PURIFICATION AND CHARACTERIZATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE FROM CHICKEN LIVER

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1. Introduction

It is widely recognized that 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, mevalonate: NADP oxidoreductase, EC 1.1.1.34), which catalyzes the reduction of hydroxymethylglutaryl CoA to mevalonate, is the rate-limiting enzyme in hepatic cholesterogenesis. Over the last few years a number of reports and reviews have detailed the biochemical and physiological properties of this enzyme [1–7]. Studies of hepatic HMG-CoA reductase have been carried out principally with the rat species. A number of procedures have been reported for solubilization and characterization of HMG-CoA reductase from liver microsomes [8–16]. Two laboratories have recently the isolation of highly purified HMG-CoA reductase from rat liver [9,15]. The procedures used in these studies, however, required multiple fractionation steps, were time-consuming, and had a relatively low yield of purified enzyme. The present report describes the purification of HMG-CoA reductase from chicken liver. The isolation procedure for HMG-CoA reductase solubilized from liver microsomes, outlined in this report, is relatively simple and can be completed within six to eight hours. The purified enzyme is obtained in high yield and is electrophoretically homogeneous.

2. Materials and methods

2.1. *Assay of HMG-CoA reductase activity*

Assay of HMG-CoA reductase activity was performed in 1.5 ml microfuge tubes (Brinkman Instruments) in 0.22 ml buffer containing 100 mM KH_2PO_4 (pH 7.2), 10 mM dithiothreitol (DTT), 4.5 mM NADP, 45 mM glucose 6-phosphate, 0.6 units of glucose 6-phosphate dehydrogenase (Sigma) and 0.4 mM DL-HMG-[3-^{14}C]CoA (New England Nuclear, 1000 dmp/nmol). The samples were incubated in a Dubanoff shaker for 30 min at 37°C, and the reaction terminated by the addition of 0.02 ml 10 N HCl. The samples were incubated for an additional 30 min at 37°C, centrifuged at 12 000 × *g* (Beckman Instruments, microfuge) for 1 min, and 0.1–0.2 ml aliquots were applied to columns (0.5 × 4 cm) of BioRex 5 resin (100–200 mesh, chloride form, Bio-Rad) for elution of [^{14}C]mevalonolactone as previously reported [17,18].

2.2. *Preparation of microsomes*

Livers from laying hens were obtained from a local abattoir, transported to the laboratory on ice, and used within two hours. The tissue (6.8 kg) was homogenized in a Waring blender (CB-2) for 1 min in 3 vol. (3 ml buffer/g wet wt tissue) of buffer containing 100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4, 30 mM EDTA and 2 mM DTT, pH 7.2 (buffer A). The homogenate was centrifuged at 12 000 × *g* (Sorval RC 2-B) for 15 min. The supernatant solution was recentrifuged to clarify the homogenate and the pellets discarded. The microsomes were separated from the supernate by centrifugation in an RK continuous flow centrifuge (Electronucleonics, Inc.) at 90 000 × *g* in rotor #RK3. Sixteen liters of supernate were processed in approximately four hours. Following centrifugation, the microsomal pellet (550 g) was removed and resuspended in 1.8 liters of buffer A. The microsomes were recentrifuged at 100 000 × *g* (Beckman L2-65B) for 90 min. The isolated microsomes were

frozen, either on dry ice or by storage in a freezer at −20°C.

2.3. *Solubilization of HMG-CoA reductase*

Solubilization of HMG-CoA reductase was performed by a freeze–thaw technique as previously reported [18]. In brief, solubilization was performed as follows: washed, frozen microsomes (−20°C) were permitted to thaw at room temperature. These microsomal pellets were suspended in ice cold buffer A (4 ml/g tissue), homogenized (one stroke up/down in 30 s with a Potter-Elvehjem homogenizer, #C50901, Arthur Thomas Co.) and centrifuged at 100 000 × *g* for 90 min. The clear supernatant was frozen on dry ice and stored at −20°C.

