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PARTIAL PURIFICATION AND CHARACTERIZATION OF OXIDOSQUALENE-LANOSTEROL CYCLASE FROM BAKER'S YEAST

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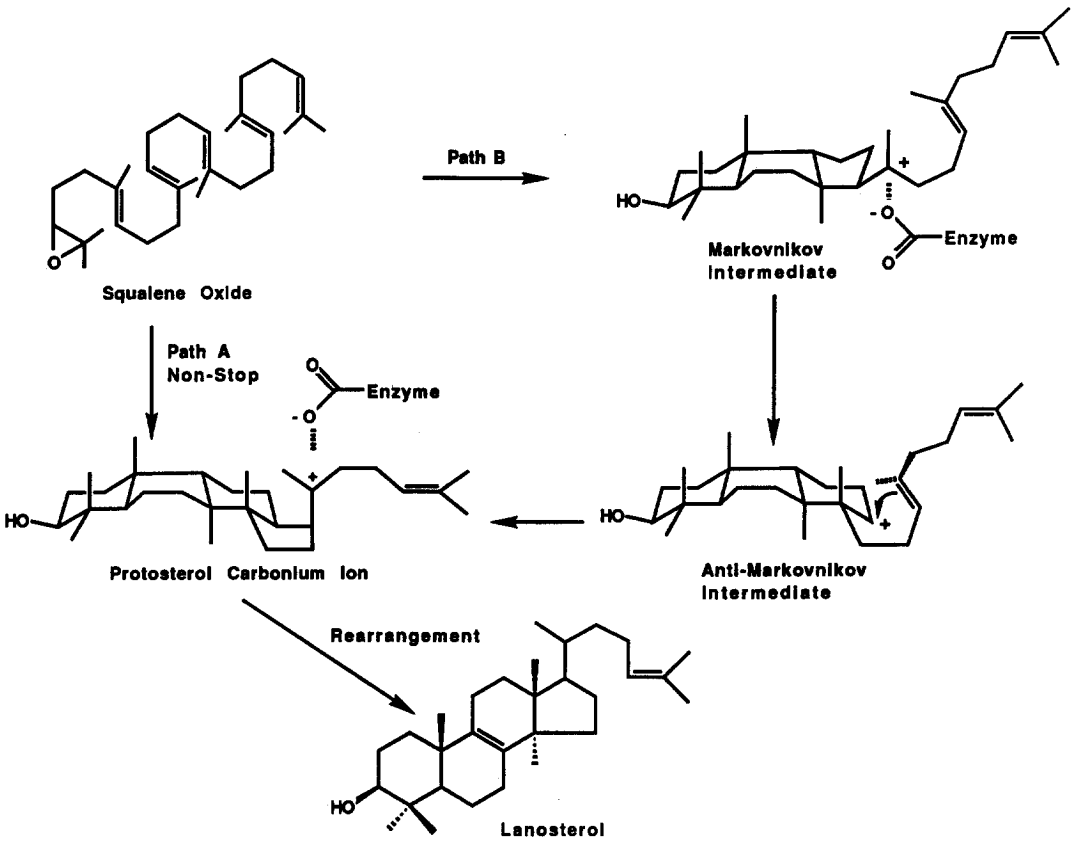
Abstract: Partial (120-fold) purification of oxidosqualene-lanosterol cyclase from yeast is described. The enzyme derived from the microsomal fraction converts 1 mM S-squalene oxide to lanosterol in 5 h and has a pH optimum of 6.2, lower than that (pH 7.2) of the hog-liver cyclase catalyzing the same reaction. Although the yeast cyclase is stimulated by high concentrations of potassium phosphate buffer, high concentrations of potassium or sodium chloride inhibit activity. The concentration range of Triton X-100 for optimal activity is 0.7-1.2%.

