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ISOLATION OF IMMUNOCHEMICALLY DISTINCT FORM OF CYTOCHROME P-450 FROM MICROSOMES OF TULIP BULBS

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SUMMARY A highly purified cytochrome P-450 was obtained from the microsomes of tulip bulbs(*Tulipa gesneriana L.*). The molecular weight(Mr=52,500) and amino acid composition of this plant cytochrome P-450 are similar to those reported for rat livers. On the contrary, Ouchterlony double diffusion analyses indicated that cytochrome P-450 isolated from tulip bulbs shares no common antigenic determinants with those of 9 other plants, in spite of the presence of comparable contents of cytochrome P-450 and/or *trans*-cinnamate 4-monooxygenase with tulip bulbs.

The involvement of cytochrome P-450 in the synthesis of secondary metabolites of plant tissues is now well established (1,2). The majority of the substrates are physiological compounds, for example, *trans*-cinnamate, several fatty acids, terpenol nerol and geraniol. It is unknown whether a system equivalent to the drug-induced hepatic monooxygenase having broad specificity is present in higher plants. So far, no purified preparation of plant cytochrome P-450 had been obtained, because of the difficulty to isolate organella and enzymes from plant tissues due to the rigid cell wall, presence of phenolic compounds(3) and low concentration of microsomal cytochrome P-450 (4). So far, the optical and magnetic properties of plant cytochrome P-450 in the microsomes have been reported to be similar to those of animal sources(5).

In the present study, we have succeeded in isolating an almost electrophoretically homogeneous preparation(more than 98 %) of cytochrome P-450 from the microsomes of tulip bulbs.

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We confirmed a close similarity of molecular weight and amino acid composition of plant P-450 to those of animal ones. It was, however, revealed that there are distinct immunochemical specificity among cytochrome P-450 of individual plants.

MATERIALS AND EXPERIMENTAL PROCEDURES

The procedure of purification of cytochrome P-450 from tulip bulbs(*Tulipa gesneriana L.*, Balalaika) was essentially similar to those described by Saito and Strobel(6),which was employed for cytochrome P-450 from rat liver microsomes,with minor modifications. The details of the purification will be published elsewhere. Briefly, tulip bulbs were purchased directly from a local cultivator. Bulbs were homogenized in a blender with an equal volume of 0.1 M sodium phosphate buffer, pH 7.4, in the presence of 10 mM mercaptoethanol, 5 mM EDTA, 0.6 M mannitol, 1 % bovine serum albumin, 0.15 mM spermine and 0.5 mM spermidine. Microsomes were prepared and washed as described previously(7). Microsomes from approx. 5 kg of tulip bulbs stored at -70°C were employed in one purification procedure. Microsomes were solubilized in 0.1 M Tris-HCl, pH 7.7, containing 1 mM dithiothreitol, 0.5% sodium deoxycholate(w/v), 0.1 mM EDTA and 20 % glycerol(v/v) in the presence of 1.5 % Emulgen 911(Kawo Chemicals,Tokyo) (v/v) at 4°C. Supernatant obtained by centrifugation was loaded on a DEAE-Sephadex A-25 column following polyethylene glycol precipitation, and then fractionated by DEAE-cellulose (DE-52) column chromatography(6). The fractions having absorbance at 417 nm were pooled, dialyzed and finally chromatographed by a hydroxylapatite column (Bio-Gel HT, BIO RAD Lab., Calif.). The final preparation was analyzed by SDS-polyacrylamide gel electrophoresis(8). The profile of densitometric scanning of purified cytochrome P-450 , by Joyce-Loeble Microdensitometer 3CS, after staining with coomasie brilliant blue is shown in Fig.1.

Purified cytochrome P-450 was extensively dialyzed against 10 mM ammonium acetate solution, lyophilized and hydrolyzed in 5.7 N HCl for either 24, 48 or 72 h in sealed evaculated tubes at 110°C. The amino acid content was determined on a HITACHI Model 835 Amino Acid Analyzer (Tokyo). Half cystine was determined as cysteic acid(9).

Ouchterlony double diffusion analyses were carried out as follows: purified cytochrome P-450 preparation(400 µg of protein) was mixed with an equal volume of Freund's complete adjuvant, and approx. 2 ml of the suspension was injected into femoral muscles of rabbit (male, approx. 3 kg). Half of the initial amount of cytochrome P-450 was injected 4 weeks later into the rabbit i.m. The antibodies against cytochrome P-450 were purified by affinity chromatography(10). Immuno-double diffusion analysis was carried out in 1.2 % agarose containing 25 mM Veronal buffer, pH 8.6. Microsomes from various species of higher plants were analyzed after solubilization in the same buffer employed for purification procedure.