2.4. *Heat treatment*

Heat fractionation of freeze–thaw solubilized enzyme was performed at 65°C for 10 min. The sample was cooled to room temperature and centrifuged (Sorval RC 2-B) at 35 000 × *g* for 15 min. Thirty ± 5% of the protein and 100 ± 2% of the enzyme activity remained in the supernate. Enzyme purified by heat fractionation is designated heat treated enzyme.

2.5. *Affinity chromatography*

Affinity chromatography employing agarose-hexane-coenzyme A has been utilized to partially purify rat-liver HMG-CoA reductase* and has been recently incorporated into the purification procedures for the isolation of reductase from rat microsomes [19] and yeast [20]. In the present studies, we have employed dextran-blue affinity chromatography for the final step in purification of chicken HMG-CoA reductase. While these studies were in progress, Tormanen et al. independently reported the partial purification of rat-liver HMG-CoA reductase by agarose-blue dextran [16].

For the final purification of heat-treated chicken enzyme by agarose-blue dextran, cyanogen bromide activated agarose 4B (Pharmacia, Lot No. B15467) was coupled with blue dextran, 2000 (Pharmacia) by the method of Ryan and Vestling [21]. Agarose-blue dextran was equilibrated and washed with five column volumes of buffer A. The ratio of heat-treated enzyme to affinity gel was experimentally determined by adjusting the ratio of enzyme to gel which would permit the elution of the column with 0.3 M KCl without any significant release of enzyme activity. In a typical experiment, 50 ml heat-treated enzyme was applied to a column (4 × 9 cm) of agarose-blue dextran. The column was eluted sequentially with 100 ml buffer A, 200 ml 0.3 M KCl containing 2 mM DTT and 200 ml of buffer B (buffer A containing 0.3 M KCl). The buffer B column effluent was collected in 10 ml fractions and enzyme activity was determined in 0.1 ml aliquots of each fraction. Eighty to 85% of the enzyme activity eluted between 70 ml and 150 ml. Regeneration of resin between samples was achieved by washing with five column volumes of 3 M KCl and reequilibration in buffer A. The blue-dextran resin can be used repeatedly and is stable when stored at 4°C in 3 mM sodium azide for three to four months.

A yellow non-dialyzable, non-proteinous material occasionally co-purified with HMG-CoA reductase on agarose-blue dextran chromatography. The yellow contaminant varied with different liver preparations. The contaminant could be removed from the final purified enzyme by a modification of the elution scheme from agarose-blue dextran as follows: following elution with 200 ml 0.3 M KCl containing 2 mM DTT, the column was washed with 100 ml 5 mM NADPH in buffer A (concentrations of NADPH greater than 5 mM will elute enzymatic activity). This latter buffer removes the yellow contaminant from the column resin, and the enzyme can be released with buffer B as outlined above.

HMG-CoA reductase, purified by affinity chromatography on agarose-blue dextran, was dialyzed against 10 mM NH_4HCO_3 (pH 8.0). The protein content was determined by the method of Lowry et al. [22] and the homogeneity of the purified enzyme assessed by electrophoresis.

2.6. *Acrylamide electrophoresis*

2.6.1. Disc-gel electrophoresis

Polyacrylamide gel electrophoresis was performed by a modification of the procedure of Reisfeld and Small [23]. The upper tray buffer contained 5.4 mM Tris glycine (pH 8.0), and the lower tray buffer was 64 mM Tris HCl (pH 7.2). The stacking buffer was

* Beg, Z. H., Anderson, P. J. and Gibson, D. M. (1974) unpublished results

92 mM Tris phosphate (pH 6.7). All buffers contained 1 mM DTT and the gels were 5 X 75 mm. Electrophoresis was performed at 2 mA/gel for 3.5 h. Following electrophoresis, the gels were either cut into 2 mm slices (gel slicer, NIH Model #7173-173) for determination of enzyme activity or stained in 0.1% Coomassie Blue (R250) in methanol/water/acetic acid (10:10:1, v/v/v) for 30 min at 60°C and destained in a diffusion destainer (Bio-Rad, model 172) containing 7.5% acetic acid and 5% methanol.