Oxidosqualene cyclase (O.S.C.) (2,3-epoxysqualene lanosterol-cyclase EC:1:1:14), the enzyme which mediates the conversion of (S)-2,3-epoxysqualene to lanosterol via "protosterol" intermediates (Scheme 1) is found in mammalian liver, yeast and several plant species.[1] While the liver enzyme is located in the microsomal fraction, yeast enzyme activity has been reported to reside in the supernatant after high-speed centrifugation, suggesting a soluble protein.[2] Methods for preliminary purification of O.S.C. from hog-liver have been described,[3] but no report on the purification of the yeast enzyme has appeared. It has also been found that enzyme activity was completely lost during most conventional purification procedures.[2] In this paper we disclose some characteristics of yeast cyclase and describe a partial purification of the enzyme. The availability of purified enzyme will facilitate studies on the central problem of the cyclization mechanism, *viz.* whether a distinction can be made between a non-stop anti-Markovnikov mechanism (path A, Scheme 1) or a two step mechanism implicating an intermediate (path B). The subsequent methyl migrations are predicted to take place without an isolable intermediate.Yeast cyclase is found to be localized in the microsomal fraction and the behavior of the enzyme during hydroxyapatite and ion exchange chromatography is somewhat different from that of the hog-liver enzyme. Although it has been reported that yeast cyclase is strongly inhibited in solutions of high ionic strength,[2] we find that the activity of partially purified O.S.C. is stimulated by high concentrations of potassium phosphate buffer.



SCHEME 1

Following the report that the enzyme activity of yeast cyclase is stimulated by sonication,[4,5] we first adjusted the sonication intensity until the *conversion ratio* of [R,S]-squalene oxide to lanosterol reached 35-40%. At this stage 95% of the cyclase was found to be in the supernatant after centrifugation at 20,000 G. However 80% of activity was found in the microsomal pellet at 180,000 G (Fig. 1), indicating that yeast cyclase is a membrane-bound protein, a result contrasting with the report by Shechter *et al.*,[2] who used a French press to extract the crude enzyme. The enzymatic activity of the microsomal fraction was retained after storage for 1 month at -20°.

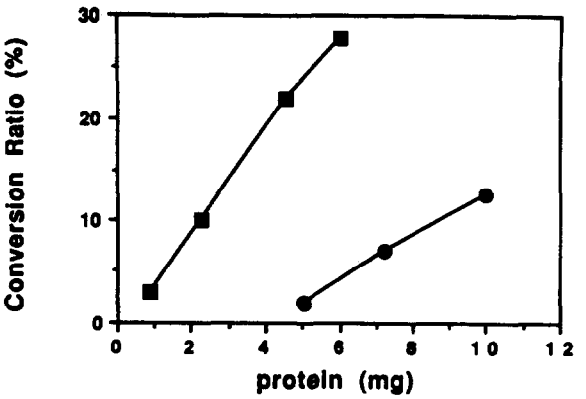


Figure 1. Distribution of cyclase between microsomal and soluble fractions: (●) soluble fraction, (■) microsomal fraction.

Maximum conversion by the supernatant of the first centrifugation was achieved after 4 hr at 37° and 9 hr at 25°. Activity was highest below pH 7.4, and decreased rapidly at pH > 8.0, indicating denaturation in basic media. When microsomal pellets were stirred or sonicated in phosphate buffer (pH 7, 0.1M) containing 0.1-0.6 M KCl or NaCl and then centrifuged, all activity remained in the microsomal fraction, indicating that the enzyme is not a peripheral protein but binds to the membrane.

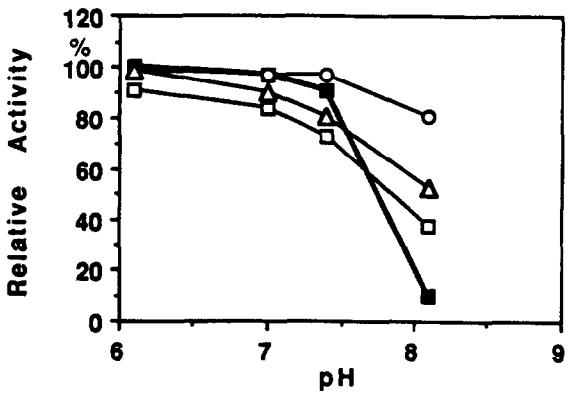


Figure 2. Effect of incubation pH on enzyme activity. Microsomal pellets were suspended and homogenized in phosphate buffer solutions at different pHs. An equal volume of suspension was added to each incubation tube, and 2 mg of [R,S]-squalene oxide with 7 mg of Triton X-100 dissolved in 1 ml of 0.1 M phosphate buffer was added. Microsomal suspensions were stored at 5-7° for (○) 1 day, (△) 2 days, (□) 3 days, and (■) 4 days prior to testing. For pH 8.1, the mixture was adjusted to pH 7 using 0.1 N HCl prior to assay.

