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Short communication

Purification of cytochrome P-450 from adult pig testis by hydroxylapatite and deoxycorticosterone affinity column chromatography

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Abstract

Adult testicular cytochrome P-450 was purified by a two-step procedure utilizing hydroxylapatite and deoxycorticosterone affinity column chromatography. Cytochrome P-450 was determined to have an isoelectric point of 6.5 on analytical isoelectric focusing. The purified cytochrome P-450 was found to be homogeneous and its molecular mass was estimated to be 52 000 on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The carbon monoxide difference spectrum with a peak at 448 nm exhibited the absorption spectrum of a typical cytochrome P-450. A 1000-fold purification was achieved with a yield of 5%.

Keywords: Cytochromes; Enzymes

1. Introduction

In liver microsomes, cytochrome P-450 catalyzes the hydroxylation of a broad range of substrates such as drugs, carcinogens, hydrocarbons, and endogeneous substrates such as fatty acids and steroids [1–4]. In testicular and adrenal microsomes, cytochrome P-450 plays an important role in steroidogenesis. Nakajin et al. [5–7] and Suhara et al. [8] have reported the purification and characterization of cytochrome P-450 from neonatal pig testis microsomes, although a few reports have been made of the purification and characterization of adult pig testis [9,23]. It is more difficult to purify cytochrome P-450 from adult pig testis because adult pig testis contains more contaminating proteins than neonatal pig testis. Furthermore, cytochrome P-450 content in adult pig testis microsomes is only one-tenth of that of liver microsomes [10]. The purification process for cytochrome P-450 is very complicated, involving five or more column chromatographic steps for the final preparation [5,11–13]. Furthermore, a reduction in the number of steps will improve the yield.

In the present paper, we describe a two-step procedure for the purification of cytochrome P-450. As the first step, hydroxylapatite column chromatography is performed only once and then, as the second step, the partially purified cytochrome P-450 is completely purified by deoxycorticosterone (DOC) affinity (DOC-substituted EAH-Sepharose 4B) col-

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umn chromatography. The combination reduced purification to a two-step procedure.

2. Experimental

2.1. Reagents

Sodium dodecyl sulfate (SDS) steroid, and acrylamide were purchased from Sigma (St. Louis, MO, USA). Immobiline Dry Plate pH 4–7 polyacrylamide gel, ampholine pH 3–10, SDS marker proteins, p*I* marker proteins, and EAH-Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). Thin-layer plates (Silica Gel 60) were purchased from Merck (Darmstadt, Germany). Emulgen 913 was kindly supplied by Kao (Tokyo, Japan). Glycerol, EDTA, dithiothreitol, sodium dithionite, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide and sodium cholate were purchased from Nakalai (Kyoto, Japan). All other chemicals used were analytical grade.

2.2. Preparation of adult pig testis microsomes

Testes of adult pigs were supplied by a local slaughterhouse and stored at −15°C. The frozen testes (about 5 kg) were partially thawed, decapsulated, sliced and then washed with 20 l of a 0.9% NaCl solution. Microsomes were prepared according to the method of Omura and Sato [14] with slight modifications. The slices were homogenized with four volumes of a 1.15% KCl solution in a Waring-type blender for 5 min. The homogenate was then centrifuged at 12 000 *g* for 30 min and the resulting supernatant was further centrifuged at 78 000 *g* for 90 min. The firmly packed pellet of microsomes was resuspended in 2 l of 100 m*M* phosphate buffer containing 100 μ*M* EDTA and 100 μ*M* dithiothreitol. Each suspension was centrifuged at 78 000 *g* for 90 min to obtain the microsomal pellet.

2.3. Solubilization and ammonium sulfate fractionation of testicular cytochrome P-450

The microsomes were suspended in 1.5 l of 100 m*M* phosphate buffer containing 20% (v/v) glycerol and 1% (w/v) sodium cholate. After stirring for 90 min at 4°C, the mixture was centrifuged at 105 000 *g* for 90 min. The supernatant was fractionated with ammonium sulfate to obtain the fraction that precipitated at between 35 and 55% saturation [2,9].

