Isolation of Plant Cytochrome P-450 and NADPH: Cytochrome P-450 Reductase from Tulip Bulbs (*Tulipa fosteriana L.*) Oxidizing Xenobiotics

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Abstract. Cytochrome P-450 and NADPH: cytochrome P-450 reductase were solubilized by detergents from microsomal fraction of tulip bulbs *Tulipa fosteriana L.* and purified to electrophoretic homogeneity. The purification was achieved by anion-exchange column chromatography, hydroxyapatite-column chromatography and affinity chromatography. The two enzyme showed relative molecular weights of about 54,200 and 77,600 for cytochrome P-450 and NADPH: cytochrome P-450 reductase, respectively. The purified enzymes were characterized by their absorption spectra and by kinetic characteristics. The interaction with endogeneous as well as exogenous substrates was studied by differential spectroscopy. Both enzymes in the presence of dilauroyl phosphatidylcholine and NADPH were able to oxidize xenobiotics (N-nitroso-N-methylaniline and N-nitroso-N-dimethylamine) in the reconstitution experiments.

Key words: Cytochrome P-450 — NADPH:cytochrome P-450 reductase — Xenobiotics metabolism in plant — *Tulipa fosteriana*

Introduction

The number of cytochrome P-450-dependent enzymes found in higher plants increases steadily. These microsomal activities in plants are prevalently active in plant secondary metabolism and in the oxidation of xenobiotics. Similar to the microsomal system in animals, the cytochrome P-450-dependent monooxygenases

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in plants serve as terminal oxidases of an electron transport chain and depend on NADPH:cytochrome P-450 reductase as an electron donor (for review see Higashi 1985; Riviere and Cabanne 1987).

Purification of cytochrome P-450 (cyt P-450) from plant tissues and characterization of the purified activities have been reported in a very few cases (Higashi et al. 1983, 1985; Gabriac et al. 1985; Kochs and Grisebach 1989; O'Keefe and Leto 1989; Gabriac et al. 1991; Kochs et al. 1992). Moreover, the coexistence of different cyt P-450 subspecies in the same plant, assumed from induction experiments (Benveniste et al. 1982; Kochs et al. 1987) and inhibitor studies (Kochs et al. 1992), could be demonstrated only recently at the protein level by the separation of one enzyme (cinnamate 4-hydroxylase) from the other cyt P-450 (3,9-dihydroxypterocarpan 6a-hydroxylase) from soybean on hydroxyapatite (Kochs and Grisebach 1989) and by the chromatographic separation of cinnamate 4-hydroxylase of Jerusalem artichoke from several other cyt P-450 isoforms by monitoring substrate binding spectra (Gabriac et al. 1991).

Similarly, isolation and characterization of NADPH:cyt P-450 reductases from plant tissues has been reported only in very few cases (Benveniste et al. 1986, 1991).

It was possible only in the soybean system to measure good activities in the purified cyt P-450 protein fractions after reconstitution with NADPH:cyt P-450 reductase and phospholipids (Kochs et al. 1992). Only endogenous compounds have been studied as substrates of isolated plant cytochromes P-450 since the attempts to convert xenobiotics *in vitro* were unsuccessfull (Higashi 1985, 1988; Riviere and Cabanne 1987; Kochs et al. 1992).

In this paper we report on the isolation and characterization of cyt P-450 and NADPH:cyt P-450 reductase from microsomes of tulip bulbs. The enzymes were characterized by their absorption spectra and kinetic data. The isolated cyt P-450 isoform is able to convert exogenous substrates when reconstituted with purified reductase and with dilauroyl phosphatidylcholine.

Abbreviations: ΔA_{sat}, differences between the actual minimal and maximal absorbances; cyt P-450, cytochrome P-450; cyt $P\text{-}450_b$, cyt $P\text{-}450_c$, cytochrome P-450 isolated from livers of rats pre-treated with phenobarbital; cyt $P\text{-}450_{TB}$, cytochrome P-450 of tulip bulbs; EDTA, ethylenediaminetetraacetate; FMN, flavin mononucleotide; K_m, Michaelis constant; K_s, spectral dissociation constant; NDMA, N-nitrosodimethylamine; NMA, N-nitrosomethylaniline PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis.

Materials and Methods

Chemicals and subcellular fraction

Chemicals were obtained from the following sources: acrylamide, N,N,N′,N′-tetramethylendiamine, N,N′-methylen(bis)acrylamide, cytochrome c, $NADPH^+$ and NADPH from Fluka, Switzerland; dithiothreitol from Koch-Light, UK; Emulgen 911 from Atlas Co., Japan and all other chemicals were reagent grade or better. N-nitrosomethylaniline (NMA) and N-nitrosodimethylamine (NDMA) were synthesized as described previously (Druckrey et al. 1967). Microsomes from rat livers were prepared as described by Kimura et al. (1982) from rats pre-treated with phenobarbital as described by Stiborová et al. (1988).

Microsomes of bulbs, roots and shoots of tulip plants *Tulipa fosteriana L.* were prepared from tissues without any inducing pretreatment. Plant tissues were sliced (1 mm thick) and washed by distilled water (2 × 200 ml). 200 g of plant tissue slices were homogenized in 300 ml of 0.1 mol/l sodium phosphate (pH 7.4) containing 0.6 mol/l mannitol, 1% bovine serum albumin, 10 mmol/l 2-mercaptoethanol, 5 mmol/l EDTA and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF). The homogenates were filtered and centrifuged for 20 min at 15,000 ×*g* (at 4 °C). The resulting supernatants were collected and centrifuged for 90 min at 125,000 ×*g* (at 4 °C). The microsomal pellets were washed by homogenization buffer (see above) and centrifuged for 60 min at 165,000 × *g*. The microsomal pellets were resuspended in 0.1 mol/l sodium phosphate (pH 7.4) containing 30% (v/v) glycerol and stored at −70 °C until used without apparent loss of activity over several months.

Purification of enzymes

Cytochrome $P\text{-}450_b$ and $P\text{-}450_e$ were isolated from liver microsomes of rats pre-treated by phenobarbital by procedure described previously (Anzenbacher et al. 1984).

Solubilization of plant microsomes

Plant microsomes were solubilized with Triton X-100, Triton X-114 and Emulgen 911. Microsomes were mixed with 0.1 mol/l potassium phosphate (pH 7.4) containing 30% (v/v) glycerol, 1.5 mmol/l 2-mercaptoethanol, 1 mmol/l PMSF and 0.1 mmol/l EDTA to obtain final protein concentration 2.0–2.5 mg/l. Solubilization was carried out under a stream of nitrogen. 2% (w/v) Triton X-114 or Triton X-100 was used as detergent. During solubilization (20 min), the slurry was sonicated three times for 30 s and centrifuged for 60 min at 125,000 × *g* (at 4 °C). Similarly as described by Gabriac et al. (1991), two phases were formed spontaneously during centrifugation when Triton X-114 was used; the upper, detergent-rich phase contained 75% of the spectrophotometrically detectable cyt P-450. This upper phase was carefully separated from the lower detergent-poor phase. The detergent-rich phase was diluted five times with 10 mmol/l potassium phosphate (pH 7.7), containing 25% (v/v) glycerol, 1.5 mmol/l 2-mercaptoethanol, 0.4% Emulgen 911 and 1 mmol/l EDTA before being loaded onto a column of DEAE-Sepharose CL 6B. If Triton X-100 was used, only one-phase supernatant and pellet were formed during centrifugation. 1.5% (v/v) Emulgen 911 was used as another detergent for solubilization of microsomes. Sonication and centrifugation of the solution were carried out as in the procedure, in which Triton X-100 or Triton X-14 were used (see above).