Protein was determined by the method of Lowry *et al.*(11). Determination of cytochrome P-450 was carried out as described by Omura and Sato(12).

RESULTS AND DISCUSSION

The purity of the final preparation of cytochrome P-450 from tulip bulbs, as judged by SDS-gel electrophoresis(Fig. 1), is more than 98 % and enough to analyze its amino acid composition. The molecular weight of the purified cytochrome P-450 was calculated as 52,500. The specific content of cytochrome P-450 per mg protein increased approx. 30 fold as compared with the initial solubilized microsomal solution. The absolute spectra of various forms of oxido-reduction and carbon monoxide difference spectra and also the behavior of this hemeprotein on several column chromatography were not significantly different from those reported for rat livers(6) (data not shown here).

The amino acid compositions of purified cytochrome P-450 from tulip bulb microsomes and average values of individual amino acid residues of 12 different cytochrome P-450 preparations from rat livers reported by others(13,14,15) are listed in Table 1. The relatedness of amino acid composi-

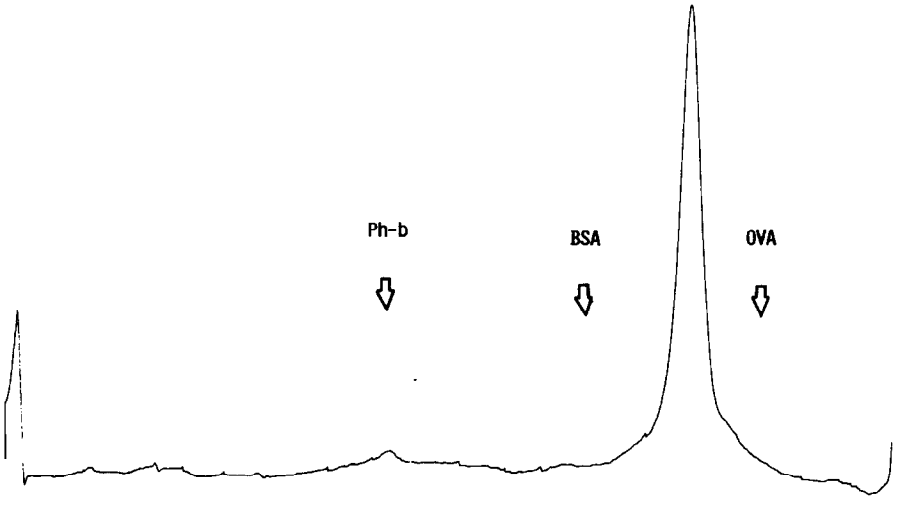

Fig.1 Densitometric scanning of purified cytochrome P-450 from tulip bulbs. The final preparation was boiled in sample buffer and loaded on SDS-polyacrylamide gel electrophoresis(8). Marker: OVA, ovalbumin(chicken egg); BSA,bovine serum albumin; Ph-b, phosphorylase b(rabbit muscle).

Table 1. Amino acid composition of cytochrome P-450 from tulip bulbs and rat livers

	(A) Tulip bulbs		(B) Rat livers *		
	No. of residues	%	No. of residues	%	$(n_{iA} - n_{iB})^2$ **
Asx	48	10.0	41	8.8	49
Thr	27	5.6	26	5.5	1
Ser	37	7.7	31	6.7	36
Glx	42	8.8	46	9.9	16
Pro	27	5.6	27	5.7	0
Gly	42	8.8	36	7.7	36
Ala	36	7.5	25	5.3	121
Cys/2	5	1.0	6	1.2	1
Val	34	7.0	27	5.7	49
Met	7	1.5	11	2.4	16
Ile	19	4.0	26	5.6	49
Leu	45	9.4	53	11.4	64
Tyr	16	3.3	14	3.0	4
Phe	29	6.0	31	6.7	4
Lys	39	8.1	29	6.3	100
His	7	1.5	13	2.8	36
Arg	20	4.2	23	5.0	9
Trp	ND	-	2	0.4	-
Total	(N_A) 480		(N_B) 467		(591)
Asx, Glx		18.8		18.7	
Arg, Lys, His		13.8		14.1	
Gly,Ala,Val,Leu,Ile		36.7		35.6	
Phe,Tyr, (Trp)		9.3		10.1	

* Means of 12 determinations(refs.13,14,15), ** To test the significance of relatedness between protein A and B, the equation $S\Delta n = 1/2\ \Sigma(n_{iA}-n_{iB})^2$ was employed according to Cornish-Bowden(16). The number of the ith type of amino acid in protein A is designated as n_{iA} and those of B as n_{iB}. $S\Delta n$ was 295.5 and this value is located between 0.42N and 0.93N [$N=1/2(N_A + N_B)$].

