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Purification of a Single Major Form of Microsomal Cytochrome P-450 from Tulip Bulbs (*Tulipa gesneriana* L.)

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Microsomal cytochrome P-450 from tulip bulbs (*Tulipa gesneriana* L., Balalaika) was purified to an almost electrophoretically homogeneous preparation. The specific content of cytochrome P-450 in the final preparation was 6.68 nmol/mg protein, which was 30-fold enriched from that of the solubilized fractions of microsomes. The molecular weight of purified cytochrome P-450 by SDS-gel electrophoresis is 52,500. The Oxidized form of the purified cytochrome P-450 had absorption peaks at 392, 552, and 645 nm and the absolute reduced CO spectrum peaked at 448 nm. Judged spectrally, the purified cytochrome P-450 is in high spin in the oxidized state. Antiserum against this cytochrome P-450 previously has shown to be highly specific for its antigen but showed a single precipitin line with solubilized microsomal proteins from tulip bulbs of several other cultivars. The physiological role of this cytochrome P-450, however, is unknown in these dormant tulip bulbs.

In 1969, a mixed function oxidase in the extract of higher plant tissues was found by Frear *et al.*[1] and Murphy and West.[2] Later, the optical and magnetic properties of the microsomal cytochrome P-450 from a variety of higher plants were studied.[3,4] It was suggested from these results that the cytochrome P-450 system in higher plants is analogous to that in mammalian microsomes. Until an experiment of ours, no purified preparation of plant cytochrome P-450 had been obtained due to the difficulty of isolating organelles and enzymes from higher plant tissues because of the rigid cell wall, the presence of phenolic compounds,[5] and a low concentration of microsomal cytochrome P-450.[3] We have succeeded in isolating an almost electrophoretically homogeneous preparation (more than 98%) of cytochrome P-450 from the microsomes of tulip bulbs. Some properties of the purified hemoprotein had been reported briefly.[6] In this paper, the details of purification procedures and the optical characteristics of the purified cytochrome P-450 will be described. As far as we know, this is the first case of a highly purified cytochrome P-450 extracted from a higher plant.

MATERIALS AND METHODS

Tulip bulbs (*Tulipa gesneriana*, L., Balalaika) were purchased directly from a local cultivator (Toyama Kakikyukon Center) and stored in a cold room until use. Tulip bulbs were homogenized in a blender with a one to one half volume (w/v) of 0.1 M sodium phosphate buffer, pH 7.4, containing 10 mM mercaptoethanol, 5 mM EDTA, 0.6 M mannitol, 1% bovine serum albumin (w/v), 0.15 mM spermine, and 0.5 mM spermidine for 1 min in the cold room (2～4°C) and passed through 2 layers of cheesecloth to remove fibrous materials. The filtrate was centrifuged at 10,000 × *g* for 20 min. The supernatant obtained was centrifuged at 40,000 rpm for 90 min in a 70 Ti rotor (Beckman). Microsomes were resuspended in a smaller volume of the initial homogenizing buffer, centrifuged at 40,000 rpm in a 50 Ti rotor (Beckman) for 1 hr after which the microsomal pellets were stored at −70°C. These

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procedures were repeated until a sufficient amount of microsomes for purification was obtained.