Chromatography on DEAE-Sepharose CL 6B and hydroxyapatite

Solubilized microsomal fractions were chromatographed on a DEAE-Sepharose CL 6B column (30 × 2 cm). Under the conditions used, two pools of cyt P-450 could be separated; pool *A* did not bind the gel and it was eluted from the column immediately with 0.1 mol/l potassium phosphate (pH 7.4) containing 1.5 mmol.l/l 2-mercaptoethanol, 0.4% Emulgen 911, 0.4% Triton X-100, 25% glycerol, 1 mmol/l EDTA and 0.1 mol/l mannitol (pool *A*). The residual portion of cyt P-450 (pool *B*) was eluted from the column using the linear gradient of potassium phosphate (0.1–0.5 mol/l) in above mentioned buffer and it was separated from NADPH:cyt P-450 reductase. The fractions containing the highest concentrations of enzymes (cyt P-450 – pool *B* and reductase) were pooled separately. Fractions containing cyt P-450 were then dialyzed against 10 mmol/l potassium phosphate (pH 7.4) containing 20% glycerol, 1.5 mmol/l 2-mercaptoethanol. 0.1 mmol/l EDTA, 0.1 mol/l mannitol, 0.2% Triton X-100 and 0.2% Emulgen 011. Cyt P-450 (pool B) was then chromatographed on a column of hydroxyapatite (30 × 1 cm) using the linear gradient of KCl (0–0.5 mol/l) in 0.1 mol/l potassium phosphate (pH 7.4) containing 1.5 mmol/l 2-mercaptoethanol, 0.4% Emulgen 911, 0.4% Triton X-100, 25% glycerol, 1 mmol/l EDTA and 0.1 mol/l mannitol. Isolated cyt P-450 was stored in 10 mmol/l potassium phosphate (pH 7.4) containing 20% glycerol at −70 °C.

Chromatography of NADPH:cytochrome P-450 reductase on 2′,5′-ADP Sepharose 4B

Fraction containing NADPH:cyt P-450 reductase obtained by chromatography on DEAE-Sepharose CL 6B was dialyzed against 10 mmol/l potassium phosphate (pH 7.8) containing 0.1 mmol/l EDTA , 1.5 mmol/l 2-mercaptoethanol, 20% glycerol and 0.2% Emulgen 911. Detergent was then removed by adsorption on silica gel (0.02 g/l) as described (Anzenbacher et al. 1984). Reductase was applied on a column of 2′,5′-ADP Sepharose 4B (15 × 1 cm). The column of 2′,5′-ADP Sepharose 4B was washed with 0.3 mol/l potassium phosphate (pH 7.8) and 10 mmol/l potassium phosphate (pH 7.8) containing 0.1 mmol/l EDTA, 1.5 mmol/l 2-mercaptoethanol, 20% glycerol and 0.2% Emulgen 911. The reductase was then eluted from the column with 0.5 mmol/l $NADP^+$ dissolved in 10 mmol/l potassium phosphate (pH 7.4) containing 0.02% Emulgen 911. Reductase was stored after addition of FMN (2.5 μmol/l) at −70 °C.

Analytical methods

Quantitative determination of cyt P-450 and cyt P-450 was carried out according to Omura and Sato (1964). The concentration of NADPH:cyt P-450 reductase was estimated as described earlier (Vermilion and Coon 1978). The activity of NADPH:cyt P-450 reductase was measured according to Sottocasa et al. (1967) using cytochrome c as the substrate (i.e. as NADPH:cytochrome c reductase). The incubation of reductase with FMN and dialysis were carried out as described by Benveniste et al. (1986).

Polyacrylamide slab gel electrophoresis was performed in the presence of 0.1% (w/v) SDS with 8% (w/v) acrylamide as described by Laemmli (1970). Bovine serum albumin, catalase, ovalbumin, cytochrome c and phosphorylase were used as standards.

Protein concentrations were estimated according to Bradford (1976) or Lowry et al. (1951) with bovine serum albumin as a standard.

In vitro binding of substrates to cyt P-450 was monitored by differential spectroscopy (Jefcoate 1978). Tested compounds were directly added to the sample cuvette containing oxidized microsomes or purified fractions, an identical volume of solvent being added to the reference. 30 seconds after mixing, absorption spectra were recorded between 370 and

500 nm on a spectrophotometer SPECORD M-42 (Carl Zeiss, Germany). Results were expressed as A (380–425 nm).ml^{-1} obtained after addition of substrate. When shifts of minima or maxima of absorbance were observed, ΔA between the actual minima and maxima were estimated (ΔA_{SAT}). Spectral dissociation constants (K_s) were determined using double-reciprocal plots of the absorbance differences ΔA (380–425 nm) versus substrate concentrations.

Kinetic analyses were carried out using the non-linear least-square methods described previously (Cleland 1983).

The assay media for N-demethylation reactions of NMA and NDMA contained in 1 ml: 60 pmol/l cyt P-450 isolated from tulip bulbs, 90 pmol/l NADPH:cyt P-450 reductase from the same source, 0.05 mol/l potassium phosphate (pH 7.4), 7.5 mmol/l $MgCl_2$, 8 mmol/l semicarbazide, 4 mmol/l glucose-6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase, 60 μg of D,L-dilauroyl phosphatidylcholine and 0–50 mmol/l NMA or NDMA dissolved in dimethylsulfoxide (DMSO). After 1 min preincubation the reaction was started by addition of $NADP^+$ (final concentration was 0.5 mmol/l). The mixture was incubated 10 min at 37 °C. The reaction was determined by addition of 20% trichloroacetic acid and the amount of formaldehyde formed was measured as described by Nash (1953).

For the reconstitution experiments, either 30–250 pmol/l of the NADPH:cyt P-450 reductase and a constant amount of 125 pmol/l of P-450 in one ml were used, or 20–200 pmol/l of cyt P-450 were tested with a constant amount of the NADPH:cyt P-450 reductase (150 pmol/l). The NMA or NDMA concentration was 3 mmol/l.

The assay media for demethylation reactions of NMA and NDMA catalyzed by microsomes contained in one ml 50 mmol/l potassium phosphate (pH 7.4), 0.2 nmol/l of cyt P-450 (measured in microsomal preparations containing 2–3 mg/l protein), 0.4 units of glucose-6-phosphate dehydrogenase, 4 μmol/l glucose-6-phosphate, 7.5 μmol/l $MgCl_2$, 8 μmol/l semicarbazide and 0.015–0.6 μmol/l NMA or 0.15–15 μmol/l NDMA (dissolved in DMSO). The following procedure was the same as that described for the reconstitution experiments (see above).

Results

Distribution of cytochrome P-450 in tulip plants and its solubilization

We studied the content of cyt P-450 in tissues of tulip plants cultivated for seven days in distilled water. The content of cyt P-450 was determined in plant microsomes sedimented by centrifugation (see Materials and Methods).