2.6.2. Urea disc-gel electrophoresis

Polyacrylamide gel electrophoresis in 8 M urea was performed as outlined above except that all buffers contained 8 M urea. Samples for electrophoresis were preincubated in 8 M urea and 10 mM DTT for 30 min at 37°C.

2.6.3. Sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis

SDS gel electrophoresis was performed by the procedure of Neville and Glossmann [24] with the following modifications:

(a) DTT (10 mM) replaced 2-mercaptoethanol as the reducing agent.

(b) Acrylamide gel concentration was increased from 10.0–12.5%.

(c) Samples for analysis contained 10 mM DTT–0.5% SDS and were heated to 100°C for 2 min prior to electrophoresis.

2.7. *Isoelectric focusing*

2.7.1. Thin-layer isoelectric focusing (TLIF)

TLIF was performed on glass plates (20 X 20 cm) layered with 50 ml of a suspension of 15% Sephadex G-75 (superfine, Pharmacia) containing 1% (v/v) ampholin (pH 3.5 - 10, LKB Instruments, Inc.), 0.015% DTT, 0.038% EDTA, and 0.05% lysine and arginine. The plate was initially electrophoresed for 3 h at 200 V, after which the voltage was increased to 800 and the electrophoresis continued for an additional 5 h. Following isoelectric focusing, 1 cm^2 segments of gel suspension were removed from the plate by a spatula and transferred to glass tubes containing 0.5 ml buffer A for measurement of HMG-CoA reductase activity. Aliquots of solution were also dialyzed against 10 mM NH_4HCO_3, concentrated by ultrafiltration (10 S & S collodion bags, #100, Schleicher and Schuell) and analyzed by SDS gel electrophoresis. Identical 1 cm^2 segments of gel suspension adjacent to the sample aliquots were removed, transferred to glass tubes containing 1 ml distilled water, and the pH determined on a Radiometer pH meter (model PHM 51).

2.7.2. Acrylamide gel isoelectric focusing (AGIF)

AGIF was performed as described by the MRA Corporation [25], utilizing a 7.5% acrylamide concentration and 2% ampholytes (pH 3–10, LKB Instruments, Inc.). Following electrophoresis the gels were placed in a trough containing 0.25 M KCl and the pH determined every 0.5 cm by a microelectrode (MI-408, Microelectrodes, Inc.) attached to a Beckman pH meter (Zeromatic, model 96). After pH measurements, the gels were stained for 30 min at 60°C with 0.2% Fast Green (FCF, Fisher) in ethanol/acetic acid/water (40:10:50, v/v/v). Destaining was performed by diffusion as outlined above in ethanol/acetic acid/water (10:10:80, v/v/v).

3. Results

3.1. *Purification*

A three-step procedure for the purification of HMG-CoA reductase from avian liver has been developed (table 1). Liver microsomes were isolated from laying hens by homogenization, followed by continuous flow centrifugation. Isolated microsomes were recentrifuged and stored at −20°C. A new freeze–thaw procedure for solubilization of HMG-CoA reductase has been developed which permits the solubilization of 75–85% of the microsomal enzyme ([18], table 1). Partial purification of HMG-CoA reductase was achieved by heat treatment of freeze–thaw solubilized enzyme. No loss of enzyme activity occurred during heating, and 70–75% of the total protein was precipitated. Heat-treated enzyme was further purified by agarose-blue dextran chromatography. The enzyme was released by high ionic strength, with an overall yield of 49% from the microsomal suspension. The specific activity of the purified enzyme was 1739 nmol/min/mg (table 1).