Schecter *et al.*[2] report that Triton X-100, a neutral detergent, stimulates enzyme activity, while anionic deoxycholate does not. We investigated the effects of various types of detergents on enzymatic activity in detail in order to find optimum conditions for solubilizing the enzyme. Figure 3 shows the effect of the nonionic detergents Triton X-100 and Tween #40. Triton X-100 stimulated enzyme activity markedly compared with Tween #40, optimal concentration of Triton X-100 being around 0.7%. Increasing detergent concentration beyond this point gradually decreased enzyme activity. In order to test the solubilizing efficiency of Triton X-100, the microsomal fraction was treated with increasing concentrations of detergent and activities were measured both in the supernatants and in the precipitates after separation by centrifugation at 140,000 G

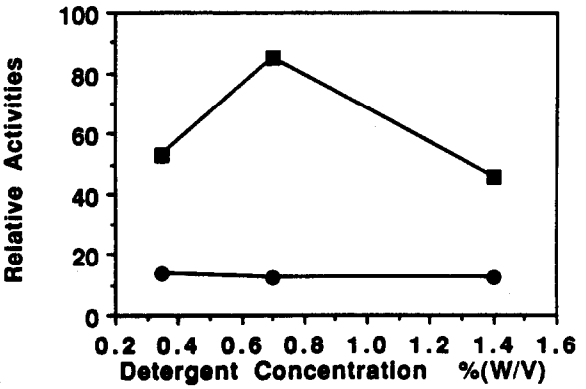


Figure 3. Effect of Triton X-100 (■) and Tween #40 (●) on microsomal enzyme activity. Conversion ratio of 35% corresponds to 100% relative activity. Microsomal pellets were first suspended in the original volume of phosphate buffer. Incubation tubes contained 1 ml of homogenized suspension and 2 mg squalene oxide dissolved in the given concentration of detergent.

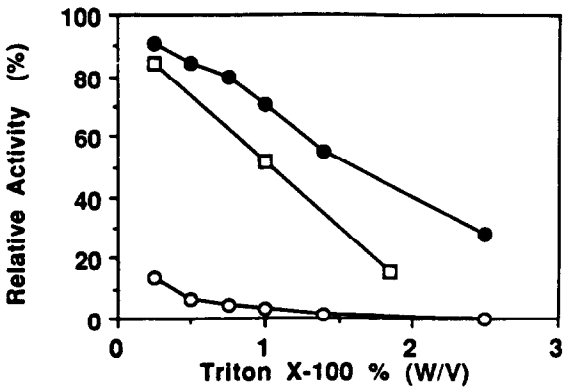


Figure 4. Solubilization of the microsomal enzyme with Triton X-100. One ml of the sonicated cell suspension was added to squalene oxide (2 mg) and Triton X-100 (7 mg) in phosphate buffer (1 ml), and incubated overnight anaerobically at 37°, giving a conversion ratio of 37% which corresponds to 100% relative activity. The microsomal pellets were treated with the given concentration of Triton X-100 in a Potter-Elvehjem homogenizer and then stirred for 2 h at 6°. The soluble and insoluble fractions were then separated by centrifugation at 45000 rpm for 90 min and bioassayed. Mixture prior to centrifugation (□), insoluble fraction (○), soluble fraction (●).

(Fig. 4). At detergent concentration of 1%,the enzyme was almost completely solubilized, but further increase of the detergent concentration caused the cyclase activity to decrease. Activities of the solubilized supernatant were higher than those of mixtures of the microsomal fraction and Triton X-100 prior to centrifugation.