The tulip bulbs contained a larger amount of cyt P-450 than the roots. The shoots of tulip plants do not contain any measurable content of cyt P-450 (Table 1). The tulip bulbs were therefore used for further experiments.

It was postulated previously that the most important factor for successfull isolation of plant cyt P-450 is using a suitable detergent for solubilization (Higashi et al. 1983, 1985; Gabriac et al. 1991). Three detergents were tested in our laboratory; Triton X-100, Triton X-114 and Emulgen 911. Triton X-114 was found to be the best detergent, as cyt P-450 solubilized with this detergent exhibited the highest specific content (Table 2).

The nature of detergents used affected the measurable content of cyt P-450 in

Table 1. The content of cytochrome P-450 in tulip tissues

Plant tissue	Total cyt P-450 (nmol/l)	Specific content of cyt P-450 (nmol/mg)
Roots	1.35	0.030
Bulbs	28.00	0.045
Shoots	0	0

The experimental conditions for isolation of microsomes and determination of cytochrome P-450 or protein concentrations are described in the text (see Materials and Methods).

Table 2. Solubilization of cytochrome P-450 from microsomes of tulip bulbs in Triton X-114 (A), Triton X-100 (B) and Emulgen 911 (C)

	Total cyt P-450 (nmol/l)	Specific content of cyt P-450 (nmol/mg)	Recovery of cyt P-450 (%)
(A)			
Microsomes	28.00	0.046	100.00
Detergent phase	21.00	0.150	75.00
Aqueous phase	3.62	0.026	12.93
(B)			
Microsomes	27.00	0.045	100.00
Solubilizate	21.50	0.060	79.63
(C)			
Microsomes	34.50	0.048	100.00
Solubilizate	29.50	0.080	85.50

Experimental conditions are described in the text.

microsomes. Only 80% of cyt P-450 remained detectable when microsomes contained Triton X-100 or Triton X-114, while 100% of cyt P-450 remained detectable with Emulgen 911 (Table 2).

Similarly as it was postulated by Gabriac et al. (1991), plant microsomes solubilized by Triton X-114 in the presence of 30% glycerol were spontaneously separated into two phases during centrifugation of solubilized supernant from membrane residues. The upper, detergent-rich phase (red) contained around 75% of the whole amount of cyt P-450 (Table 2), as well as most of the NADPH:cyt P-450 reductase (not shown). The lower, detergent-poor phase contained a lower amount

of cyt P-450 (Table 2).

The phase partitioning as the result of solubilization by Triton X-114 resulted in a threefold increase of specific content of cyt P-450 (Tables 2 and 3).

Table 3. Purification of cytochrome P-450 from microsomes of tulip bulbs

Step	Total cyt P-450 (nmol)/l	Specific content of cyt P-450 (nmol/mg)	Purification (fold)	Recovery (%)
Microsomes	27.00	0.045	1.0	100.0
Triton X-114 rich phase	21.00	0.150	3.3	77.7
DEAE-Sepharose Pool *A*	11.48	0.930	20.7	42.5
Pool *B** (peak in Fig. 1)	4.70	0.830	18.4	17.4
Hydroxyapatite Peak 1 (in Fig. 2)	3.25	5.20	115.5	12.1

* Pool *B* was only used for chromatography on hydroxyapatite, pool *A* was removed. For details see the text.

Isolation of cytochrome cyt P-450 and its spectral properties

Solubilization and phase partition in Triton X-114 was followed by chromatography on a column of DEAE-Sepharose CL 6B (Fig. 1). The presence of 0.4% (w/v) of Emulgen 911 and Triton X-100 in the buffers used for column equilibration and proteins elution cased that 42% of total cyt P-450 did not bind to DEAE-Sepharose CL 6B and was eluted from the column (assigned as pool A in Table 2). This fraction, however, contained a larger amount of other (ballast) proteins and only a low amount of cyt P-450 binding xenobiotic substrates (NMA, NDMA) (data not shown); therefore it was not used for further enzyme purification. Cyt P-450 retained on the DEAE-Sepharose CL 6B column (pool B) was separated from NADPH:cyt P-450 reductase from the column using the linear gradient of potassium phosphate (0.1–0.5 mol/l) (Fig. 1).

Chromatography on a column of hydroxyapatite was used as the final purification step (Fig. 2). The final preparation had a specific content of about 5 nmol/l cyt P-450 per mg protein and the recovery of cyt P-450 was about 12% (Table 3).

SDS-electrophoresis on polyacrylamide gel was used for the estimation of purify of the final cyt P-450 preparation and for the determination of its molecular mass.

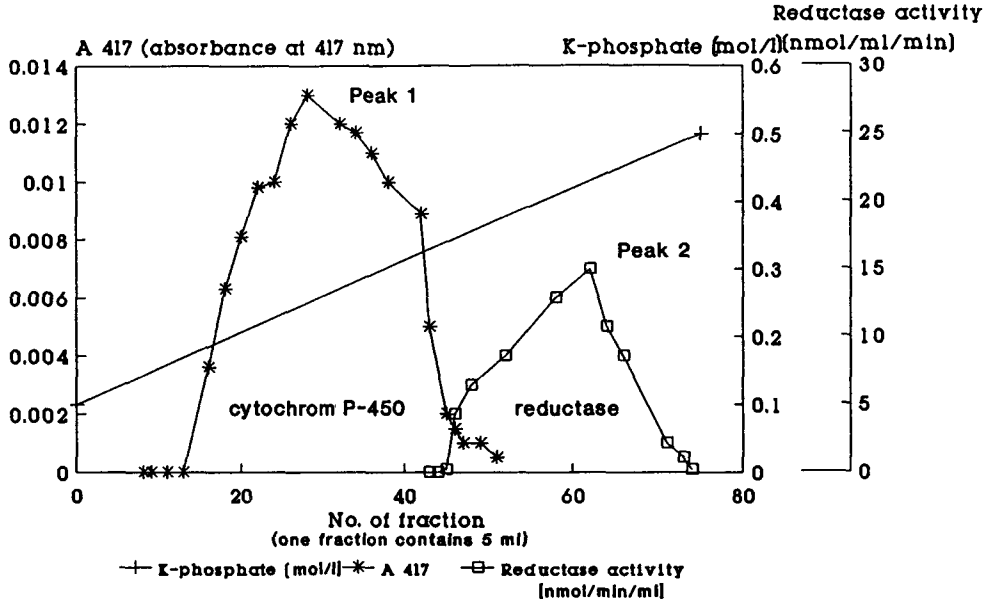


Figure 1. Chromatography of the Triton X-114 rich fraction on DEAE-Sepharose CL 6B. Elution profile of cyt P-450 (measured as the absorbance at 417 nm (∗), Peak 1) and NADPH:cyt P-450 reductase (nmol/min/ml (□), Peak 2). The concentration gradient of potassium phosphate (+) was used for separation of both enzymes.

Isolated isoform of cyt P-450 is an electrophoretically homogeneous protein with a relative molecular mass of about 54,200 (Fig. 3).