3.2. *Properties*

Homogeneity of the isolated enzyme was assessed

Table 1
Purification of chicken-liver HMG-CoA reductase

Fraction	Total Protein (mg)	Total activity (nmol/min)	Specific activity[a] (nmol/min/mg)	Purification (-fold)	Yield (%)
Microsomal suspension	959	252	0.26	1	100
1. Solubilized fraction	116	196	1.69	6.5	78
2. Heat fractionation (65°C, 10 min)	28.5	197	6.91	26.5	78
3. Affinity chromatography (agarose-blue dextran)	0.071	123.5	1739	6688	49

[a] nmol mevalonate formed/min/mg protein under the assay conditions described in the text

Frozen microsomal pellet (13.3 g wet wt) was thawed and homogenized in 50 ml buffer A for 30 s. The suspension was centrifuged at 100 000 × *g* (90 min) and the soluble fraction (50 ml) was used for purification of the enzyme. The purification scheme was carried out as described under Materials and methods.

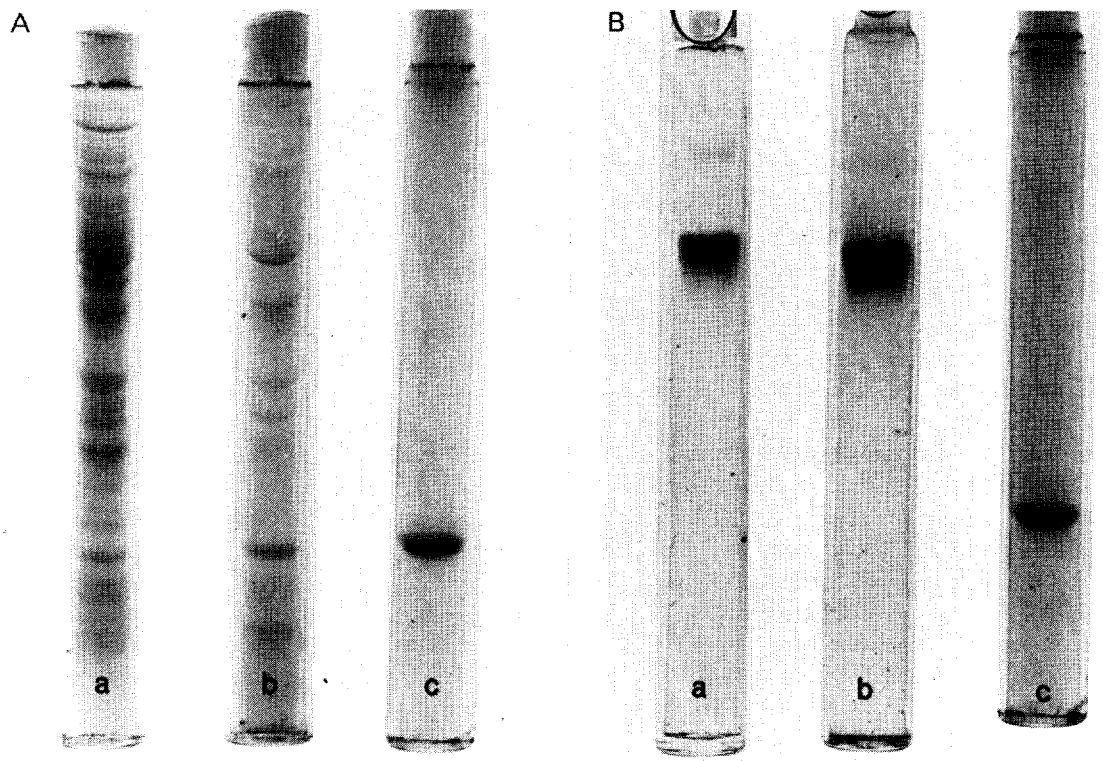

Fig.1

by polyacrylamide gel electrophoresis. SDS electrophoresis of enzyme at various stages of purification, including freeze–thaw solubilized enzyme, heat-treated, and agarose-blue dextran released enzyme, is shown in fig.1A. The purified enzyme was electrophoretically homogeneous by SDS electrophoresis (fig.1Ac) and by polyacrylamide gel electrophoresis in aqueous buffers and in 8 M urea (fig.1B).