2.4. Synthesis of DOC-21-hemisuccinate

DOC-21-hemisuccinate was prepared according to Kuhn et al. [15] starting with DOC and succinic anhydride. One gram of succinic anhydride was added per gram of DOC and dissolved in 30 ml of pyridine. The mixture was stirred for 3 h at 70°C. After removing the solvent in a rotary evaporator, the residue was dissolved in 300 ml of chlorform–ethyl alcohol (4:1). Part of the solvent was used for thin-layer chromatography (Fig. 1, lane 2). The thin-layer chromatography plate was developed using chloroform–acetone (95:5), dried by hot air, and then exposed to 50% (v/v) sulfuric acid to localize

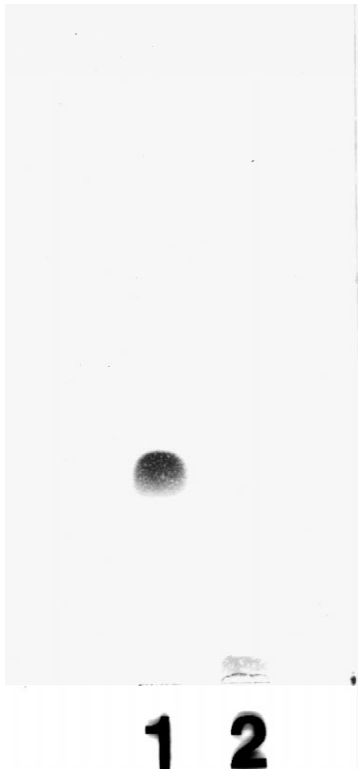

Fig. 1. Thin-layer chromatogram obtained using chloroform–acetone (95:5) solvent system. Lane 1, synthesized deoxycorticosterone-21-hemisuccinate; lane 2, deoxycorticosterone. Two samples were simultaneously chromatographed and revealed by 50% (v/v) sulfuric acid. The thin-layer chromatography plate was run (8.5 cm) in a glass chamber (25×25×12 cm) saturated with solvents.

the spot. When DOC was coupled with succinic anhydride, DOC-21-hemisuccinate was observed at the Rf value position of 0.041 (Fig. 1, lane 2). DOC was located at Rf value of 0.32 (Fig. 1, lane 1). Most of the solvent was removed by three washes with distilled water. Sodium sulfide anhydride was added to the solvent and the mixture was dried in a rotary evaporator. The crude product was dissolved in a minimum amount of H_2O–ethyl alcohol (1:10) and DOC-21-hemisuccinate was crystalized from H_2O–methyl alcohol (1:10), then recrystalized from ethyl alcohol.

2.5. Coupling of DOC-21-hemisuccinate to EAH-Sepharose 4B (DOC affinity column chromatography)

DOC-substituted EAH-Sepharose 4B was prepared by a modified method of Cuatrecasas et al. [16,17] as follows. One hundred grams of EAH-Sepharose 4B (Pharmacia) was swollen in 2000 ml of 0.5 *M* NaCl solution and washed with 500 ml of distilled water (adjusted with 0.1 *M* HCl to pH 4.5). The moist EAH-Sepharose 4B was placed in 100 ml of diethylene dioxide (1,4-dioxane) containing 4.4 g DOC-21-hemisuccinate (10 m*M*) and 100 ml of H_2O (pH 4.5). DOC-21-hemisuccinate was coupled to 1,6-diaminohexyl (EAH)-substituted Sepharose 4B using the carbodiimide-promoted condensation reaction previously described [18,19]: 5.5 g of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (0.13 *M*) was added to 20 ml of H_2O, (pH 4.5), manually inverted for 60 min, and stirred for 24 h at 4°C. The resulting DOC-substituted EAH-Sepharose 4B was washed successively with 2000 ml of 1,4-dioxane, 10 l of 80% (v/v) ethyl alcohol, 1000 ml of acetone, 2000 ml of distilled water, and 2000 ml of 10 m*M* potassium phosphate buffer, pH 7.5, containing 20% (v/v) glycerol. One milliliter of DOC affinity sediment contained 3.5 to 5 μ*M* of DOC, which was analyzed with sulfuric acid after alkaline hydrolysis.

2.6. The cytochrome P-450 partially purified from testicular microsomes on an hydroxylapatite column chromatography (the first step)

The precipitate (between 35% and 55% ammonium sulfate) was dissolved in 10 m*M* phosphate buffer, pH 7.5, and then subjected to the hydroxylapatite column chromatography (10×1.5 cm I.D.) (Fig. 2). Cytochrome P-450 was eluted with a 600 ml linear gradient of 10 to 300 m*M* potassium phosphate buffer, pH 7.5. The peak of cytochrome P-450 was eluted with 100 to 200 m*M* potassium phosphate buffer, pH 7.5. The eluted solution was collected and then dialyzed against 10 m*M* phosphate buffer, pH 7.5, containing 0.05% (w/v) Emulgen 913 overnight. Samples of the partially purified cytochrome P-450 were subjected to DOC-