The effect of CHAPS, a zwitterionic detergent with a critical micellar concentration (cmc) much higher (1%) than that of Triton X-100 (0.015%), was also examined. At concentrations below cmc, solubilization was not effective, but activity was transferred to the supernatant at and above cmc. Activity was lowered on addition of CHAPS but removal of excess detergent by dialysis of material solubilized at or below cmc restored activity to a level higher than that of the original material, indicating that denaturation had not occurred. At concentrations above cmc, activity was irreversibly reduced. The ionic detergent sodium cholate had an effect similar to that of CHAPS, solubilizing the enzyme at the cmc (0.6%), but had a much greater denaturation effect. Hence, Triton X-100 was used for all further studies.

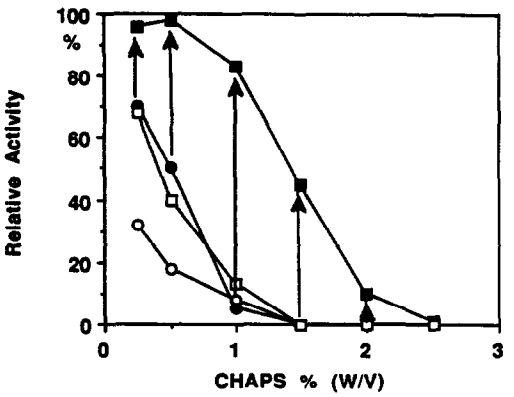


Figure 5. Solubilization of the microsomal enzyme with CHAPS in 0.1 M phosphate buffer (pH 7.0). Symbols and experimental conditions were the same as those in Fig. 4. The CHAPS soluble fraction was added to squalene oxide (2 mg) and Triton X-100 (7 mg) in 1 ml 0.1 M phosphate buffer and the soluble fractions dialyzed overnight against 0.1 M pH 7 phosphate buffer to remove excess CHAPS (■). Activity exhibited by one ml of the sonicated broken cells corresponds to 100%.

Attempts to purify the yeast cyclase by many methods, including ammonium sulfate fractionation, gel filtration, and chromatography on DEAE cellulose or hydroxyapatite with or without Triton X-100, have been reported, but activity was invariably lost.[2] We found that under certain conditions, enzyme solubilized with Triton X-100 retained activity. Gel permeation chromatography using Sephadex G-200 and G-150 with 0.06% Triton X-100 in 0.1M phosphate buffer pH 6 or 7 gave cyclase near the void volume with some loss of activity and no gain in specific activity. Efforts to purify the cyclase with DEAE exchange chromatography failed when sodium chloride was used for elution, irrespective of pH. When the same separation was carried out using 10 mM phosphate buffer, pH 6.5-7, and fractions were eluted with 0.20-0.25 M sodium chloride, activity was increased 5.5 fold, but was lost on standing at 4° for 2-3 days or at -20° overnight. However, when hydroxyapatite was used for purification, the extract adsorbed on the column in 10 mM phosphate buffer and eluted using a 0.25-0.35 M phosphate buffer gradient, in contrast to the hog-liver cyclase which is not retained in this system,[8] yeast cyclase activity increased by 4.6 fold with a recovery of activity of 76%. Approximately 40-60% of cyclase activity was lost on standing for 4 days at 6-9°. Addition of dithiothreitol (1mM) and phenylmethylsulfonylfluoride (1 mM) did not stabilize the enzyme. These studies indicated that sodium chloride had a detrimental effect on enzyme activity and that phosphate buffer was a superior medium. Experiments performed to determine the effect of phosphate buffer and sodium chloride concentrations indicated that reasonably high concentrations of phosphate both activated and stabilized activity which could be retained for months at -20° - or -80° if extracts were stored in phosphate buffer solution of high concentration.

With CM Sepharose, almost all proteins solubilized with Triton X-100 were not retained, resulting in little loss of activity but little gain in specific activity, in contrast to hog liver cyclase, which completely loses activity under these conditions.[6] The lack of affinity of yeast cyclase for the column suggests that the enzyme is negatively charged even at low pH (<6). Pentyl-agarose chromatography was also found to be ineffective. However, using gradient elution with phosphate buffer, we succeeded for the first time in the application of DEAE Sephacel chromatography with an average purification factor of 2.5 and with a recovery of 60%, active fractions eluting at phosphate buffer concentrations of 0.25-0.35M. For final purification, gel permeation HPLC was performed using Macrosphere GPC 300 with a phosphate buffer Triton X-100 mobile phase. Good separation was obtained resulting in a 6.5 fold increase in activity over DEAE sephacel chromatography. A summary of the purification methods is shown in Table 1. We found it preferable to solubilize 1.8 g of microsomal protein in 200 ml of phosphate buffer containing Triton X-100 (1%), which gave a 60% recovery of activity. Recovery could be increased to 85% using much larger volumes of detergent solution.

