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PURIFICATION OF SQUALENE EPOXIDASE FROM RAT LIVER MICROSOMES

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SUMMARY: Squalene epoxidase was purified from rat liver microsomes by DEAE-cellulose, alumina C_γ gel, hydroxylapatite, CM-Sephadex C-50 and Cibacron Blue Sepharose 4B in the presence of Triton X-100. The specific activity was increased 50 fold with a yield of about 10 %. On SDS-polyacrylamide gel electrophoresis, the preparation gave one major band and one minor band with apparent molecular weights of 47,000 and 27,000 daltons, respectively. The protein of 47,000 was the most probable candidate for squalene epoxidase. Squalene epoxidase activity could be reconstituted in the squalene epoxidase preparation with the addition of NADPH-cytochrome P-450 reductase, FAD, and Triton X-100.

INTRODUCTION

The epoxidation of squalene is known to be the first step among several oxygenase-catalyzed reactions in cholesterol biosynthesis (1). Our previous reports confirmed that two microsomal components are required for squalene epoxidase activity, one of which was identical with NADPH-cytochrome P-450 reductase (2,3). Though a terminal oxidase of squalene epoxidation has been partially characterized (2), no evidence has been found for the participation of a cytochrome P-450 species (4,5). In addition, the Triton X-100 solubilized enzyme system required FAD for enzyme activity (2,3).

Despite extensive efforts, squalene epoxidase has not yet been purified to homogeneity. By using Cibacron Blue Sepharose 4B as an affinity adsorbent (6,7), we have developed a purification method for squalene epoxidase. This communication reports the method and some properties of the purified squalene epoxidase.

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METHODS

Solubilization of rat liver microsomes with Triton X-100 and initial DEAE-cellulose chromatography of the supernatant have been previously described (2).

Purification of NADPH-cytochrome P-450 reductase: The reductase fraction eluted in the initial DEAE-cellulose chromatography was further purified on a 2',5'-ADP Sepharose 4B column according to Yasukochi and Masters (8). The specific activity of the purified reductase was 48 μmol of cytochrome c reduced/min/mg of protein.

Preparation of squalene epoxidase: Buffers designated as A and B were potassium phosphate, pH 7.4 and Tris-HCl, pH 7.4, respectively. All buffers contained 0.5 % Triton X-100, 1 mM EDTA and 1 mM DTT through all purification procedures. The first DEAE-cellulose fraction (300-400 ml), which was contaminated with NADH-ferricyanide reductase and cytochrome b_5, was treated with alumina C$_\gamma$ gel (40 ml/g) by a batch method and a squalene epoxidase active fraction was eluted with 200 mM Buffer A (150 ml) after extensive washing with 70 mM Buffer A. The dialyzed alumina C$_\gamma$ gel fraction was applied to a hydroxylapatite column (bed volume, 40 ml) and then eluted with a linear gradient consisting of 50 mM Buffer A (150 ml) and 300 mM Buffer A (150 ml). The dialyzed hydroxylapatite fraction was applied to a CM-Sephadex C-50 column (bed volume, 25 ml) and then eluted with a linear gradient consisting of 10 mM Buffer A, pH 7.2 (100 ml) and 300 mM Buffer A, pH 7.2 (100 ml). The dialyzed CM-Sephadex C-50 fraction was applied to a second DEAE-cellulose column (bed volume, 10 ml) previously equilibrated with 20 mM Buffer B. Squalene epoxidase was recovered as the unadsorbed fraction. The fraction was then applied to a Cibacron Blue Sepharose 4B column (bed volume, 7 ml). After extensive washing of the column with 0.2 M KCl-20 mM Buffer B, squalene epoxidase was eluted with 1.0 M KCl-20 mM Buffer B. The Blue Sepharose 4B fraction was dialyzed against 20 mM Buffer B and stored at -70° until use.

Enzyme assay: The reaction mixtures containing 20 μmol of Tris-HCl buffer, pH 7.5, 1 μmol of NADPH, 0.01 μmol of FAD, 12 nmol of 11-^{14}C squalene (20,000 cpm) dispersed with the aid of Tween 80, 0.2 unit of NADPH-cytochrome P-450 reductase, and the squalene epoxidase fraction (10-100 μg of protein) in a final volume of 1.0 ml were incubated for 30 min at 37° . Radioactive sterols and 2,3-oxidosqualene were separated from squalene by silica gel thin layer chromatography and counted as previously described (4). Specific squalene epoxidase activity is expressed as the sum of nanomoles of product formed/30 min/mg of protein.