The absolute absorption spectrum of isolated native plant cyt P-450(Fe^{3+}), that of reduced cyt P-450(Fe^{2+}) in the complex with CO and that of reduced cyt P-450(Fe^{2+}) in the complex with metyrapon were measured and compared with the same spectra of isoenzymes of cyt P-450 isolated from rat livers (cyt P-450_b, cyt P-450_e). Table 4 shows the values of absorption maxima of these cyt P-450 isoenzymes. After reduction, isolated plant cyt P-450 shows an absorption maximum at 450.5 nm in the presence of carbon monooxide. Amounts of cyt P-450 were, hence, estimated from these CO-difference spectra by the method described for liver enzymes previously (Omura and Sato 1964). The similarities of the spectra of plant cyt P-450 preparation with those of animal ones are evident (Table 4). However, there are also differences in their spectra. Oxidized cyt P-450 of tulip bulbs exhibited a shift to 385 and to 640 nm in comparison with maxima of mammalian cytochromes P-450 (417 and 565 nm) signalizing also high spin component spectra (the mixed spin state of the plant enzyme).

The interactions of plant cyt P-450 with the compounds, which are the endoge-

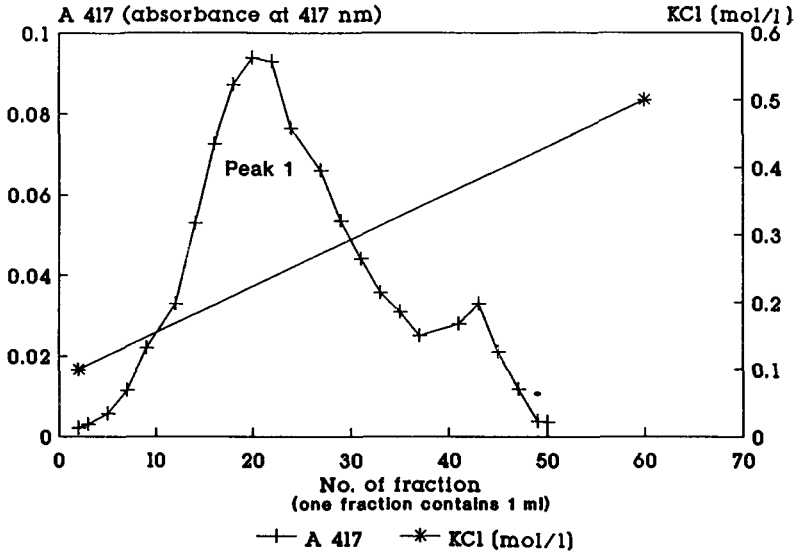
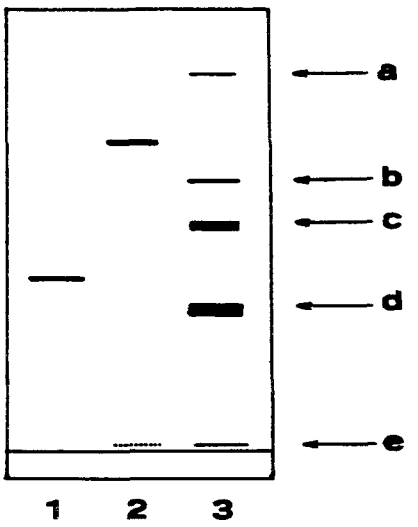


Figure 2. Chromatography of the plant cyt P-450 fraction obtained by DEAE-Sepharose CL 6B (pool B) on hydroxyapatite

Figure 3. SDS-polyacrylamide gel electrophoresis of cyt P-450 purified by the isolation procedure (Lane 1), isolated NADPH cyt P-450 reductase (Lane 2) and standards (Lane 3) Phosphorylase (94,000) (*a*), bovine serum albumin (67,000) (*b*), catalase (60,000) (*c*), ovalbumin (43,000) (*d*) and cytochrome c (12,4000) (*e*) were used as standards



nous substrates of cyt P-450 or with those, which could be the potential exogenous substrates of this enzyme were studied by differential spectroscopy The binary complexes of enzyme with cinnamic acid, lauric acid or N-nitrosomethylaniline ex-

Table 4. The absorption maxima of cytochrome P-450 isolated from tulip bulbs (cyt P-450$_{TB}$) and rat livers (cyt P-450 b, cyt P-450$_e$)

Isoenzyme	Absorption maxima of oxidized cyt P-450 (Fe^{3+})	Maxima of the cyt P-450(Fe^{2+})-CO complex (nm)	Maxima of the cyt P-450(Fe^{2+}) -metyrapon complex
cyt P-450$_b$	358.6	449.8	445.0
	417.9	543.2	
	534.4		
	567.8		
cyt P-450$_e$	360.3	450.6	446.2
	416.8	544.3	
	531.5		
	564.2		
cyt P-450$_{TB}$	345.0	450.5	447.5
	385.0	548.0	
	530.0		
	640.0		

The absorption spectra of the cytochrome P-450 and those of complexes of their reduced forms with CO or metyrapon were measured in 10 mmol/l potassium phosphate (pH 7.4) on a SPECORD M-42 spectrophotometer (Carl Zeiss, Germany).

Table 5. Spectral characteristics resulting from the binding of substrates to plant cytochrome P-450 isolated from tulip bulbs

Substrate	K_S (μmol/l)	ΔA_{SAT}	Values of extremes (nm)	
			maxima	minima
Cinnamic acid	1.72 ± 0.14	0.03 ± 0.0046	390	421
Lauric acid	0.09 ± 0.05	0.02 ± 0.0020	390	418
NMA	448.00 ± 23.00	0.005 ± 0.0013	389	425
NDMA	745.00 ± 21.00	0.005 ± 0.0010	412	380

Spectral dissociation constants (K_s) and differences between the actual minimal and maximal absorbance (ΔA_{SAT}) were estimated as described in Materials and Methods. The numbers in the table (K_S and A_{SAT}) are averages and standard deviations of three parallel experiments.

hibited high spin forms. It was shown in the substrate binding spectra with a maximum at around 390 nm and a minimum at around 420 nm (Table 5). Table 5 also shows the values of spectral dissociation constants (K_s) and the absorbance