Microsomes from approximately 2.5 kg of tulip bulbs were used for one purification experiment. Microsomes were solubilized in Buffer S [0.1 M Tris-HCl, pH 7.7, containing 1 mM dithiothreitol (DTT), 0.2% sodium cholate (w/v), 0.1 mM EDTA, 20% glycerol (v/v)]. The microsomal protein concentration was approximately 10 mg per ml. Ten percent Emulgen 911 (w/v, Kawo Chemicals, Tokyo) was added slowly while stirring at 4°C to a final concentration of 1.5% (w/v), that is, approximately 15 mg of Emulgen/mg protein.[7] Clear solubilized supernatant after centrifugation at 40,000 rpm for 1 hr was put on a DEAE-Sephadex A-25 column (2.8 × 40 cm), previously equilibrated with Buffer DS (0.1 M Tris-HCl, pH 7.7, containing 1 mM EDTA, 0.1 mM DTT, 30% glycerol, and 0.15% Emulgen 911). Usually two columns under the same conditions were prepared and used simultaneously to reduce the time for this step. The loaded columns were eluted with the same Buffer DS at 90 ml/hr and monitored at 417 nm. The fractions having absorbancy at 417 nm in the unadsorbed fractions were pooled, and an addition of 50% solution of polyethylene glycol (PEG, molecular weight 7,800 to 9,000) to a final concentration of 16% saturation of PEG (w/v) was made; the solution was centrifuged at 40,000 rpm for 90 min at 4°C.

The pellets obtained after PEG-precipitation were resuspended in a small volume of 10 mM potassium phosphate buffer, pH 7.4, containing 0.5% sodium cholate (w/v), 0.2% Emulgen 911, 1 mM EDTA, and 30% glycerol (Buffer A) and centrifuged at 9,000 rpm for 10 min. The next step, DEAE-cellulose column chromatography, was done at room temperature (22°C). The supernatant obtained as above was put on a Whatman DE-52 column (2.8 × 40 cm) equilibrated with Buffer A. After washing the loaded column with 200 ml of Buffer A, the column was eluted with 800 ml of a 0 to 0.3 M KCl linear gradient in Buffer A. The fractions with absorbancy at 417 nm were pooled and dialyzed overnight against 2 liters of 10 mM potassium phosphate buffer, pH 7.2, containing 0.5% sodium cholate, 0.2% Emulgen 911, 1 mM EDTA, 0.1 mM DTT, and 20% glycerol (Buffer H) at 4°C.

The dialyzed enzyme solution was put onto an hydroxyapatite column (2.8 × 10 cm) equilibrated with Buffer H, at 4°C and eluted with a linear gradient containing the same additional components as Buffer H. The fractions with absorbancy at 417 nm were pooled and stored at −70°C. Little degradation has occurred under these conditions, as judged spectrally, after more than 6 months.

The final preparation was analyzed by SDS-polyacrylamide gel electrophoresis by the procedure of Laemmli.[8] Protein was measured with a Bio-Rad Protein Assay (Bio-Rad Laboratories, Calif.). Measurement of cytochrome P-450 was done as described by Omura and Sato.[9] Spectrophotometric analysis of the purified cytochrome P-450 was done with a Carry 17D (Varian, Calif.).

RESULTS

Purification of microsomal cytochrome P-450

Stability of microsomal cytochrome P-450 from tulip bulbs in the presence of Emulgen 911 was examined before large scale purification. Microsomal pellets from tulip bulbs were homogenized in Buffer S containing 0, 0.2, 1.5, and 3.0% Emulgen 911. As described in MATERIALS AND METHODS, the microsomal protein concentration was approximately 10 mg per ml. Each tube was left overnight at 4°C and its content of cytochrome P-450 was measured the next morning. Unexpectedly, the cytochrome P-450 of tulip bulbs was found to be quite stable under these conditions, that is, only a small percentage of cytochrome P-450 was reduced in the presence of 3% Emulgen 911 in the Buffer S. We decided to use 1.5% Emulgen 911 at the first step of solubilization and therefore approximately 15 mg of Emulgen/mg protein.

The results of the typical purification experiments are shown in Table I. The content of cytochrome P-450 in the frozen microsomes was about a half of the freshly prepared micro-

TABLE I. PURIFICATION OF MICROSOMAL CYTOCHROME P-450 FROM TULIP BULBS

Purification step	Total protein (mg)	Total P-450 (nmol)	Specific content (nmol/mg prot.)	Yield (%)
Solubilized Fraction	1,540	342.5	0.22	100
DEAE-Sephadex A-25	811	100.9	0.12	29.5
Polyethylene Glycol	266	60.9	0.23	17.8
DEAE-Cellulose (DE-52)	26.9	34.6	1.29	10.1
Hydroxyapatite	3.7	25.0	6.68	7.3

