Highly Variable Distributions of *trans*-Cinnamate 4-Monooxygenase in a Variety of Plant Tissues

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Abstract : Microsomes from both avocado mesocarps and tulip bulbs contained about six times more cytochrome P-450 than those of either potato tubers or cauliflower buds. The specific activity of *trans*-cinnamate 4-monooxygenase (EC 1.14.13.11) per P-450 in potato microsomes, however, was found to be 9 fold and 200 fold higher than avocado and tulip microsomes, respectively. Little activity of *trans*-cinnamate 4-monooxygenase was detected in microsomes of cauliflower buds, although the content of cytochrome P-450 in this tissue is comparable to potato tubers. On the other hand, *trans*-cinnamate 4-monooxygenase activity in Jerusalem artichoke tuber microsomes correlated with the increase of cytochrome P-450 after wounding of tuber tissues. Less heterogeneity of substrate specificity was observed among the microsomes from various plant tissues with exogenous compound, that is, benzphetamine. These results suggested the presence of multiple forms of cytochrome P-450 in plant microsomes and therefore the content of cytochrome P-450 and *trans*-cinnamate 4-monooxygenase activity was not proportional in a variety of plant tissues.

Key words : cytochrome P-450, *trans*-cinnamate 4-monooxygenase, plant microsomes, isozymes.

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Introduction

Contents and enzymatic properties of cytochrome P-450 in the microsomes from various plant tissues were studied to see whether a single cytochrome P-450 or multiple forms exist in higher plants. The activity of *trans*-cinnamate 4-monooxygenase (EC 1.14.13.11), which is known to be a microsomal cytochrome P-450 linked monooxygenase, is induced by the wounding of Jerusalem artichoke tuber tissues (Benveniste *et al.*, 1977). The cytochrome P-450 content correlated with the changes in monooxygenase activity (Reichhart *et al.*, 1980). It is, however, yet unknown whether a single or multiple forms of cytochrome P-450 increased after induction.

In the animal cells, especially in the liver, the microsomal cytochrome P-450 metabolize a variety of xenobiotics with various chemical structures. Low substrate specificity of cytochrome P-450 is suitable for detoxication of a number of xenobiotics. Reconstituted monooxygenase with a highly purified form of cytochrome P-450 still shows relatively broad substrate specificity in the case of animals (Koop *et al.*, 1981; Lu & West, 1978). On the other hand, the presence of specific-forms for endogenous compounds, such as cholesterol metabolite (Murakami *et al.*, 1982) and prostagrandin (Powell, 1978) have

been reported with animal sources.

Recently we have compared enzymatic activities in the microsomes of various higher plants for chemical carcinogens (Higashi *et al.*, 1981) and endogenous compounds. There was a high substrate specificity for cinnamic acid, which is the physiological substrate for the microsomal cytochrome P-450 in a variety of plant tissues. The results suggest that a specific form of cytochrome P-450 is functioning as *trans*-cinnamate 4-monooxygenase and substrate specificities of various forms of cytochrome P-450 in the plant appear to be narrow and less overlapping at least for endogenous substrate, although further investigations are required to confirm this possibility.

Experimental Procedures

Cauliflower buds (*Brassica oleracea*), potato tubers (*Solanum tuberosum*), avocado mesocarps (*Persea americana*) and tulip bulbs (*Tulipa gesneriana*) were purchased locally. *Symplocarpus spadix* (*Symplocarpus renifolius*) was a generous gift from Dr. T. Kashimoto, Iwate University Medical School. Jerusalem artichoke (*Helianthus tuberosus*) was cultivated on a farm of the University and their tubers were stored in a cold room (2–4°C). Jerusalem artichoke and potato tubers and cauliflower buds were sliced and incubated in the dark at 25°C in a flask with water as described by Benveniste *et al.* (1977). After 16 h of incubation, the disc or sliced materials were washed and homogenized in 2 volumes of 0.1 M sodium phosphate buffer, pH 7.4, containing 10 mM mercaptoethanol, 1 mM EDTA, 0.6 M mannitol and 0.4% bovine serum albumin (Higashi *et al.*, 1981). Microsomes were prepared as described previously (Higashi *et al.*, 1981).

