SOLUBILIZATION AND PARTIAL PURIFICATION OF HEPATIC 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE[1]

Renu A. Heller and R. Gordon Gould, Department of Medicine
Stanford University School of Medicine, Stanford, California, 94305

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SUMMARY: This paper describes an effective method for the solubilization of microsomal HMG-CoA reductase from rat liver. Exposing the microsomes to a freeze-thaw treatment solubilized 80% of the microsomal reductase activity. Subsequently, a 25-fold purification has led to an enzyme preparation with a specific activity of 10-14 nmoles MVA per min per mg of protein and an increased stability.

3-Hydroxy-3-methylglutaryl Coenzyme A reductase catalyzes the conversion of HMG-CoA[2] to mevalonic acid, which is the rate-limiting step in the biosynthesis of cholesterol from acetate in liver (1,2).

Purification of the reductase from high-speed supernatant fractions of cell-free extracts of yeast (3,4) and Pseudomonas (5) has been described, but in mammalian liver the enzyme is associated with the microsomal fraction and is insoluble. Acetone powder extracts of hepatic microsomes were reported to solubilize the reductase activity (6), although later application of the same technique in our and in other laboratories has not been successful (7). Kawachi and Rudney (7) and Higgins et al. (8) have reported solubilization by using high concentrations of deoxycholate; however, the yield and specific activities of the solubilized and purified enzyme preparations are low. In view of these limitations and because of the desirability of obtaining a soluble preparation of this enzyme with as little loss in activity as possible we undertook the development of new methods of solubilization.

MATERIALS AND METHODS

Microsomes were prepared from livers of six male Sprague-Dawley rats

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2. Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-Coenzyme A; MVA, mevalonic acid; DTT, dithiothreitol.

fed ad libitum and weighing 300-350 gm. The rats were housed under daily light schedules of lights on from 4 p.m. to 4 a.m. for at least 3 weeks. Livers were excised from rats sacrificed at 10 a.m. at which time HMG-CoA reductase activity was at its peak (9). Livers were weighed and homogenized in a Potter-Elvehjem homogenizer in 3 volumes by weight of buffer containing 0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4, 0.03 M EDTA, and 1 mM DTT at pH 7.2, and the same buffer was used throughout the purification procedure described. The homogenate was centrifuged twice for 15 min at 10,000 x g, and the pellets discarded. The supernatant was centrifuged for one hour at 100,000 x g, and the microsomal pellet resuspended in 1/3 the original volume of buffer and centrifuged again at 100,000 x g for one hour. The resulting microsomal pellet was transferred to a pyrex tube and frozen in a dry ice-alcohol bath, a modification of the method of Shapiro (10), and stored at -20°C. Enzyme activity from freshly prepared microsomes was assayed by suspending an aliquot taken from the microsomal pellet in buffer before it was frozen.

Assays of HMG-CoA reductase activity were carried out in a total volume of 1.0 ml containing 1 μmole DTT, 30 μmoles EDTA, 70 μmoles NaCl, 2 enzyme units of glucose-6-phosphate dehydrogenase, 30 μmoles glucose-6-phosphate, 3 μmoles NADP, and 300 nmoles DL-HMG-3-^{14}C-CoA (specific activity 460 dpm/nmole). All incubations were carried out at 37°C. Under these conditions of assay HMG-CoA reductase activity is expressed as nanomoles of MVA formed per min per mg of protein.

The reaction was stopped by addition of 0.5 ml of 10% NaOH and mevalonate extracted as described previously by Edwards and Gould (9). Protein was measured by the biuret technique (11) using bovine serum albumin as the standard. To prevent interference by DTT in the biuret protein determination the proteins were precipitated with 5% trichloroacetic acid and redissolved in 0.5 M NaOH. This procedure was repeated twice to assure removal of all DTT. Prior to carrying out the biuret reaction the microsomal proteins were solubilized by the addition of 10% deoxycholate solution.