Analytical methods: The protoheme content was determined by the conventional pyridine ferrochrome method (9). Cytochrome P-450 species were determined by the method of Omura & Sato (9) using an extinction coefficient of 110 $mM^{-1}cm^{-1}$ and by its soret band at 418 nm using an extinction coefficient of 107 $mM^{-1}cm^{-1}$. Protein was determined by the method of Lowry et al.(10) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Purification and molecular weight of squalene epoxidase

The data for the purification of squalene epoxidase are summarized in Table I. The over all recovery of squalene epoxi-

TABLE I

SUMMARY OF PARTIAL PURIFICATION OF SQUALENE EPOXIDASE

	Protein	Squalene Epoxidase Total Act.	Specific Act.	Yield
	mg	nmol/30min	nmol/30min/mg	%
First DEAE-cellulose	782	1251.2	1.6	100
Alumina C_γ gel	159	699.6	4.4	55.9
Hydroxylapatite	63	598.5	9.5	47.8
CM-Sephadex C-50	15	298.5	19.9	23.8
Second DEAE-cellulose	6.7	244.6	36.5	19.5
Blue Sepharose 4B	1.3	108.6	83.5	8.7

dase yielded 8.7 % and the specific activity was increased 50 fold. Alumina C_γ gel achieved a complete removal of cytochrome b_5 and a hydroxylapatite column was effective in removing contaminating NADH-ferricyanide reductase. Squalene epoxidase is quantitatively bound to Blue Sepharose. We were unable to elute squalene epoxidase from Blue Sepharose with either ATP or NAD alone at 10 mM in 0.5 % Triton X-100. However, we found that squalene epoxidase could be desorbed from the resin by KCl (all at over 0.6 M in 0.5 % Triton X-100), suggesting that squalene epoxidase is adsorbed to Blue Sepharose by ionic interactions. Its affinity to Cibacron Blue Sepharose 4B column suggested that squalene epoxidase might have dinucleotide binding sites (6).

The patterns of SDS-polyacrylamide gel electrophoresis in each step are shown in Fig. 1 (Left). The final preparation stored in buffer containing 0.5 % Triton X-100 revealed one major and one minor band estimated to have apparent molecular weights of 47,000 and 27,000 daltons, respectively. To obtain further purification, the Blue Sepharose 4B fraction (1.2 mg of protein) was treated with 2.4 mg of sodium cholate and then applied to a CM-Sephadex C-50 column. As shown in Table II, squalene epoxidase was recovered in three fractions. The loss of activity in these procedures may be explained by the fact that ionic detergents inhibit the enzyme

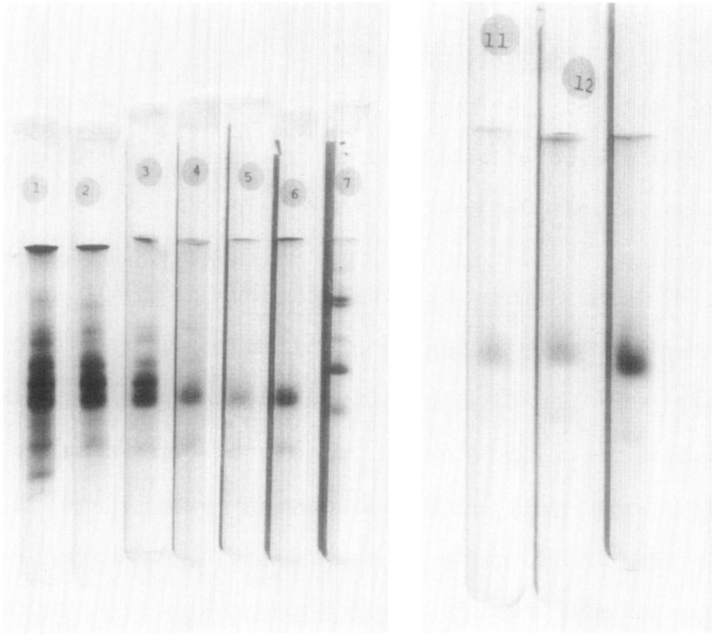

Fig. 1 SDS-polyacrylamide gel electrophoresis of squalene epoxidase fractions.
(Left) 50 µl of samples in the purification steps were subjected to electrophoresis on 5 % gel in the presence of 0.1 % SDS. 1, First DEAE-cellulose fraction; 2, alumina Cγ gel fraction; 3, Hydroxylapatite fraction; 4, CM-Sephadex C-50 fraction; 5, Second DEAE-cellulose fraction; 6, Cibacron Blue Sepharose 4B fraction; 7, Standard proteins (phosphorylase b, rat serum albumin, ovoalbumin, carbonic anhydrase and soybean trypsin inhibitor).
(Right) 50 µl of fraction I, II, and III from the second CM-Sephadex C-50 chromatography were subjected to electrophoresis on a 5 % gel in the presence of 0.1 % SDS. From left to right, fraction III, II and I.