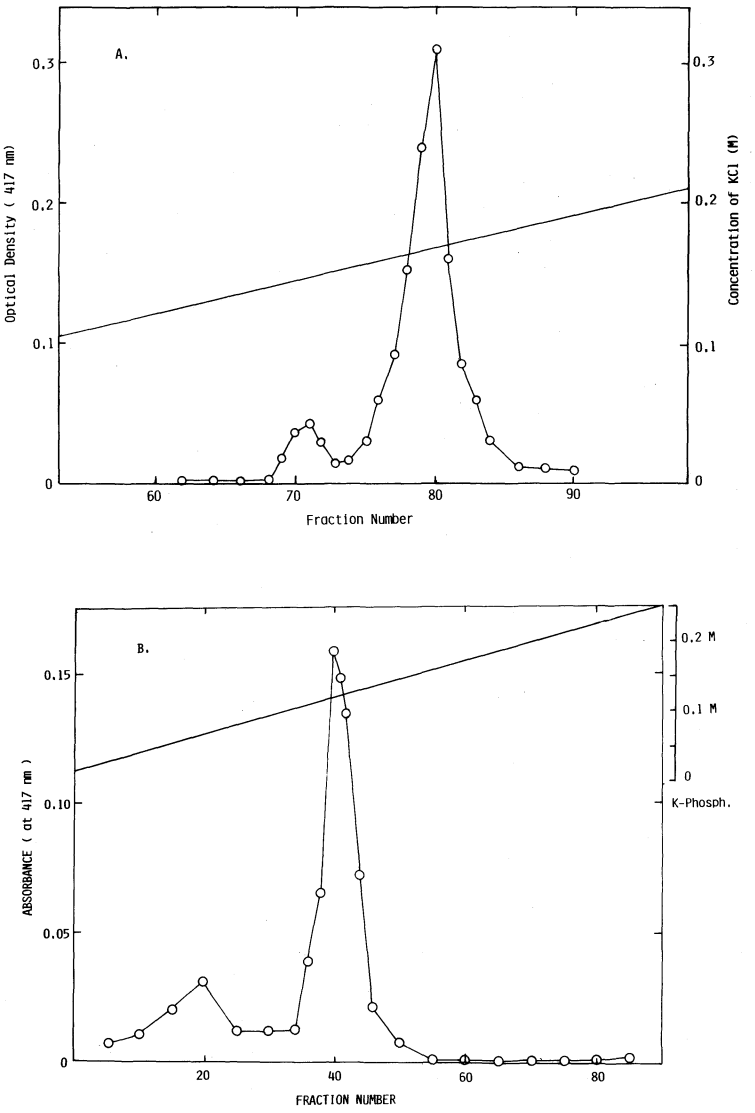

FIG. 1. Chromatography of Solubilized Microsomes.

(A) DEAE-cellulose (DE-52) column chromatography was done at room temperature with a linear gradient of 800 ml of 0 to 0.3 M KCl in Buffer A (see the text). (B) Hydroxyapatite column chromatography was done at 4°C with a linear gradient of 200 ml of 30 to 250 mM potassium phosphate buffer (pH 7.4) containing 0.5% Na-cholate, 0.2% Emulgen 911, 1 mM EDTA, 0.1 mM DTT, and 20% glycerol. Since both buffers employed for Figs. 1A and 1B showed high absorbancy at 280 nm, the profiles of total proteins were not determined.

somes. Furthermore, the specific activity of the preparation after the DEAE-Sephadex A-25, which was used to remove cytochrome b_5, was not increased but rather decreased during this step. The effective step of the purification was DEAE-cellulose (DE-52) column chromatography at room temperature. This step took more than 7~8 hr but did not enhance the degradation of cytochrome P-450 significantly. The components with absorbancy at 417 nm were eluted as two peaks during this DEAE-cellulose column chromatography (Fig. 1A). However, the fraction eluted earlier (fraction 70 to 72) was approximately one-tenth of the major one and antibodies against the purified cytochrome P-450 from the second peak did