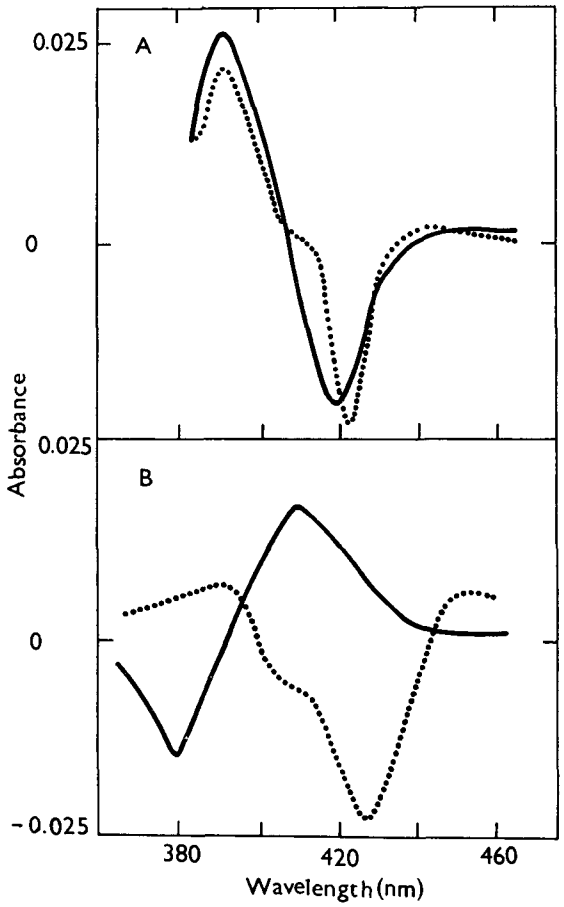


Figure 4. Substrate difference spectra of purified plant cyt P-450 with 12 μmol cinnamic acid (*A*, ·····), 10.2 μmol/l lauric acid (*A*, ——) 0.3 μmol/l NMA (*B*, ·····), and 1 mmol/l NDMA (*B*, ——). The substrate spectra of cyt P-450 (0.9 pmol/l) were measured at 20 °C in 20 mmol/l potassium phosphate (pH 7.4) on a SPECORD M-42 spectrophotometer (Carl Zeiss, Germany). The total volume in the cuvettes was 0.5 ml. The reference cuvette contained the same buffer without cyt P-450.

differences (ΔA_{SAT}) for these compounds. Among the compounds tested, the highest affinity of cyt P-450 protein was shown to be for lauric acid and cinnamic acid. The dissociation constant determined for cinnamic acid (1.72 μmol/l) is close to the value of 1.5 μmol/l determined by Gabriac et al. (1991) for this substrate in the Mn-induced microsomes of Jerusalem artichoke.

Typical type I binding spectrum was obtained for cinnamic acid and lauric acid (Fig. 4).

We moreover found that also compounds exogenous for plants (namely NMA and NDMA) interacted with isolated plant cyt P-450 NMA elicited a spectrum, which was close to type I binding spectrum and NDMA the reverse type of spectrum (see maxima in Table 5 and Fig 4) Both exogenous substrates were also oxidized by cyt P-450 isolated from tulip bulbs when reconstituted with NADPH cyt P-450 reductase *in vitro* (see below)

Table 6. Purification of NADPH cytochrome P-450 reductase from microsomes of tulip bulbs

Step	Total activity* (nmol/min)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
Microsomes	2666 50	2 71	1 00	100 0
Triton X-114				
Rich phase	1850 72	3 77	1 39	69 4
DEAE-Sepharose				
CL 6B (peak 2 in Fig 1)	2775 00	26 73	9 86	104 1
2′,5′-ADP Sepharose 4B				
Peak 1 (Fig 5)	2100 0	77 50	28 60	78 7
Peak 2 (Fig 5)	672 0	22 32	8 24	25 2

* NADPH-dependent reduction of cytochrome c was monitored (for experimental details see Materials and Methods)

Isolation of NADPH cytochrome P-450 reductase and its spectral properties

The homogenous preparation of NADPH cyt P-450 reductase of tulip bulbs was isolated from the plant microsomes by the slightly modified method described earlier (Benveniste et al 1986, 1991) The isolation procedure involved detergent solubilization, phase partition, chromatography on a column of DEAE-Sepharose CL 6B (the second peak in Fig 1 eluted with 0 3 mol/l K-phosphate) and the affinity chromatography on 2′,5′-ADP Sepharose 4B (Fig 5 Table 6) A linear gradient of KCl used for elution of proteins (solubilized microsomes) from the column of DEAE-Sepharose CL 6B achieved the separation of cyt P-450 from reductase successfully The fraction containing reductase was, however, contaminated by cytochrome b_5 (data not shown) Affinity chromatography on 2′,5′-ADP Sepharose was hence used It should be mentioned that the presence of detergents in the enzyme fractions after the chromatography on DEAE-Sepharose CL 6B caused that NADPH cyt P-450 reductase did not interact with the affinity carrier (2′,5′-ADP Sepharose 4B) and under these conditions it was eluted from the column

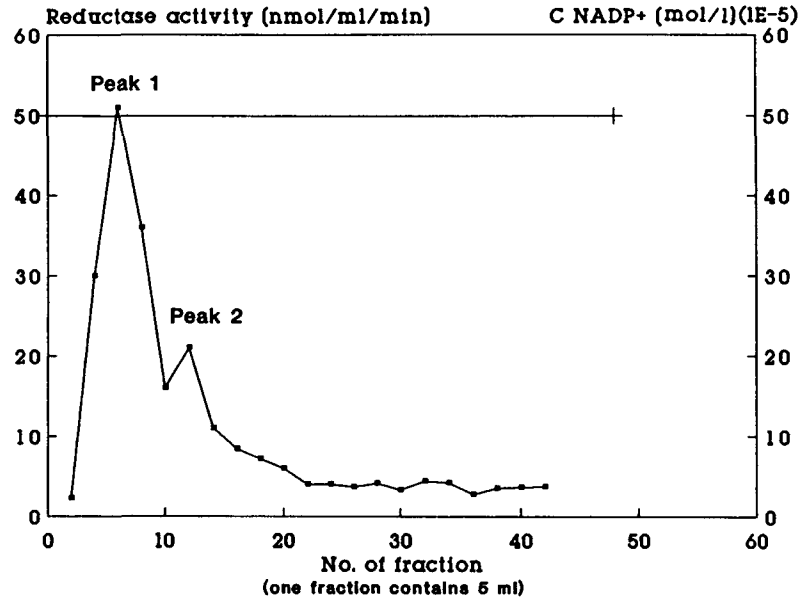


Figure 5. Chromatography of plant NADPH:cyt P-450 reductase on 2′,5′-ADP Sepharose 4B. (·) Reductase activity, (+) Concentration of $NADPH^+$.

immediately. The detergents, hence, had to be removed from the enzyme preparations. The absorption on silica gel was used for these purpose (Anzenbacher et al. 1984). On the other hand, the presence of 0.2% Emulgen 911 was necessary for further enzyme purification. At the concentration of this detergent of 0.2% (w/v), NADPH:cyt P-450 reductase was selectively retained on the affinity gel, whereas residual cytochrome b_5, which was present in the sample after chromatography on DEAE-Sepharose, was excluded.

NADPH:cyt P-450 reductases, which interacted with 2′,5′-ADP Sepharose 4B, were eluted from the column after elution of other proteins (eluted with 0.3 mol/l potassium phosphate) with 0.5 mmol/l $NADP^+$ as two peaks of the reductase activity (Fig. 5). The major peak 1 (Fig. 5) of the higher specific activity (Table 6) was used for its further characterization and the reconstitution experiments.

The major isoenzyme is electrophoretically homogeneous and its molecular mass was estimated to be 77,600 (see Fig. 3; SDS-PAGE). The final preparation of NADPH:cyt P-450 reductase had a specific activity of 77.5 nmol/min/mg (Table 6).