Electrophoresis of the purified enzyme preparation was carried out by using the Ortec 4200 electrophoresis system[3]. Acrylamide gel slabs, 6%, were made using 0.375 M tris-sulfate at pH 9.0 as the gel buffer and 0.065 M tris-borate at pH 9.0 as the tank buffer. Protein was precipitated with 12.5% trichloroacetic acid and stained with 0.25% Coomassie brilliant blue.

RESULTS AND DISCUSSION

Kinetic Properties of HMG-CoA reductase: Using the assay system described above, MVA formation was found to be linear up to 6 mg of protein per assay. The reaction proceeds at a constant rate for 20 min with microsomes as the source of the enzyme and 50 min with soluble enzyme at 37°C using less than 5 mg of the enzyme protein.

Preparation of soluble HMG-CoA reductase: All attempts to solubilize HMG-CoA reductase were carried out on the microsomal pellet. Efforts to solubilize the enzyme with low concentrations of taurocholic acid, changes in pH, in the presence or absence of salt, or quick freeze-thawing were unsuccessful. However, we have found that the activity can be obtained in soluble form by slow freezing and thawing. The freshly prepared microsomal pellet was frozen in a dry ice-alcohol bath by reducing the temperature at a rate of 8-10°C per min down to -50°C. The pellet was then thawed, suspended in buffer, and centrifuged at 100,000 x g for one hour and the supernatant removed for assay. About 80% of the activity was present in the supernatant and 20% in the residual pellet. For all experiments the microsomal preparations were frozen as described above and left overnight at -20°C. Soluble enzyme was prepared the following day.

Purification of the solubilized reductase: The described purification procedure has been repeated five times with consistent results. Data from a typical preparation are presented in Table I. The specific activity of the reductase in freshly prepared microsomes ranges between 0.4 to 0.7 nmoles min^{-1} mg^{-1}. Solubilization leads to a 3-4 fold purification so that the

3. Ortec, Incorporated, 100 Midland Road, Oakridge, Tennessee, 37830.

TABLE I

PURIFICATION OF HMG-CoA REDUCTASE FROM RAT LIVER

Fraction	Total activity (nmoles MVA min^{-1})	Total protein (mg)	Specific activity (nmoles MVA min^{-1} mg^{-1} protein)	Relative specific activity	Yield (%)
Microsomal suspension	368	659	0.558	1	100
Frozen-thawed suspension	110	538	0.204	0.36	37
Soluble supernatant	80	40	2.0	3.5	27
35-50% $(NH_4)_2SO_4$ fraction	66	13	5.1	9.1	22
Agarose fraction	59	---	13.6	24.3	19

The solubilization and purification procedure is described under Results. Assay for HMG-CoA reductase is described under Methods. The pellet obtained from a 1 hr 100,000 x g centrifugation of the frozen-thawed suspension contained 34 nmoles min^{-1} of enzyme activity and had a specific activity of 0.076 nmoles min^{-1} mg^{-1}. The yield of enzymatic activity from the agarose column is based on the total recovery while the specific activity is that of the peak tube representing 23% of the total recovered activity.

specific activity of the enzyme in the soluble supernatant is around 2 nmoles min^{-1} mg^{-1}. Freshly prepared microsomes can be frozen by the technique described above and stored in a -20°C freezer as a source of soluble enzyme.

Further purification was achieved by fractionating the clear supernatant with the addition of solid $(NH_4)_2SO_4$. Precipitates were collected between 0-35; 35-50; 50-75; and 75-100% concentrations of $(NH_4)_2SO_4$ by centrifugation at 20,000 x g for 10 min and dissolved in buffer. The protein from the supernatant fractionated equally between the above 4 fractions; however, 82-90% of the HMG-CoA reductase activity was recovered in the 35-50% frac-