Table 1

Purification of Yeast Cyclase

Source	Protein	Activity Recovered (%)*	Specific Activity	Purification Factor
Microsome	1.72 g	100	4500	1
Solubilization	0.67 g	60	3000	1.5
Hydroxyapatite	113 mg	45.6	650	6.9
DEAE Sephacel	28.6 mg	27.0	260	16.2
HPLC (GPC 300)	3.1 mg	20.0	40	112.5

*Specific activities are expressed in terms of protein (mg) necessary for 20% conversion ratio. Assays were performed in 0.3 M phosphate buffer.

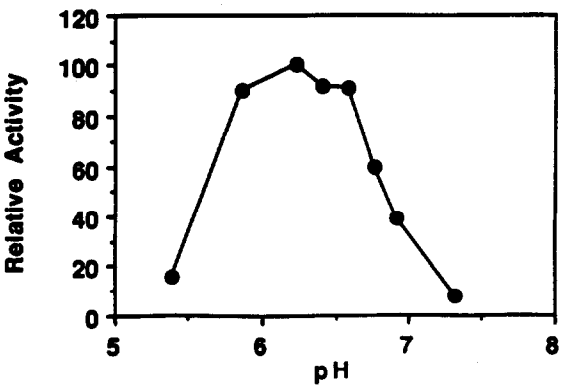


Figure 6. Determination of optimal pH for cyclase activity. The partially purified enzyme from hydroxyapatite was employed. Assays were performed in 0.3 M potassium phosphate buffer at each pH.

Several chemical modifications were carried out in order to access the catalytic site(s) of yeast cyclase. As in the case of hog-liver cyclase,[6] N-ethylmaleimide inhibits enzyme activity, indicating involvement of a thiol group. The yeast cyclase was also strongly inhibited by cysteine-modifying reagents such as p-chloromercuribenzenesulfonic acid and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). The yeast cyclase is negatively charged at pH 6 as described above, and addition of N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline reagent (EEDQ), a carboxylic group modifier, inhibited activity slightly (20%). The failure of diethylpyrocarbonate and phenylmethylsulfonylfluoride to inhibit the yeast cyclase indicated that histidyl and serine hydroxyl groups respectively are not necessary for activity. The yeast cyclase was not inactivated by EDTA (1 mM). These findings coincide with those for liver cyclase. Hog liver cyclase is stable for 40 days at 4°,[6] but yeast cyclase is unstable at this temperature and becomes even more unstable as the purification proceeds. This instability may have caused the difficulties encountered by others in the purification of this enzyme.

Table 2

Effects of Various Reagents on Yeast Cyclase

Reagent	Concentration mM	Functional Group Inhibited	Cyclase Inhibition (%)
p-Chloromercuri-benzenesulfonic acid	5	Cys-SH	100
	0.5		96
DTNB	10	Cys-SH	100
	1		95
N-Ethylmaleimide	5	Cys-SH	81
	1		54
EEDQ	1	-COOH	21
	0.25		10
Diethylpyrocarbonate	4	His-imidazole	0
	0.25		0
Phenylmethylsulfonyl fluoride	1	Ser-OH	0

EEDQ: N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline.
DTNB: 5,5'-dithio-bis(2-nitrobenzoic acid).

Electrophoresis of the hog liver cyclase in the presence of sodium dodecyl sulfate indicated a major band at 90 kDa. SDS-PAGE electrophoresis of yeast cyclase, however, displays no bands at this region, although bands at approximately 40 kDa and 55 kDa are enriched as purification proceeds. Very recently the cyclase of squalene oxide-cycloartenol was purified[11] and the molecular weight found to be 55 kDa from SDS-PAGE electrophoresis, while from HPLC a value of 100 kDa was estimated, indicating dimerization in solution.

