard had parent molecular ions at *m/e* 410, base peaks at *m/e* 69, intense ion fragments at *m/e* 81 (82.4%) and 137 (37.8%) and they also contained prominant M-15 (*m/e* 385), M-43 (*m/e* 367), and M-69 (*m/e* 341) fragments. The MS of the squalene obtained in this study was very similar to the previously published data [6, 7]. Squalene was confirmed as the principal radioactive product of the cell-free system by GC–RC.

Identification of the minor radioactive products of the *Rhizopus* cell-free preparation was not made. The two unknown compounds, **1** (R_f, 0.38) and **2** (R_f, 0.62), did not correspond to lanosterol, ergosterol, squalene 2,3-oxide, or farnesol.

Good incorporation of mevalonate-[2-^{14}C] into squalene was obtained at pH values between 6.5 and 7.5 with the optimum at 7. Little radioactivity was found in the squalene fraction at pH values less than 6 and greater than 8.5. The optimum pH for squalene synthesis by the *Rhizopus* preparation is similar to that used in studies involving higher plant [8], yeast [9] and rat liver [10] systems.

Temperature optima for synthesis of both the total hexane-soluble compounds, and squalene was 20°, which is considerably lower than that (30°) reported for other cell-free systems [8–10].

In a time course study incorporation of mevalonate-[2-^{14}] into total hexane-soluble products and squalene was linear through the first hour of incubation. Extending the incubation period to 5 hr resulted in no further increase in activity. An almost linear increase in radioactivity up to 40 min incubation was observed for unknown **2** while a detectable increase in unknown **1** occurred after only 20 min incubation.

It is well established that ATP, Mg^{2+} or Mn^{2+} and reduced pyridine nucleotides are required for the incorporation of mevalonic acid into squalene [11, 12]. The first and second steps in squalene biosynthesis are the phosphorylation of mevalonic and 5-phosphomevalonic acids, respectively, which require ATP. The result of deleting ATP from the cell-free preparation of *Rhizopus* was as expected; no incorporation of mevalonate-[2-^{14}C] into squalene occurred (Table 1).

Table 1. Cofactor requirements for incorporation of mevalonate-[2-^{14}C] into squalene by a cell-free preparation of *R. arrhizus**

Cofactor deleted	Squalene	% of control
Control†	38 910	100
−ATP	—	0
−Mn^{2+} (+Mg^{2+})	—	0
−Mg^{2+} (+Mn^{2+})	23 040	59
−Mn^{2+} (−Mg^{2+})	—	0
−NADH (+NADPH)	12 990	33
−NADPH (+NADH)	38 970	100
−GSH	40 580	104

*The results in this table are those of a typical experiment which contained three replications.

†Incubation medium containing complete cofactor mixture and each flask contained a total of 4 ml.

‡No detectable radioactivity above background.

Enzymes that catalyse the phosphorylation reactions require a divalent cation for activity. Tchen [13] showed that Mn^{2+} was the most effective cation at lower concentrations in a yeast preparation, but at higher levels Mg^{2+} was more effective. This was also true for tobacco tissue culture preparations [8]. Loomis and Battaile [14] showed that, compared to other divalent cations including Mg^{2+}, Mn^{2+} was the most effective cation in stimulating mevalonic kinase from pumpkin seedlings. Bloch *et al.* [15] found that Mg^{2+} was most effective in stimulating phosphokinase activity and Mn^{2+} and Ca^{2+} were least effective in yeast. In each of the studies cited above, Mn^{2+} or Mg^{2+} was the most effective cation in stimulating enzyme activity, but other divalent cations such as Zn^{2+}, Co^{2+} and Fe^{2+} could substitute for them in a less effective manner.

In this study, only Mn^{2+} and Mg^{2+} were considered as cofactors for enzymes catalyzing the incorporation of mevalonate-[2-^{14}C] into squalene. When Mn^{2+} was omitted from the incubation medium containing Mg^{2+}, little or no incorporation of mevalonate-[2-^{14}C] into squalene was observed (Table 1). However, when Mg^{+2} was deleted from the incubation medium containing Mn^{2+} variable results were obtained depending on the experiment, but never more than 40% reduction in radioactivity in squalene was observed compared to a control (Table 1). Lyophilized *Rhizopus* extract was used in each of the above experiments, but fresh extract was used in subsequent experiments to determine concentration curves for the cations. Concentration curves for both Mn^{2+} and Mg^{2+} are very similar to those reported for tobacco tissue culture preparations [8]. Optimum Mn^{2+} concentration for incorporation of mevalonate into squalene was 2 mM but, unexpectedly, Mg^{2+} in the absence of Mn^{2+} stimulated incorporation and was linear in the concentration range 0.5 to 6 mM. With lyophilized extracts, Mg^{2+} did not stimulate incorporation in the absence of Mn^{2+}. Presumably alteration of enzymes occurred during lyophilization whereby Mg^{2+} was no longer effective as cofactor.

The final step in the formation of squalene is the tail-to-tail condensation of two farnesyl pyrophosphate molecules which is a reductive reaction requiring NADPH (and Mg^{2+}) in yeast. Presqualene pyrophosphate accumulates when NADPH is omitted from a yeast cell-free preparation with *trans*-farnesyl pyrophosphate as the substrate [10]. No evidence for this intermediate was found in the *Rhizopus* system incubated in the absence of the reduced cofactors. Using a preparation of solublized and purified squalene synthetase from yeast, Shechter and Bloch [9] showed that both NADH and NADPH can serve as electron donors in the condensation reaction with the latter being more efficient. An absolute requirement for a specific pyridine nucleotide could not be shown for the *Rhizopus* preparation used in this study because [^{14}C] squalene was produced when both NADH and NADPH were omitted from the incubation medium. This was true even with dialyzed extract. However, NADH appeared to be the more effective electron donor in this system (Table 1).