Absorption spectra of NADPH:cyt P-450 reductase and those of its complexes with NADPH are shown in Fig. 6. Because of the lability of the prosthetic group of this enzyme (FMN) (Benveniste et al. 1986, 1991), the absorption spectra were measured in purified reductase, which was incubated in the presence of FMN and

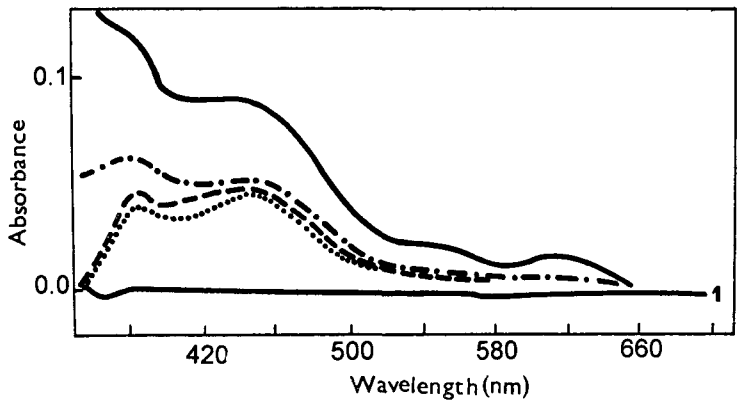


Figure 6. Absorbance spectra of purified plant NADPH:cyt P-450 reductase. The reductase concentration was 10 pmol/l in 20 mmol/l potassium phosphate (pH 7.4) at 20 °C on a SPECORD M-42 spectrophotometer (Carl Zeiss, Germany). The total volume in the cuvettes was 0.5 ml. The reference cuvette contained the same buffer, without reductase. An equal absorbance baseline (———, 1) was established with buffer in the two cuvettes. Reductase without any addition (———), aerobically NADPH-reduced reductase (15 μmol/l NADPH) (— · — · —·), aerobically NADPH-reduced reductase after reoxidation with 85 μmol/l (— — — —) and 190 μmol/l $K_3Fe(CN)_6$ (· · · · ·).

then dialyzed to eliminate the excess of unbound FMN. The reductase exhibited in the visible spectrum a peak at 455 nm and a shoulder at 380 nm. After reduction with NADPH under aerobic conditions, the peak at 455 nm decreased, and a peak at 380 nm clearly appeared. Reoxidation of the enzyme by $K_3Fe(CN)_6$ resulted in typical flavoprotein spectra (Fig. 6).

The reductive properties of NADPH:cyt P-450 reductase are expressed by the ability of the enzyme to reduce cytochrome c.

NADPH, as substrate of this two-substrate enzyme, is the electron donor. Cytochrome c is the second substrate of the reductase; it serves as an electron acceptor. Fig. 7 illustrates the double-reciprocal plots of the initial velocity of cytochrome c reduction by various amounts of NADPH at different cytochrome c concentrations (Fig. 7*a*) and its secondary graph (Fig. 7*b*). The results show that the enzyme exhibites the sequential mechanism during the formation of the ternary complex cytochrome c – NADPH – NADPH:cyt P-450 reductase. Michaelis constants for NADPH:cyt P-450 reductase with respect to NADPH and cytochrome c are 6.25 μmol/l and 4.7 μmol/l, respectively. These values, which are lower than those determined for NADPH:cyt P-450 reductase isolated from Jerusalem Artichoke (Benveniste et al. 1986, 1991), illustrate the high affinity of both substrates to the enzyme of tulip bulbs.

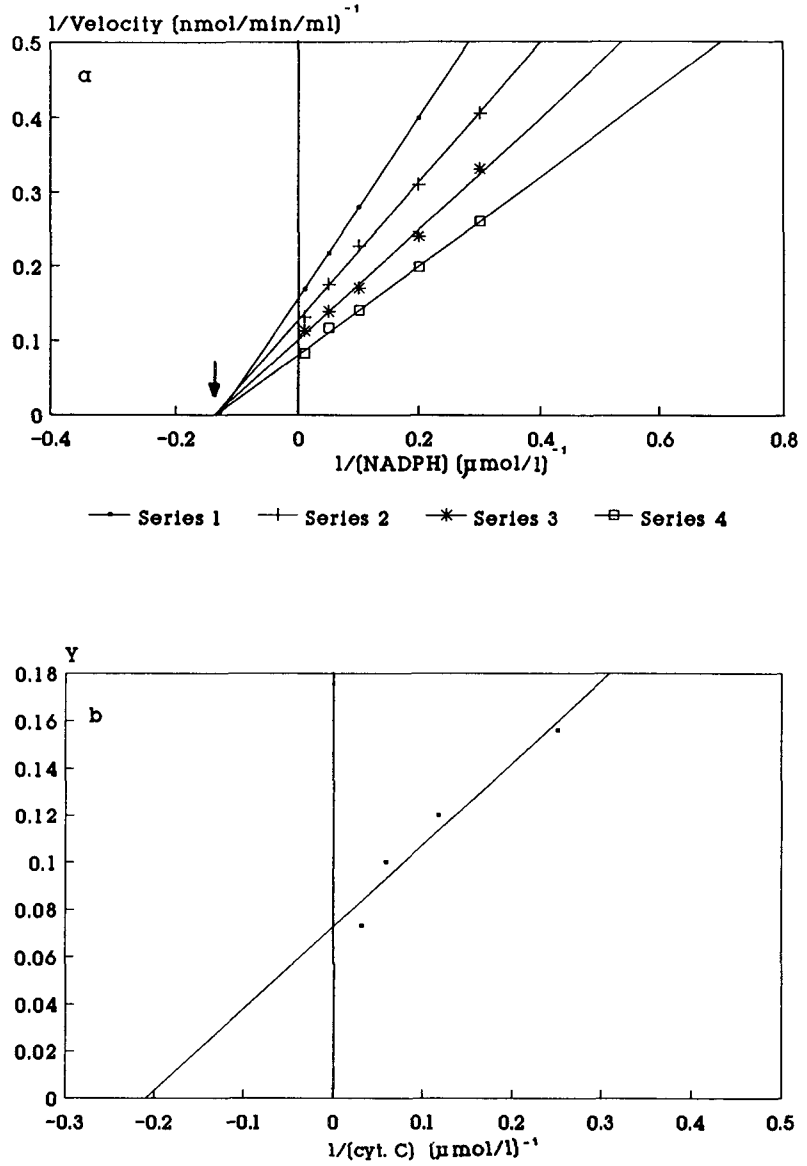


Figure 7. Double-reciprocal plots of the initial rate of cytochrome c reduction by various amounts of NADPH at different concentrations of cytochrome c. (*a*) Primary graph for the estimation of K_m for NADPH at concentration 4 μmol/l, 8.5 μmol/l, 17 μmol/l and 32 μmol/l cytochrome c (Series 1, 2, 3 and 4, respectively). (*b*) Secondary graph for the estimation of K_m for cytochrome c. (Y – intercepts on ordinate of the primary graph). NADPH:cytochrome c reductase activity was measured at 20 °C in 20 mmol/l potassium phosphate (pH 7.4) containing 2.5 μmol/l FMN. The total volume in cuvettes was 0.5 ml.

Table 7. Dependence of the rate of NMA and NDMA demethylation on cytochrome P-450 in the reconstituted enzyme assay

Cyt P-450 (pmol/l)	The rate of demethylation of NMA (nmol/l HCHO formed per min)	NDMA
0	0	0
20	0.070 ± 0.007	0.058 ± 0.005
30	0.100 ± 0.009	0.083 ± 0.008
50	0.113 ± 0.009	0.094 ± 0.010
70	0.130 ± 0.010	0.108 ± 0.011
130	0.130 ± 0.011	0.108 ± 0.010
200	0.120 ± 0.011	0.106 ± 0.010

Experimental conditions are described in the text. The assay contained 150 pmol/l of NADPH:cytochrome P-450 reductase. Numbers are mean values and standard deviations of three parallel experiments.