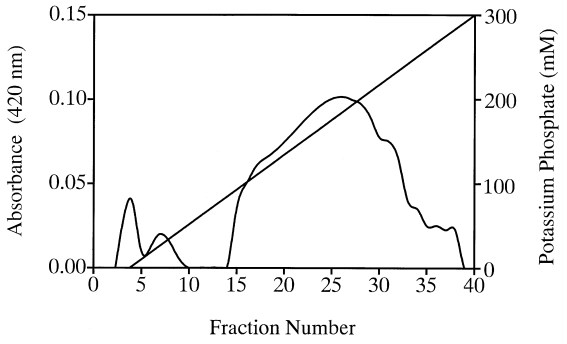

Fig. 2. Elution profile on hydroxylapatite column chromatography (the first step). The ammonium sulfate precipitate was dissolved in 10 m*M* phosphate buffer, and then subjected to hydroxylapatite column chromatography. Cytochrome P-450 was eluted with a 600 ml linear gradient of 10 to 300 m*M* potassium phosphate buffer, pH 7.5.

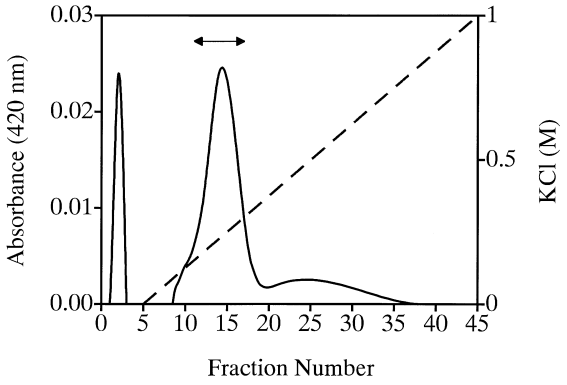

Fig. 3. Elution profile on deoxycorticosterone affinity column chromatography (the second step). The preparation obtained from the hydroxylapatite was further purified by DOC affinity column chromatography (10×1.0 cm I.D.). Cytochrome P-450 was eluted with a 700 ml linear gradient 0 to 1 *M* KCl in 10 m*M* phosphate buffer, pH 7.5. The peak fractions (horizontal arrow) of cytochrome P-450 were eluted with 200 to 300 m*M* KCl in 10 m*M* phosphate buffer, pH 7.5, containing 0.05% (w/v) Emulgen 913 and 20% (v/v) glycerol.

substituted EAH-Sepharose 4B column chromatography (hereafter called DOC affinity column) (Fig. 3).

2.7. DOC affinity column chromatography (the second step)

DOC-substituted EAH-Sepharose 4B was packed in a column (10×1.0 cm I.D.), equilibrated with 10 m*M* phosphate buffer, pH 7.5, containing 0.05% (w/v) Emulgen 913. The product of the hydroxylapatite column chromatography was further purified by DOC affinity column chromatography (Fig. 3). The column was washed with the same buffer.

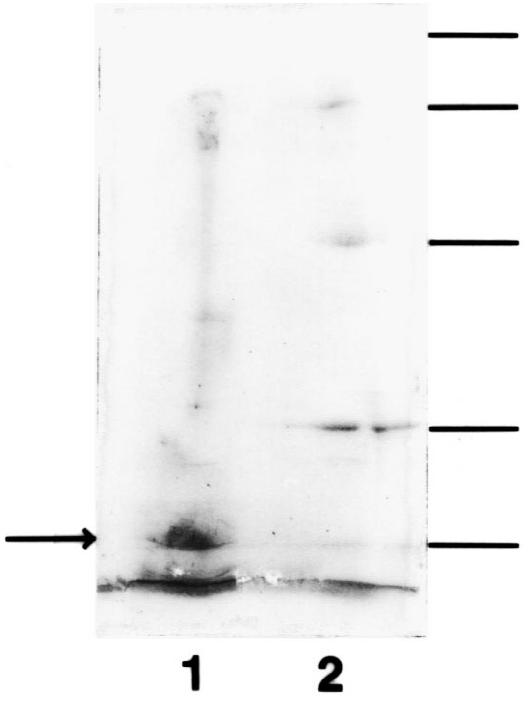

Fig. 4. Analytical IEF on an Immobilized pH gradient, 4.0–7.0. After deoxycorticosterone affinity column chromatography was performed, the peak fractions were applied to lane 1. After analytical IEF, the gel was stained with Coomassie Blue R 250. The major protein band exhibits an apparent isoelectric p*I* of 6.5. The position of cytochrome P-450 is indicated by an arrow. p*I* Marker proteins of known p*I*'s were applied to lane 2, from top to bottom, glucose oxidase, 4.14; soybean trypsin inhibitor, 4.55; β-lactoglobulin A, 5.20; bovine carbonic anhydrase B, 5.85; human carbonic anhydrase B, 6.55. The five bars at the right, from top to bottom, indicate the positions of p*I* 4.14, 4.55, 5.20, 5.85, and 6.55, respectively.