In order to demonstrate that the protein contained within the single electrophoretic band on acrylamide gel electrophoresis (fig.1) was associated with enzyme activity, purified reductase was fractionated by an additional technique, thin-layer isoelectric focusing. Following isoelectric focusing, sequential aliquots of gel containing enzymatic activity were analyzed by SDS electrophoresis. All fractions ran as a single band on SDS electrophoresis in a position identical to that visualized in the purified enzyme preparation (fig.2). In addition, assay of enzymatic activity of sequential 2 mm slices of acrylamide gel following electrophoresis revealed the peak activity (60%, three slices) coincident with the single electrophoretic band.

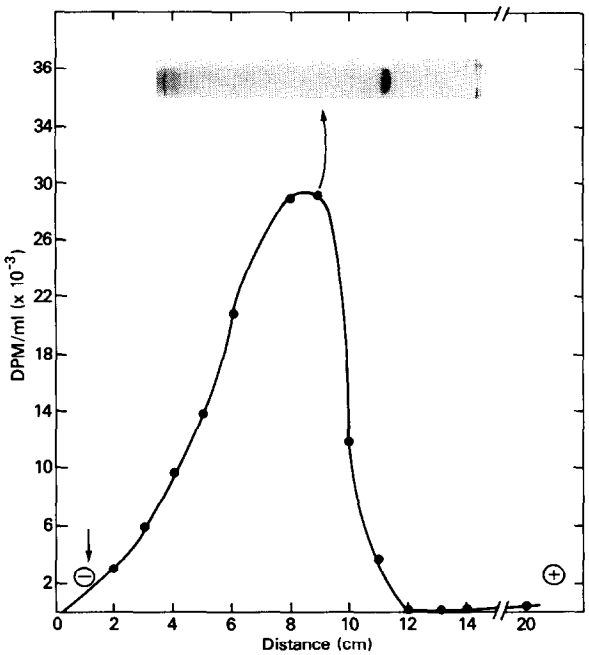

Fig.2. Enzyme activity profile of purified HMG-CoA reductase on thin-layer isoelectric focusing. Aliquots of gel containing enzymatic activity run as a single band on SDS gel electrophoresis. An aliquot of gel containing peak enzymatic activity (the fraction at the tail of the arrow) was analyzed by SDS gel electrophoresis and is illustrated in the inset (for details, see text).

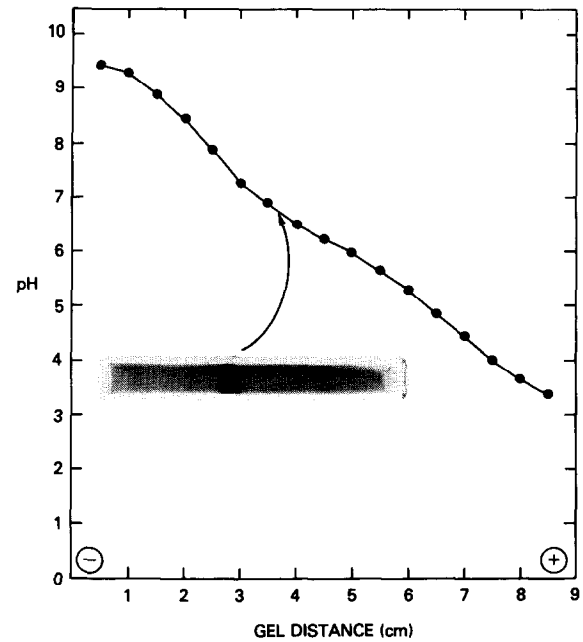

Fig.3. Isoelectric focusing of HMG-CoA reductase in 7.5% acrylamide gels containing 2% carrier ampholytes. The pH gradient, established during the 18 h run, was measured by inserting a microelectrode in the gel every 0.5 cm as outlined in Materials and methods. The protein (see inset) migrated as a single band with an apparent pI of 6.7 ± 0.2.