tions between these two groups of cytochrome P-450 were calculated according to the method of Cornish-Bowden(16). The value $S\Delta n$ (see the footnote to Table 1) is 295.5 and located between 0.42N and 0.93N. According to his "weak" test, amino acid compositions of cytochrome P-450 from tulip bulbs and rat livers are related. We tentatively calculated the total percentages of amino acid groups with side chain containing acidic groups or their amides, basic groups, aliphatic side chains, and aromatic rings (Table 1). These rough estimations of the characteristics of amino acid remarkably resembled each other.

The formation of 4-hydroxy-cinnamate, a precursor of flavonoids, lignin and phenolic compounds, is catalyzed by the *trans*-cinnamate 4-monooxygenase[EC 1.13.14.11]. This activity is probably the most important cytochrome P-450 isozyme in plants. Previously we have measured the contents of both cytochrome P-450 and *trans*-cinnamate 4-monooxygenase in microsomes from Jerusalem artichoke(*Helianthus tuberosus*), potato tubers(*Solanum tuberosum*), avocado mesocarps(*Persea americana*), and cauliflower buds(*Brassica oleracea*). It was observed that specific activities of *trans*-cinnamate 4-monooxygenase per cytochrome P-450 were highly variable among these plants, that is, these enzyme activities were not proportional to cytochrome P-450 content in microsomes(Karasaki *et al.*, manuscript in preparation). It was, therefore, interesting to examine whether these variations of specific activities are due to qualitative differences of individual P-450 or quantitative differences of a common *trans*-cinnamate 4-monooxygenase.

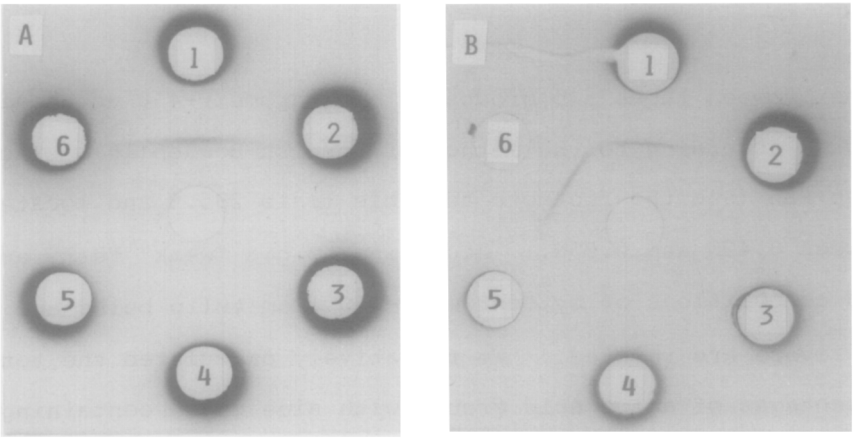

Fig.2 Ouchterlony double diffusion studies. The wells in plate A contained the followings: 1, microsomes of tulip bulbs; 2, artichoke tubers; 3,cauliflower buds; 4,avocado mesocarps; 5, potato tubers; 6, cauliflower leaves. Plate B: 1, microsomes from tulip bulbs; 2, lily bulbs; 3, allium bulbs; 4,narcissus bulbs; 5. gladiolus bulbs; 6, purified preparation of P-450 from tulip bulbs.

Antiserum against purified cytochrome P-450 from tulip bulbs formed a single precipitin line with either crude extract of the microsomes from tulip bulbs or purified preparation of its antigen(Fig.2). However, no precipitin lines were detected with other microsomes from plants described above. Next, we have prepared microsomes from related species, such as lily(*Lilium lancifolium*), allium(*Allium schoenoprasum*), narcissus(*Narcissus tazetta*) and gladiolus(*Gladiolus gandavensis*). Again, none of the microsomes from these bulbous plants shared antigenicity with purified cytochrome P-450 from tulip bulbs(Fig. 2B), although there existed comparable cytochrome P-450 in these microsomes(data not shown).

There are some reports in which rabbit anti-(rat cytochrome P-450) serum reacted with those of mouse liver(10,17) and even with chick-embryo liver(18). It is unknown yet whether these cross-reactions are exceptional cases in animal cytochrome P-450 or not. In the present study, we have demonstrated that the plant cytochrome P-450 had high specificity of immunochemical properties among them, in spite of the presence of comparable activity of *trans*-cinnamate 4-monooxygenase with tulip bulbs.

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