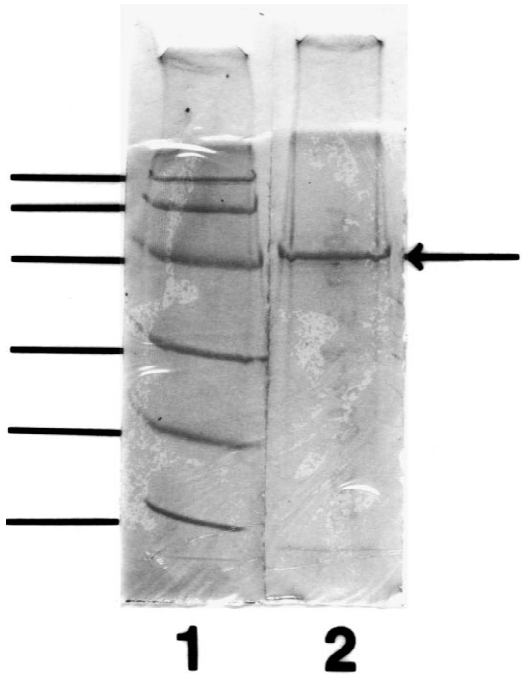

Fig. 5. SDS polyacrylamide gel electrophoresis. The sample obtained on DOC affinity column chromatography was applied to lane 2. The position of cytochrome P-450 is indicated by an arrow at the left. Lane 1 contained protein standards of known molecular masses: phosphorylase b, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 000; and α-lactoalbumin, 14 400. Migration was from top to bottom.

Cytochrome P-450 was eluted with a 700 ml linear gradient 0 to 1 *M* KCl in 10 m*M* potassium phosphate buffer, pH 7.5. The peak fractions of cytochrome P-450 were eluted with 200 to 300 m*M* KCl in 10 m*M* potassium phosphate buffer, pH 7.5 and dialyzed against 10 m*M* phosphate buffer, pH 7.5, containing 0.05% (w/v) Emulgen 913 and 20% (v/v) glycerol overnight. Samples of the purified cytochrome P-450 were subjected to analytical isoelectric focusing (IEF) (Fig. 4), SDS polyacrylamide gel electrophoresis (Fig. 5), and carbon monoxide (CO) difference spectrum (Fig. 6).

2.8. Analytical IEF in Immobiline, pH gradient 4–7, on an analytical scale in a 0.5 mm thick gel

Immobiline Dry Plate polyacrylamide gel was reswollen with distilled water containing 1% (w/v)

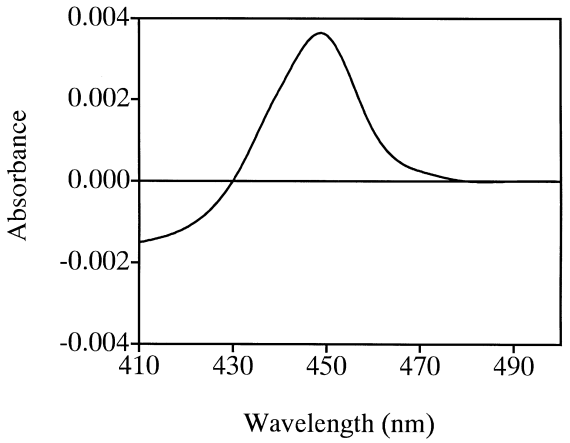

Fig. 6. The CO difference spectrum of the purified cytochrome P-450 (41 n*M*) from pig testis microsomes.

ampholine pH 3–10, 0.05% (w/v) Emulgen 913 and 20% (v/v) glycerol. IEF, with an Immobiline pH gradient, was carried out in LKB Multiphor II system. The purified cytochrome P-450 obtained from DOC affinity column chromatography was applied to a well in the gel near the anodal strip. Separation was carried out at 15°C for 10 h. The maximal values set for the power supply were 3400 V, 25 mA, and 10 W. After IEF, the gel was placed in a mixture of 10 g sulfosalicylic acid, 20 ml methanol, and 180 ml distilled water for protein fixing. Finally, it was stained with Coomassie Blue R 250 to visualize the protein bands (Fig. 4).