Table 8. Dependence of the rate of NMA and NDMA demethylation on NADPH:cytochrome P-450 reductase in the reconstitute enzyme assay.

NADPH:cyt P-450 reductase (pmol/l)	The rate of demethylation of NMA (nmol/l HCHO formed per min)	NDMA
0	0	0
30	0.062 ± 0.006	0.048 ± 0.005
70	0.084 ± 0.008	0.060 ± 0.006
125	0.118 ± 0.010	0.090 ± 0.008
150	0.130 ± 0.013	0.108 ± 0.010
200	0.130 ± 0.012	0.108 ± 0.010
220	0.104 ± 0.010	0.102 ± 0.010
250	0.080 ± 0.008	0.078 ± 0.008

Experimental conditions are described in the text. The assay contained 125 pmol/l of cyt P-450. Numbers in the table represent mean values and standard deviations of three parallel experiments.

Reconstitution of cytochrome P-450-dependent activities

For reconstitution experiments, purified cyt P-450 protein was mixed with plant NADPH:cyt P-450 reductase and with dilauroyl phosphatidylcholine. Tables 7 and 8 show the dependence of the enzyme activity to demethylate exogenous substrates (NMA and NDMA) on cyt P-450 and on NADPH:cyt P-450 reductase,

Table 9. Michaelis constants of NMA and NDMA determined in plant microsomes of reconstituted plant enzyme assay

System	K_m (mmol/l)	
	NMA	NDMA
Microsomes	0.0448 ± 0.003	0.151 ± 0.033
Cyt P-450-dependent reconstitution system	0.0440 ± 0.001	0.142 ± 0.027

Experimental conditions are described in the text except that 0–50 mmol/l NMA or NDMA was used. The numbers in the table are means and standard deviations of three experiments.

respectively. Michaelis constants determined for NMA and NDMA in the reconstituted system are comparable with those determined in microsomes (Table 9). Details of oxidation of exogenous substrates (NMA, NDMA and aminopyrine) by plant reconstituted cyt P-450 – dependent system are described elsewhere (Hansíková et al. 1994).

Mammalian microsomal NADPH:cyt P-450 reductase was also able to reduce cyt P-450 isolated from tulip bulb microsomes and NMA was N-demethylated by this reconstituted system, but with the lower rate. The rate of NMA demethylation by this system (under the same conditions as used for the plant system) was determined as about one half of that by the plant system (0.68 nmol/l HCHO formed per min). This result shows that plant and mammalian microsomal NADPH:cyt P-450 reductases play the same role. It is in accordance with previous results; catalytic interaction between NADPH:cyt P-450 reductases and cytochromes P-450 of different origins is a general phenomenon (Benveniste et al. 1991).

Discussion

To determine the properties of plant cyt P-450, which is specific to xenobiotics, the enzyme has to be isolated as a highly purified preparation. The procedure for isolation of plant cyt P-450 was described previously e.g. for enzymes from Jerusalem artichoke (*Helianthus tuberosus L.*) tuber tissues (Gabriac et al. 1985) from mesocarp of *Persea americana L.* (O'Keefe and Leto 1989), from soybean (*Glycine max L.*) cell cultures (Kochs and Grisebach 1989) and tulip bulbs (Higashi et al. 1985; Lau et al. 1993). The enzymes were usually only partially characterized and they were inactive in most cases with respect to oxidation of exogenous substrates when reconstituted with NADPH:cyt P-450 reductase (Higashi 1988; Lesot et al. 1990). Only the isoforms specific to endogenous substrates (i.e. cinnamic 4-hydroxylase or 3,9-dihydroxypterocarpan 6a-hydroxylase) were well characterized (Gabriac et

al. 1991, Kochs et al. 1992). We focus our attention on the isolation of the isoform of plant cyt P-450 having the abilities to oxidize xenobiotics *in vitro* when reconstituted with NADPH:cyt P-450 reductase.

The slightly modified isolation procedure described by Gabriac et al. (1991) used for isolation of cyt P-450 leads to the preparation of electrophoretically homogeneous enzyme with specific content of 5 nmol/mg protein. We however isolated by this procedure an isoenzyme of plant cyt P-450 different from that isolated by Gabriac et al. (1991), an isoenzyme which is specific not only for the endogenous substrates but also for exogenous ones; pool B of cyt P-450 retained on DEAE-Sepharose CL 6B, which also exhibited the xenobiotics-binding spectra, was used for the final isolation and following characterization. Another isoenzyme of cyt P-450 was isolated from tulip bulbs by Lau et al. (1993). Our results open the question of presence of several cyt P-450 isoenzymes in plant tissues as the cyt P-450 isolated here differs significantly from that obtained from tulip bulbs by Lau et al (1993). The main difference is that while the cyt P-450 prepared by Lau et al. (1993) was not reducible by NADPH and did not bind CO, the cyt P-450 preparations used in this work were both reducible and able to bind CO as any other cyt P-450. The properties (molecular weights, spectral properties) of the enzyme preparation isolated in our laboratory are comparable with those of enzymes of *Tulipa gesneriana* (Higashi et al. 1985) and *Helianthus tuberosus* (Gabriac et al. 1985). The specific contents of these cyt P-450 preparations isolated in different laboratories were, however, lower than those of cyt P-450 isolated from *Persea americana L.* (17 nmol/l per mg) (O'Keefe and Leto 1989). These results stress the importance of further studies on plant cyt P-450 systems.

Spectral properties of cyt P-450 isolated from tulip bulbs in our laboratories were similar to those of isoforms of rat liver induced by phenobarbital (cyt $P\text{-}450_b$ and cyt $P\text{-}450_c$).

We found that the isolated isoenzyme interacted not only with endogenous but also with exogenous substrates. The binding characteristics were determined spectrophotometrically. NADPH:cyt P-450 reductase isolated by the similar procedure as the enzyme from *Helianthus tuberosus* by Bonveniste et al. (1986) had relative molecular weight of 77,600, the value of which is similar to that of the enzyme from *Cantharanus roseus* (Madyastha and Coscia 1979).

The isolated reductase exhibited the ability to reduce cytochrome c with a specific activity of 77 nmol/min/mg. The enzyme from rat livers exhibites higher values of activity (nearly 60 μmol/min/mg) (Omura and Sato 1964). The lability of flavine prosthetic groups in the plant reductase protein molecule could explain this discrepancy; this lability was also observed for NADPH:cyt P-450 reductases of *Helianthus tuberosus* and this removal of flavine decreases the enzyme activity (Benveniste et al. 1986, 1991). The reductase isolated in our laboratory is electrophoretically homogeneous. The ability of this purified reductase to reconstitute

the monooxygenase activities in the presence of purified cyt P-450, a phospholipid and exogenous substrates reported in this paper confirms its biological role as electron carrier between NADPH and cyt P-450.