TABLE II

CM-SEPHAROSE C-50 CHROMATOGRAPHY OF BLUE SEPHAROSE 4B FRACTION AFTER TREATMENT WITH SODIUM CHOLATE

	Protein	Squalene Epoxidase Activity	
		Total Activity	Specific Activity
	mg	nmol/30min	nmol/30min/mg
Blue Sepharose 4B Fr.	1.2	100.2	83.5
CM-Sephadex C-50 Fr.			
I. Unadsorbed elute	0.76	27.7	29.9
II. 100 mM Buffer A elute	0.26	14.4	55.9
III. 150 mM Buffer A elute	0.20	21.3	106.5
Yield	1.2	63.3	

activity (2,11). Among the three fractions, fraction III has the highest specific activity compared to the other two fractions (II and I). The SDS-polyacrylamide gel electrophoresis of fraction III revealed only a single band with an apparent molecular weight corresponding to 47,000 daltons (Fig. 1 Right).

Properties of Blue Sepharose 4B fraction

As shown in Table III, the specific content of MW. 47,000 protein moiety in the Blue Sepharose 4B fraction was calculated to be from 13.8 to 16.7 nmol/mg of protein by densitometry of the SDS-polyacrylamide gel. Protoheme content was estimated to be from 0.7 to 0.9 nmol/mg of protein. Analyses of cytochrome P-450 species by a CO reduced minus oxidized difference spectrum revealed only 0.1 nmol per mg of protein.

Although cytochrome P-450 species in rat liver microsomes have been reported with molecular weights ranging from 53,000 to 44,000 (12,13), it seems most improbable that cytochrome P-450 is involved in squalene epoxidation. First, the specific content of protoheme does not run in parallel with the specific activity of the enzyme through the purification scheme. Secondly, carbon monoxide does not inhibit squalene epoxidation. Thirdly, glycerol

TABLE III

PROPERTIES OF BLUE SEPHAROSE 4B FRACTION

	Preparations A.	B.
Squalene Epoxidase Activity (nmol/30 min/mg)	83.5	70.5
Protoheme as pyridine hemochromogen (nmol/mg)	0.9	0.7
Cytochrome P-450 species by CO red. minus oxid. (nmol/mg)	0.1	0.1
Soret band OD 418nm (nmol/mg)	0.8	0.7
Specific Content of proteins by Densitometry (nmol/mg) MW.47,000	16.5	13.8
MW.27,000	6.0	4.2

TABLE IV

COFACTOR REQUIREMENTS

	Squalene Epoxidase Activity
	nmol/30 min
Complete[a]	3.62
minus Epoxidase fraction	0.10
minus NADPH-cytochrome P-450 reductase	0.05
minus NADPH	0.05
minus FAD	0.21

a; Mixtures for enzyme assay contained 20 mM Tris-HCl buffer, pH 7.5, 42 ug of Blue Sepharose fraction, 10^{-5}M FAD, 1 mM NADPH, 1 mM EDTA, NADPH-cytochrome P-450 reductase (0.2 unit), and 12 nmol of 11-[C^{14}] squalene. Final Triton X-100 concentration was 0.035 %.

which is known to stabilize cytochrome P-450 species (14) is lacking in buffers throughout the purification steps of squalene epoxidase.

Reconstitution of squalene epoxidase activity

Squalene epoxidase activity was dependent upon the Blue Sepharose 4B fraction and NADPH-cytochrome P-450 reductase (Table IV). In addition, free FAD is absolutely required for manifestation of activity. Under assay conditions, final concentration of Triton X-100 was 0.035 %. Different from our previous reports on the first DEAE-cellulose fraction (2,3), 0.3 % Triton X-100 is not optimal but slightly inhibitory in the Blue Sepharose 4B fraction. The Blue sepharose 4B fraction in 0.5 % Triton X-100 buffer is stable at -70° for several weeks. Removal of Triton X-100 from the Blue Sepharose 4B fraction caused a complete loss of enzyme activity and attempts to regain activity by the addition of either Triton X-100 or phospholipids were unsuccessful.

In the absence of more direct information on the identity of squalene epoxidase, its FAD requirement and affinity to Cibacron Blue Sepharose 4B strongly suggest that the prosthetic group of squalene epoxidase is dissociable FAD. The squalene epoxidase prepared in this work may be an apoprotein.

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