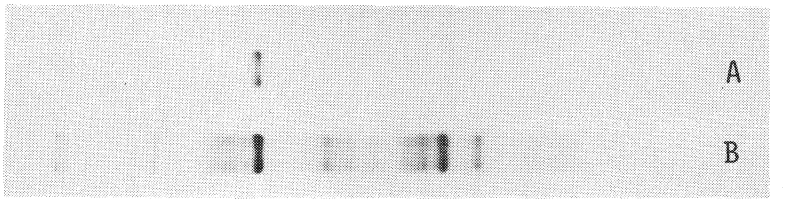

FIG. 2. SDS-Gel Electrophoresis of Crude and Purified Cytochrome P-450.

Lane A contained the mixture of fractions #39, 40, and 41 in Fig. 1-B. Lane B; total microsomal proteins from tulip bulbs. The gel used was a 12.5% Laemmli gel system. Migration was from left to right.

not show common antigenicity with the minor components of this step (data not shown).

The major fraction of cytochrome P-450 obtained after DEAE-cellulose column chromatography (fraction 77 to 83), however, still contained two different forms but these were separated by hydroxyapatite column chromatography (Fig. 1B). The minor component at this step showed a much less specific content of cytochrome P-450 and was distinct immunologically from the major one (data not shown). The recovery of the major form of cytochrome P-450 after the hydroxyapatite column was 7.3% of the total content in the initial solubilized fraction of microsomes (Table I). The specific content of the final preparation was 6.68 nmol/mg protein and this value is approximately 30-fold of the initial solubilized microsomal fraction.

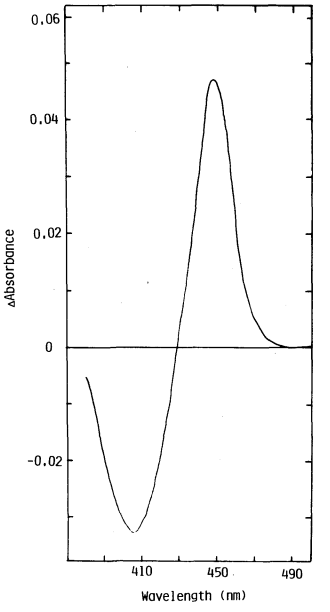

FIG. 3. CO-difference Spectrum of Microsomes of Tulip Bulbs.

Microsomes were homogenized in 0.1 M potassium phosphate buffer (pH 7.4) containing 30% glycerol. The concentration of proteins was approximately 1 mg/ml. The CO-difference spectrum of the dithionite-treated sample was measured.

Purity of the final preparation

In contrast to the multiplicity of microsomal cytochrome P-450 in mammalian livers, the enzymes in tulip bulbs seems to be less heterogeneous and could be separated easily in this study. Profiles of the total microsomal proteins and the mixture from fractions number 40, 41, and 42 in Fig. 1-B were analyzed with SDS-gel electrophoresis and shown in Fig. 2. Only one major band with a molecular weight of 52,500[6] could be observed in lane A and its purity was more than 98%, as judged by densitometry.

Spectral properties

a) *Reduced-CO difference spectrum.* As shown in Fig. 3, the absorption peak in the reduced-CO difference spectrum of the microsomes from tulip bulbs is located at 448 nm and little P-420 is detected after a rather long storage period in a cold room. The reduced-CO difference spectrum of the purified preparation was essentially identical with that of the microsomes (not shown here).

b) *Absolute spectra.* The absolute spectra of purified cytochrome P-450 are shown in Fig. 4 and are quite similar to those reported for mammalian liver.[10] The oxidized form of the