Three microsomal NADPH:cyt P-450 reductases were isolated from wound-induced Jerusalem artichoke tubers having the relative molecular weights of 80,000; 82,000 and 84,000 (Benveniste et al. 1991). Two or three proteins were also detected in the microsomes of several other plants (Benveniste et al. 1991). We detected two forms of reductase of tulip bulbs, which had been separated by affinity chromatography on 2′,5′-ADP Sepharose 4B (Fig. 5). However in the present paper we focused our attention only on one of them, i.e. the enzyme form which exhibites higher specific activity. This reductase was isolated and characterized. Nevertheless, the finding of two forms of this enzyme in tulip bulbs has confirmed the results of Benveniste et al. (1991), who postulated that multiple forms of reductase seem to be the rule in higher plants.

The results presented in this paper serve as suitable universal isolation methods for rapid purification of the active (native) plant enzymes participating in metabolism of endogeneous substrates as well as xenobiotics in plants, enzymes which are moreover fully active in the reconstitution experiments.

References

Anzenbacher P., Šípal Z., Hodek P. (1984): Purification of rat liver microsomal cytochrome P-450 by chromatography on immobilized adamantane with a new method of detergent removal. Biomed. Biochim. Acta **43,** 1343—1349

Benveniste I., Gabriac B., Durst F. (1986): Purification and characterization of the NADPH-cytochrome P-450 (cytochrome c) reductase from higher-plant microsomal fraction. Biochem. J. **238,** 365—373

Benveniste I., Lesot A., Hasenfratz M.-P., Kochs G., Durts F. (1991): Multiple forms of cytochrome P450 reductase in higher plants. Biochem. Biophys. Res. Commun. **177,** 105—112

Benveniste I., Salaun J. P., Simon A., Reichhart D., Durst F. (1982): Cytochrome P-450-dependent hydroxylation of lauric acid by microsomes from pea seedlings. Plant Physiol. **70,** 122—126

Bradford M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248—254

Cleland W. W. (1983): Statistical analysis of enzyme kinetic data. In: Methods in Enzymology (Ed. D. L. Purich), Vol. **63,** pp. 103—138, Academic Press, New York

Druckrey H., Preussman, R., Ivankovic I., Schmahl D. (1967): Organotropic carcinogenicity of 65 different N-nitroso compounds in BD rats. Z. Krebsforsch. **69,** 103—201

Gabriac B., Benveniste I., Durst F. (1985): Isolation and characterization of cytochrome P-450 from higher plants *Helianthus tuberosus.* C. R. Acad. Sci. Paris, Serie III, **301,** 753—758

Gabriac B., Werck-Reichhart D., Teutsch H., Durst F. (1991): Purification and immunocharacterization of a plant cytochrome P-450: the cinnamic acid 4-hydroxylase. Arch. Biochem. Biophys. **288,** 302—309

Hansíková H., Frei E., Schmeiser H. H., Anzenbacher P., Stiborová M. (1994): N-nitrosamines are oxidized by reconstituted cytochrome P-450 system from tulip bulbs (*Tulipa fosteriana, L*). Arch. Biochem. Biophys., in press

Higashi K. (1985): Microsomal cytochrome P-450 in higher plants. In: P-450 and Chemical Carcinogenesis (Eds. Y. Tagashira and T. Omura) Gann Monographs on Cancer Res., Vol. **30,** pp. 49—66

Higashi K. (1988): Metabolic activation of environmental chemicals by microsomal enzymes of higher plants. Mutat. Res. **197,** 273—288

Higashi K., Ikeuchi K., Karasaki Y., Obara M. (1983): Isolation of immunochemically distinct form of cytochrome P-450 from microsomes of tulip bulbs. Biochem. Biophys. Res. Commun. **115,** 46—52

Higashi K., Ikeuchi K., Obara M., Karasaki Y., Hirano H., Gotoh S., Koga Y. (1985): Purification of a single major form of microsomal cytochrome P-450 from tulip bulbs (*Tulipa gesneriana L.*). Agr. Biol. Chem. Tokyo, **49,** 2399—2405

Jefcoate C. R. (1978): Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical difference spectroscopy. In: Methods in Enzymology (Eds. S. Fleischer and L. Packer), Vol. **52,** pp. 258—279, Academic Press, New York

Kimura T., Kodama M., Nagata Ch. (1982): N-Hydroxylation enzymes of carcinogenic aminoazo dyes: possible involvement of cytochrome P-448. Jpn. J. Cancer Res. Gann **73,** 455—462

Kochs G., Grisebach H. (1989): Phytoalexin synthesis in soybean: purification and reconstitution of cytochrome P-450 3,9-dihydroxypterocarpan 6a-hydroxylase and separation from cytochrome P-450 cinnamate 4-hydroxylase. Arch. Biochem. Biophys. **273,** 543—553

Kochs G., Welle R., Grisebach H. (1987): Differential induction of enzyme in soybean cell cultures by elicitor of osmotic stress. Planta **171,** 519—524

Kochs G., Werck-Reichhart D., Grisebach H. (1992): Further characterization of cytochrome P 450 involved in phytoalexin synthesis in soybean: cytochrome P 450 cinnamate 4-hydroxylase and 3,9-dihydroxypterocarpan 6a-hydroxylase. Arch. Biochem. Biophys. **293,** 187—194

Laemmli U. K. (1970): Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **227,** 680—685

Lau S. C., Harder P. A., O'Keefe D. P. (1993): Low carbon monooxide affinity allene oxide synthase is the predominant cytochrome P450 in many plant tissues. Biochemistry USA **32,** 1945—1950

Lesot A., Benveniste I., Hasenfratz M.-P., Durts F. (1990): Induction of NADPH-cytochrome P-450(c) reductase in wounded tissues from *Helianthus tuberosus* tubers. Plant Cell Physiol. **31,** 1177—1182

Lowry O. H., Rosenbrough N. J., Farr A. L., Randall R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193,** 265—275

Madyastha K. M., Coscia C. J. (1979): Detergent-solubilized NADPH-cytochrome c (P-450) reductase from the higher plant. J. Biol. Chem. **254,** 2419—1427

Nash T. (1953): The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. J. Biochem. Tokyo **55,** 416—421

O'Keefe D. P., Leto K. J. (1989): Cytochrome P-450 from the mesocarp of avocado (*Persea americana*). Plant Physiol. **89,** 1141—1149

Omura T., Sato R. (1964): The carbon monooxide-binding pigment of liver microsomes. I – Evidence for its hemoprotein nature. J. Biol. Chem. **239,** 2370—2378

Riviere J.-L., Cabanne F. (1987): Animal and plant cytochrome P-450 systems. Biochimie **69,** 743—752

Sottocasa G. L., Kuylenstierna B., Ernster I., Bergstrand A. (1967): An electron transport system associated with the outer membrane of liver mitochondria. J. Cell. Biol. **32,** 415—438

Stiborová M., Asfaw B., Anzenbacher P., Lešetický L., Hodek P. (1988): The first identification of the benzene-diazonium ion formation from a non-aminoazo dye, 1-phenylazo-2-hydroxynaphthalene (Sudan I) by microsomes of rat livers. Cancer Lett. **40,** 319—326

Vermilion J. L., Coon M. J. (1978): Identification of the high and low potential flavins of liver microsomal NADPH-cytochrome P-450 reductase. J. Biol. Chem. **253,** 8812—8819

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