In conclusion, the prognosis for obtaining highly purified yeast cyclase is now excellent. Further studies on the purification including the development of affinity chromatography are in progress.

Experimental

Materials: Triton X-100, sodium cholate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propansulfonate (CHAPS), DEAE Sephacel and CM Sepharose were obtained from Sigma. Hydroxyapatite (Bio-gel HT) was obtained from Alltech Associates, Inc. (IL USA). 2,3-Epoxysqualene was synthesized from squalene according to the method of Nadeau *et al.*[12]

Assay Method: Squalene oxide (2 mg) was emulsified by Triton X-100 (7 mg) in 1 ml of potassium phosphate buffer, pH 6.2, 1 ml of enzyme fraction was added, the solution was flushed with nitrogen and then incubated for 15 hr at 37° under anaerobic conditions. To terminate the incubation, 3 ml of 15% methanolic KOH solution was added and the incubation tube was heated to 70° for 10 min to saponify the reaction mixture, followed by extraction with 5 ml of hexane. The product, lanosterol, was analyzed by HPLC (4.7 mm I.D. X 25 cm 5 μ silica gel, Alltech, n-hexane:2-propanol (100:2) mobile phase at 1 ml/min, UV detection at 215 nm).

Preparation of crude enzyme: Sixty g Baker's yeast (Fleischmann's Yeast Inc. CA, USA) suspended in 300 ml 100 mM phosphate buffer (pH 7.4) was stirred at 40° to homogeneity, cooled to <10° and sonicated (Heat Systems-Ultrasonic, Inc., Model 200R) using a large titanium tip (sonication intensity 180 W) for 90 min in the continuous sonication mode. After sonication, the pH of the suspension was 6.5-6.7.[4,5] Assay of 1 ml of the broken cells gave 35-40% of lanosterol from R,S-2,3-epoxysqualene. Broken cell suspensions were centrifuged at 13,000 G for 15 min to remove debris. The supernatant was further centrifuged at 140,000 - 180,000 g (for 90 min) using a preparative ultracentrifuge (Beckman, Model L3-50, SW 50.1 rotor), to separate the microsomal fraction and the soluble fraction. The microsomal fraction was then resuspended and homogenized in the original quantity of phosphate buffer using a Potter-Elvehjem homogenizer for assay. For preparation of large amounts of microsomal fraction, a 45 T1 rotor was used at 37,000 rpm for 90 min.

Enzyme purification: Material from three batches of sonicated suspension was resuspended in 0.3 M sodium chloride using a Potter-Elvehjem homogenizer and recentrifuged at 140,000 - 180,000 G for 90 min to remove peripheral protein. The microsomal fraction was then homogenized as before in 190 ml of 0.1 M phosphate buffer containing 1.0% Triton X-100 and stirred overnight. After recentrifugation at 37,000 rpm using a 45 T1 rotor, the solubilized enzyme was applied to a 2.5 X 35 cm hydroxyapatite column which had been previously equilibrated with 10 mM phosphate buffer (pH 6.2) containing Triton X-100 (0.06%). The column was washed with the same buffer containing Triton X-100, and the enzyme was eluted with a linear gradient (10 mM-0.5M phosphate buffer) containing 0.06% Triton X-100. The active milky-white fractions were combined and dialyzed against 15 mM phosphate buffer (pH 6.2) for 12 h at 5° and then applied to a DEAE Sephacel column (1.5 X 28 cm) which had previously been equilibrated with 15 mM phosphate buffer containing 0.06% Triton X-100, and washed with the same Triton X-100 solution. The enzyme was eluted with a linear phosphate buffer gradient (15 mM-0.5 M) containing 0.06% Triton X-100 and the transparent active fractions were combined and purified by gel permeation HPLC (Alltech GPC 300, 4.7 mm I.D. X 25 cm) using 15 mM phosphate buffer containing 0.06% Triton X-100 at 0.4 ml/min flow rate as mobile phase.

Protein assay: Protein was determined according to the method of Lowry modified as described by Schacterle and Pollack[7] using bovine serum albumin as standard.

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