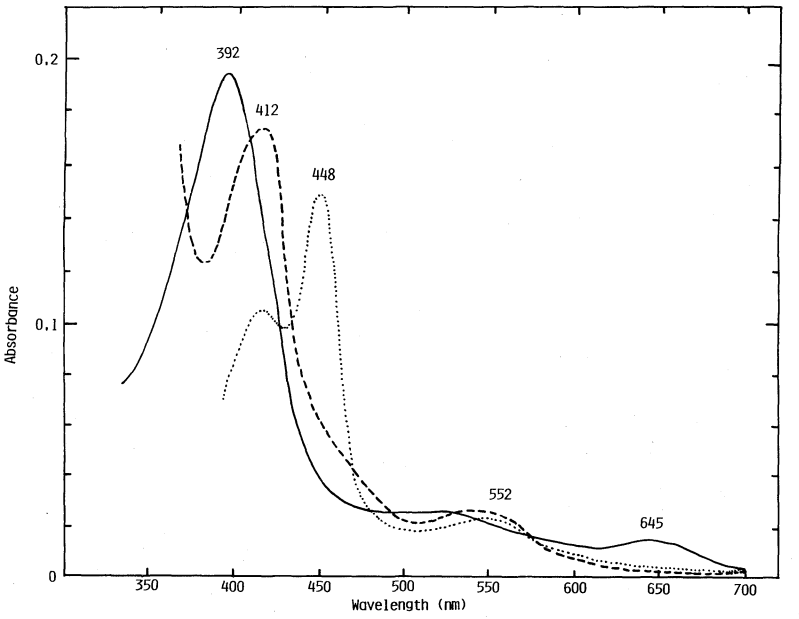

FIG. 4. Absolute Absorption Spectra of Purified Cytochrome P-450 from Tulip Bulbs.

Preparation containing 6.68 nmol cytochrome P-450 per mg protein was dissolved in buffer H (see text). The concentration of cytochrome P-450 was 1.053 μM. ——, oxidized form; ------, reduced with dithionite; · · · ·, carbon monoxide complex of reduced form.

purified fraction had absorption peaks at 392, 525, and 645 nm and the spectrum was mostly the high spin type. Upon reduction with dithionite, the Soret absorption maximum of purified fraction shifted to a longer wavelength (412 nm). The absolute reduced-CO spectrum shows the main peak at 448 nm.

Immunological studies

Antiserum against the purified cytochrome P-450 was prepared in the rabbit and purified by affinity chromatography using the purified cytochrome P-450 as a ligand.[6] Our previous studies with the antibodies against this cytochrome P-450 showed a single precipitin line with either the crude fraction of solubilized microsomes or the purified preparation of its antigen. However, no precipitin lines were detected with the microsomes from 9 other plant species including several bulbous plants.

Therefore, we attempted to examine whether cytochrome P-450 purified from tulip bulbs is present only in the cultivar Balalaika or not. We prepared microsomes from several other cultivars of the tulip. All of the three other cultivars of the tulip demonstrated a common antigenicity with the purified cytochrome P-450 from cultivar Balalaika. One example is shown in Fig. 5, in which the

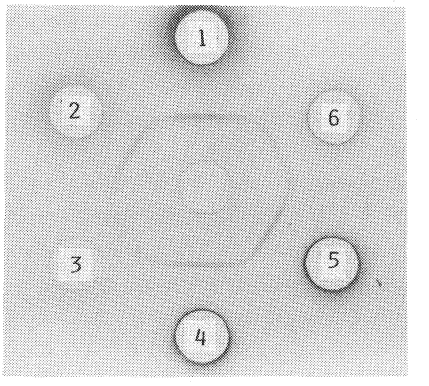

FIG. 5. Ouchterlony Double Diffusion Studies.

Purified antibodies (center well) and solubilized microsomes from cultivars Balalaika (wells 1, 2, 3) and Merry Widow (4, 5, 6) were prepared as described in the text. The concentrations of microsomal proteins were approximately 1 mg/ml for well 1 and 4, 0.3 mg/ml for well 2 and 5, 0.1 mg/ml for well 3 and 6, and 12 μl per well.

microsomes were prepared from cultivar Merry Widow. It is likely that at least there is a common form of microsomal cytochrome P-450 in the tulip bulbs examined, although its physiological role is unknown in these dormant tissues.