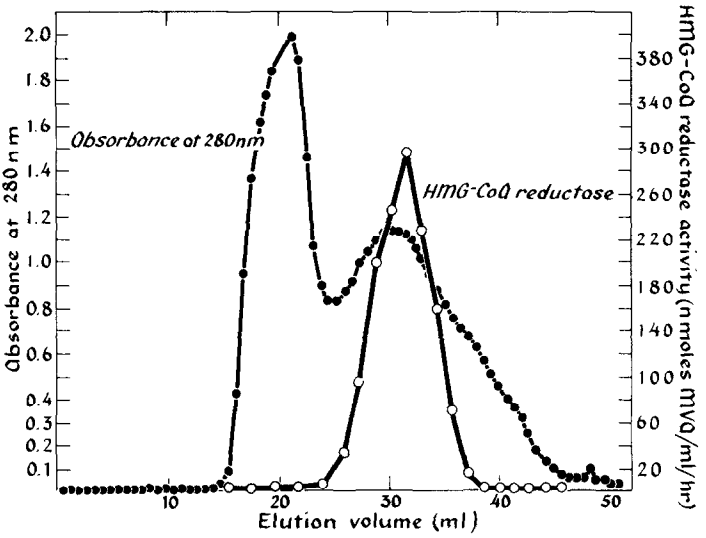

Fig. 1. Gel filtration pattern of the 35-50% $(NH_4)_2SO_4$ fraction prepared as described under Results. The column (6% agarose, 100-200 mesh, 1 cm x 53 cm, void volume = 20 ml) was equilibrated and eluted with buffer containing 0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4, 0.03 M EDTA, and 1 mM DTT at pH 7.2.

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tion and resulted in a 3-4 fold purification with a specific activity between 3 to 5 nmoles min^{-1} mg^{-1}. Alternatively, a 30 to 50% $(NH_4)_2SO_4$ precipitate recovers all the reductase activity present in the soluble supernatant, but with only a 2-fold purification. The enzyme is not stable at this stage and rapidly loses activity. In one preparation the specific activity dropped from 5.0 to 2.0 overnight.

The 35-50% $(NH_4)_2SO_4$ fraction was further purified by chromatography on 6% agarose gel column (Bio-Gel A-5m, Bio-Rad Laboratories) and the elution profile is shown in Fig. 1. Reductase activity emerged from the column well separated from the void protein and peaked at an $\frac{Ev}{E_{vo}}$ ratio of 1.6, which suggests a molecular weight of about 200,000. The pooled fractions contained 85-90% of the added activity and gave a 3-fold increase in specific activity. The enzyme as it comes off the agarose column is relatively stable and retains 50% of the reductase activity after a one-week storage in ice. This

stability may relate to the loss of proteases or inactivators during gel filtration.

Polyacrylamide gel electrophoresis of 12 to 24 μg of protein from the purified enzyme preparation was carried out. Enzyme with a specific activity of 13.5 nmoles min^{-1} mg^{-1} applied to the gels exhibited one major protein band, one minor band and 4 very faint ones.

We have reported here a very simple method for the effective solubilization of membrane-bound HMG-CoA reductase. This technique avoids use of detergents for solubilization which inhibit enzyme activity (12) and have to be eliminated before the activity can be measured. It is noteworthy that the rate of MVA formation as a function of time is linear for 50 min with soluble enzyme but only 20 min with enzyme in the crude microsomal form. We are at present unable to answer whether this data reflects increased stability of the solubilized enzyme in the presence of substrate and co-factors or is due to the loss of some membrane-bound inactivators.

Purification of the solubilized enzyme reported here results in a preparation with specific activities ranging between 10 and 14 nmoles min^{-1} mg^{-1}, which are 2-3 times higher than those previously reported (7,8). Our electrophoretic pattern of the purified enzyme as stated above showed one major band, one minor band, and 4 very faint ones. In contrast to these results, Higgins *et al.* (8) indicated that disc gel electrophoresis of 40 μg of protein with a specific activity of only 5 nmoles min^{-1} mg^{-1} exhibited 2 protein bands. This discrepancy should be taken as a caution in interpreting homogeneity of enzyme molecules as indicated by disc gel electrophoresis. Experiments to localize the enzyme activity in one or more of the protein bands seen on electrophoresis as well as further purification of the enzyme to homogeneity are underway.

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