Omitting glutathione from the incubation medium resulted in a slightly greater (*ca* 4–12%) incorporation of mevalonate-[2-^{14}C] into squalene by the *Rhizopus* system (Table 1).

In a typical experiment the three principal radioactive products of the cell-free preparation were produced in a ratio 26:3:1 for squalene, unknown **2**, and unknown **1**, respectively. The squalene had a specific activity of about 3×10^7 dpm/μmol. Evidently, almost complete loss of squalene-2,3-epoxidase activity occurred during the preparation of the cell-free extract since neither this compound nor radioactive cyclic triterpenes were detected as products.

It has been shown for plant [8, 16–18], yeast [12] and rat liver [19, 20] preparations that prenyl transferases involved in the early reactions of squalene synthesis are soluble, while the *trans*-farnesyl pyrophosphate-squalene synthetase is membrane bound in yeast [9, 21]. Thus, with mevalonate as the initial substrate, it was improbable that further purification of the *Rhizopus* preparation could be obtained without solublizing the bound enzyme(s). As expected, no incorporation of mevalonate-[2-^{14}C] was obtained with either the supernatant or particulate fractions of the S_{20} obtained after centrifugation at 104 000 *g*. However, the original activity was obtained when the two fractions from the high speed centrifugation were recombined.

Although several cell-free systems that incorporate mevalonate into squalene [22, 23] have been reported, the preparation of *R. arrhizus* described in this paper is more efficient. Enzymes that catalyze the epoxidation of squalene are presumably reduced in activity or rendered inactive during the preparative process and, hence, squalene accumulates. Accumulation of squalene in rat liver requires anaerobic incubation conditions for efficient incorporation of mevalonate-[2-^{14}C]. In one system, a supernatant (S_{10}) fraction obtained after centrifugation of a rat liver homogenate at 10 000 *g* readily incorporated mevalonate-[2-^{14}C] into squalene. Calculations from data in that report indicate that about 15% of the mevalonate incubated with the S_{10} fraction of a liver homogenate from nine rats was incorporated into squalene-[^{14}C]. Incorporation of mevalonate by the *Rhizopus* homogenate used in this study was consistently higher than 15% and was often up to 40% of the initial radioactivity supplied to the system.

EXPERIMENTAL

Materials. All cofactors, DL-mevalonic acid lactone, squalene and ergosterol used in this study were purchased from Sigma Chemical Company (St. Louis, MO). DL-Mevalonic-[2-^{14}C] acid

(DBED salt), 0.029 mCi/mg was purchased from New England Nuclear (Boston, AM). Pre-coated Si gel G TLC plates were used.

Preparation of the cell-free system. R. arrhizus was cultured in aerated 7 l. batches as in ref. [24]. *Ca* 60–80 g fr. wt of mycelium was obtained per 7 l. after 24 h growth. Mycelium was harvested in a funnel lined with cheese cloth, washed with 0.1 M K-Pi buffer (pH 7) and pressed to remove excess liquid. The mycelium was ground in a pre-cooled mortar with neutral Al_2O_3 as an abrasive and sufficient K-Pi buffer (0.1 M, pH 7) to make a thick paste. The mycelial paste was centrifuged at 20 000 g for 25 min at 5°. The supernatant (S_{20}) was decanted and either used immediately as the cell-free enzyme preparation or, more often, lyophilized and stored in an air tight container at −5°. The lyophilized extract remained active for several months.

Incubation of S_{20} with mevalonic-[2-^{14}C] acid. Each incubation flask contained 10 ml 0.08 M K-Pi buffer (pH 7), 0.15 g lyophilized S_{20} supernatant, 0.625 μCi DL-mevalonic-[2-^{14}C] acid (DBED salt), DL-mevalonic acid lactone (0.025 or 0.125 μmol) and the following cofactors: NADH (2.1 mM), NADPH (0.167 mM), glutathione (GSH) (7.5 mM), ATP (4 mM), Mn^{2+} (3.8 mM), and Mg^{2+} (3.7 mM). The final incubation medium contained 13.9 mg of protein as determined by the method of ref. [25]. Unless otherwise specified, each flask was incubated for 1 hr at 20° on a Dubnoff metabolic shaker. The reaction was terminated by bringing the incubation medium to final KOH and EtOH concns of 9 and 50%, respectively, and then heating the mixture for 30 min at 37°.

Isolation and identification of squalene-[^{14}C]. The reaction mixture was cooled and then washed 3 × with 10 ml *n*-hexane. The combined hexane washed were concd under N_2 and run on Si gel TLC developed with petrol–Et_2O–HOAc (89:10:1). Radioactive substances were detected by autoradiography or by elution from Si gel with $CHCl_3$ and counting a portion of the sample by liquid scintillation spectrometry using 5 g PPO and 0.3 g POPOP per l. of toluene as the cocktail. Squalene was also isolated in a 2% Et_2O in hexane fraction from a Si gel (60–200 mesh) column (30 × 2 cm). Two unidentified radioactive metabolites were detected on the TLC plates and were designated unknown **1** (closest to the origin) and unknown **2** (between squalene and unknown **1**). GLC analyses were conducted on a 3 m × 0.2 mm glass column packed with 3% GE SE-30 on Chromosorb Q. For GC–RC a splitter diverted 90% of the column effluent into a heated (250°) ion chamber linked to a Cary 401 Vibrating Reed Electrometer. GC–MS analyses were conducted on the same column described above.

Acknowledgements—This work was supported by grant GH-1060 from the Research Corporation. Squalene-2,3-oxide was kindly provided by Dr. H. H. Rees. We acknowledge the technical support of Ms Connie Carmack.

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