DISCUSSION

This paper describes the details of purification and some characterization of the major form of microsomal cytochrome P-450 in dormant tulip bulbs. The content of microsomal cytochrome P-450 in tulip bulbs is relatively high among various plant tissues examined,[3] although its physiological role in these dormant tissues is unknown. The activity of *trans*-cinnamate 4-monooxygenase, which is an important enzyme for secondary metabolism in a variety of higher plant tissues, has been shown to be lower in the tulip bulbs than the other plant tissue examined, such as Jerusalem artichoke and potato tubers.[11] Besides the content of cytochrome P-450 in tulip bulbs, the high stability of this hemoprotein during storage in a cold room even for several months and also during purification procedures made us decide to employ these materials for purification.

Solubilization and purification of cytochrome P-450 from bacteria[12] and a fungus[13] have been reported but no electrophoretically homogeneous preparation has been isolated from higher plants. As far as we know, our previous report is the first case.[6] We employed essentially the same procedures as those of Saito and Strobel[7] for purification of microsomal cytochrome P-450 from rat livers. The chromatographic behavior of tulip cytochrome P-450 closely resembled one of those from rat livers. Furthermore, the molecular weight and amino acid compositions are also analogous to animal ones.[6,14,15] The relatedness of the amino acid compositions of cytochrome P-450 between tulip bulbs and rat livers were calculated by the method of Cornish-Bowden.[16] According to his test, amino acid compositions of cytochrome P-450 from both tissues are related.[6] Optical properties of the purified cytochrome P-450 in this paper also demonstrated the similarity of plant cytochrome P-450 to animal ones. It should be noted, however, that the intensity of the absorption maximum of the CO complex of reduced form at 448 nm is smaller than that of the reduced Soret peak (Fig. 4). This was not due to the conversion of P-450 to P-420 because the CO-difference spectrum of the purified cytochrome P-450 showed essentially identical profiles with Fig. 3. The reason for the reduced intensity of the CO complex of reduced form at 448 nm is unknown. However, this might contribute partially to the low specific content of the final preparation (Table I). So far, no iron-sulfur proteins in the microsomal cytochrome P-450 system has been observed in higher plant tissues.

Recently, several papers have demonstrated the presence of multiple forms of microsomal cytochrome P-450 in higher plants, such as Jerusalem artichoke tubers.[17,18] In this study, we have isolated the major form of cytochrome P-450 from the microsomes of tulip bulbs. There were minor fractions with absorbancy at 417 nm during both DEAE-cellulose and hydroxyapatite column chromatographies (Fig. 1). However, they had much less specific contents of cytochrome P-450 and have not been purified and characterized further. These minor components were immunochemically distinct from the purified major form (data not shown). Judging from the profiles of SDS-gel electrophoresis of total microsomal proteins of tulip bulbs (Fig. 2 and ref. 19) and the CO-difference spectrum (Fig. 3), we consider that the final preparation of cytochrome P-450 corresponds to the major form in the microsomes of tulip bulbs.

Immunochemical studies have shown highly specific antigenicity of the antiserum against this major form of cytochrome P-450. Our previous paper[6] demonstrated that no precipitin lines could be detected with the extracts from the microsomes of a variety of higher plants examined, although there existed com-

parable contents of cytochrome P-450 in these microsomes. The possibility that we have merely isolated a peculiar form of microsomal cytochrome P-450 was ruled out through immunochemical studies with several other cultivars of tulip, as described in the text. All three cultivars of tulip bulb had at least identical antigenicity with the purified preparation in their microsomes. It is likely that there are some common physiological roles of these cytochrome P-450 in the tulip bulbs, although we have not attempted to reconstitute the monooxygenase system with our purified preparation to examine some native substances as its specific substrate.

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