The degree of purity and isoelectric point (pI) of HMG-CoA reductase was also determined by isoelectric focusing in polyacrylamide. Isoelectric focusing of reductase revealed a single major band with an apparent pI 6.7 ± 0.2 (fig.3).

Fig.1. Panel A: Sodium dodecyl sulfate acrylamide gel electrophoresis of HMG-CoA reductase in 12.5% polyacrylamide at various stages of purification: (a) freeze–thaw-solubilized enzyme; (b) heat-treated enzyme (65°C, 10 min); (c) 5 μg purified enzyme following affinity chromatography on agarose-blue dextran. Panel B: Acrylamide gel electrophoresis (6% gel; 15 μg protein) of agarose-blue dextran purified HMG-CoA reductase utilizing: (a) alkaline buffer; (b) alkaline buffer containing 8 M urea; (c) alkaline buffer containing sodium dodecyl sulfate.

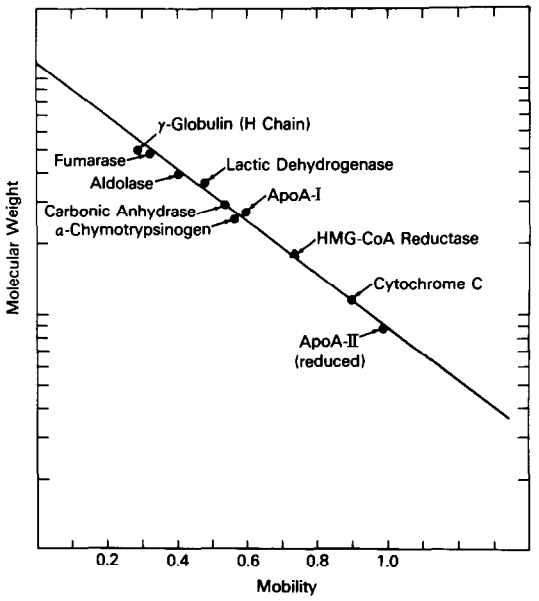

Fig.4. SDS polyacrylamide gel electrophoresis (12.5% acrylamide) of proteins of known molecular weight: γ-globulin (H chain, 50 000); fumarase (49 000); aldolase (40 000); lactic dehydrogenase (36 000); carbonic anhydrase (29 000); apoA-I (28 000); α-chymotrypsinogen (25 700); cytochrome *c* (11 700); reduced apoA-II (8400). The molecular weight of HMG-CoA reductase subunits was estimated to be 18 000 ± 2400.

The monomeric molecular weight of purified HMG-CoA reductase was estimated by SDS electrophoresis in DTT. The monomer molecular weight, when compared to proteins of known molecular weight, was 18 000 ± 2400 (fig.4).

4. Discussion

HMG-CoA reductase has been isolated from avian liver in an electrophoretically homogeneous form. The enzyme can be purified from microsomal suspensions in 6–8 h, with approximately 50% recovery. This procedure is rapid, simple and the recovery is higher than previously reported for purification of rat [9–12, 14–16] and yeast [20] HMG-CoA reductase. The isolated enzyme has a specific activity of approximately 1700 nmol/min/mg, which is significantly greater than that generally reported for the rat enzyme. A molecular weight by SDS electrophoresis of 18 000 for avian reductase is lower than the molecular weight of 50 000–65 000 reported for the rat [10,16,19] and yeast [20] enzyme. In our laboratory, rat HMG-CoA reductase, purified by sequential chromatography on agarose-blue dextran and agarose-hexane-coenzyme A also had an apparent monomer molecular weight of approx. 50 000*. The apparent monomer molecular weight by SDS electrophoresis of chicken HMG-CoA reductase is, therefore, significantly lower than that of mammalian and yeast HMG-CoA reductases. A systematic comparison of the molecular and kinetic properties of rat and chicken HMG-CoA reductases is currently under investigation.

Development of a rapid, preparative scale method for purification of HMG-CoA reductase will markedly facilitate the isolation of enzyme for detailed biochemical, kinetic and structural analysis as well as the delineation of its role in cholesterol biosynthesis.

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