2.9. Other assay methods

Cytochrome P-450 was determined by the method described by Omura and Sato [14], using a molar adsorptivity of 91 mM^{-1} cm^{-1} at 450 nm. The purified cytochrome P-450 was reduced by the addition of a few of crystaline sodium dithionite and the CO was bubbled into the reduced solution containing P-450, and then its content was determined from the reduced CO form minus reduced form difference spectrum. SDS disc gel electrophoresis was carried out according to the method of Laemmli [20]. The apparent molecular masses in the presence of SDS were determined electrophoretically by plotting logarithms of molecular mass against relative mobility, constructed with data obtained with six standard proteins. Protein concentrations were determined by the method of Lowry et al. [21] as modified by Dulley and Grieve for solutions containing detergents [22].

3. Results and discussion

The results of the purification of testicular cytochrome P-450 are summarized in Table 1. The ammonium sulfate fraction was purified to 19-fold by hydroxylapatite column chromatography (Fig. 2). The partially purified cytochrome P-450 preparation was then subjected to DOC affinity column chromatography (Fig. 3). The final preparation was purified to 1000-fold with an overall yield of 5% and amounted to 15 n*M* of P-450/mg protein. It has recently been reported by this laboratory that the cytochrome P-450 purified by the usual procedure (five or more column chromatographic steps) is capable of catalyzing 17α-hydroxylation of progesterone. The activity was shown to 10 n*M* of product/min/n*M* of P-450 [9,23]. We have confirmed that the

Table 1
Purification of cytochrome P-450[a] from pig testis microsomes

Steps	Protein (mg)	P-450 (n*M*)	Specific content (n*M*/mg)	Purification factor	Recovery (%)
Microsomes	28000	420	0.015	1	100
Hydroxylapatite	510	150	0.29	19	35
Deoxycorticosterone affinity	1.5	22	15	1000	5

[a] Cytochrome P-450 as determined by the method described by Omura and Sato [14], using a molar adsorptivity of 91 mM^{-1} cm^{-1} at 450 nm. Specific content was measured as described in Experimental.

cytochrome P-450 purified by a two-step procedure also exhibits similar activity.

DOC-21-hemisuccinate is chosen as the ligand because of its structural similarity to progesterone and because it contains a free carboxyl group which can attach the ligand to the column matrix backbone. Nakajin has shown that progesterone binds strongly to neonatal testicular cytochrome P-450 [6]. The cytochrome P-450 was eluted with a 700 ml linear gradient 0 to 1 *M* KCl in 10 m*M* phosphate buffer, pH 7.5. The peak fractions (a horizontal arrow in Fig. 3) of cytochrome P-450 were eluted with 200 to 300 m*M* KCl in 10 m*M* phosphate buffer, pH 7.5. The eluate was concentrated by membrane filtration and then dialyzed against 0.05% (w/v) Emulgen 913 and 20% (v/v) glycerol in the 10 m*M* phosphate buffer overnight. The sample was subjected to IEF on an Immobiline pH gradient, 4–7, on an analytical scale (Fig. 4, lane 1). We observed one major protein band (arrow), exhibiting an apparent isoelectric p*I* of 6.5. The purified cytochrome P-450 gave a single protein band (arrow). Contaminating protein bands did not appear to be present, though a little cytochrome P-450 remained in the sample well (the upper part). The sample from DOC affinity column chromatography was also examined by SDS electrophoresis. Adult testicular cytochrome P-450 was shown to be a single protein band and shown to have molecular weight of 52 000 (Fig. 5, lane 2). This is consistent with the molecular mass of adrenal cytochrome P-450 [11]. The sample from the hydroxylapatite column chromatography had more than ten contaminating protein bands on electrophoresis in SDS polyacrylamide gel (data not shown). As a result of DOC affinity column chromatography, the majority of the contaminating proteins were reduced to a single protein (Fig. 5). This two-step procedure also appears to be useful for investigation of all kinds of hemoproteins, especially those in steroid hormone synthesis tissues that contain a small amount of cytochrome P-450. The cytochrome P-450 gave a single protein band on analytical IEF, as shown in Fig. 4, lane 1, and on SDS electrophoresis, as shown in Fig. 5, lane 2. The CO difference spectrum of the purified cytochrome P-450 is shown to have an absorption peak at 448 nm, a typical cytochrome P-450 absorption spectrum (Fig. 6). The cytochrome P-450 was thus entirely purified by DOC affinity column chromatography.

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