trans-Cinnamate 4-monooxygenase was measured according to the radiochemical method of Potts *et al.* (1974). Benzphetamine was a generous gift from Dr. T. Omura, Kyushu University. Benzphetamine N-demethylase was measured by the determination of the formaldehyde by the procedure of Nash (1953). Each assay was carried out in triplicate and hexamine was used as the standard solution. The use of plastic-ware was avoided during the measurement of benzphetamine N-demethylase. The content of cytochrome P-450 was determined by the method of Omura & Sato (1964). Protein was determined by the procedure of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results

Microsomes were prepared from appropriate tissues of various plants and their cytochrome P-450 contents were determined. These values were somewhat varied between different experiments due to the differences of time after harvesting or conditions of storage. Each value of cytochrome P-450 content in one tissue, however, did not differ significantly beyond a certain limit and individual plant tissues had some characteristic

Table 1. Contents of cytochrome P-450 in the microsomes of various plant tissues

Source	Cytochrome P-450 (nmol / mg protein)
Cauliflower bud	
Uninduced	0.051 (0.04—0.07) ^b
Induced ^a	0.085 (0.06—0.10)
Potato tuber	
Induced ^a	0.055 (0.056, 0.054)
Jerusalem artichoke tuber	
Uninduced	0.015 (0.012 — 0.017)
Induced ^a	0.141 (0.126 — 0.157)
Symplocarpus spadix	0.058
Avocado mesocarp	0.316 (0.221 — 0.458)
Tulip bulb	0.320 (0.204 — 0.488)

^a Incubated for 14—18 h after wounding.^b Ranges of values determined.

ranges (Table 1). The profiles of carbon monoxide difference spectra of plant microsomes from uninduced materials frequently showed greater amounts of P-420 than P-450, but only the content of P-450 was calculated in this study. After incubation of discs of potatoes and artichokes and sliced materials of cauliflower buds, an increase of cytochrome P-450 was observed as reported earlier (Reichhart *et al.*, 1980; Rich & Lamb, 1977). Slicing of tulip bulbs failed to induce cytochrome P-450 in the microsomes (data not shown). Compared with those of induced sources of Jerusalem artichoke, potato and cauliflower, microsomes of both tulip bulbs and avocado mesocarps contained a large quantity of cytochrome P-450 but little P-420 even without treatment.

Using these microsomes, *trans*-cinnamate 4-monooxygenase activities were determined by radioactive assay (Potts *et al.*, 1974). Activity of *trans*-cinnamate 4-monooxygenase in microsomes of artichoke tubers increased parallel with cytochrome content after wounding of the tuber tissues (Tables 1 and 2). Microsomes from both avocado mesocarps and tulip bulbs have similar levels of cytochrome P-450 contents and similar carbon monoxide difference spectra, that is, P-448 type (data not shown here), but the *trans*-cinnamate 4-monooxygenase activity of avocado microsomes was approximately 20 fold higher than that of tulip bulbs. Furthermore, little activity of *trans*-cinnamate 4-monooxygenase was detected by the present radioactive assay in microsomes from either uninduced or induced cauliflower buds, while the highest activity of *trans*-cinnamate 4-monooxygenase was observed in potato microsomes, although the levels of cytochrome P-450 in both tissues are similar. These results suggest that there exists a *trans*-cinnamate specific form of cytochrome P-450 in plant microsomes and *trans*-cinnamate 4-monooxygenase activities are not always proportional to the total contents of microsomal cytochrome P-450.

Table 2. Activities of *trans*-cinnamate 4-monooxygenase in the microsomes of various plant tissues

Source	<i>trans</i> -Cinnamate 4-monooxygenase	
	HCHO nmol / mg protein	HCHO nmol / nmol P-450
Cauliflower bud		(%)
Uninduced	0.003	0.05 (0.03) ^b
Induced	0.003	0.05 (0.03)
Potato tuber		
Induced ^a	10.2	185.4 (100)
Jerusalem artichoke tuber		
Uninduced	0.1	6.6 (3.6)
Induced ^a	1.1	7.8 (4.2)
Avocado mesocarp	6.5	20.6 (11.1)
Tulip bulb	0.3	0.9 (0.5)

^a Incubated for 14–18 h after wounding.^b The value for potato designated as 100 per cent.

Next, we measured a common drug-metabolizing enzyme activity, that is, benzphetamine N-demethylase with microsomes from a variety of higher plants (Table 3). In this case, high substrate specificity as in cinnamate metabolism was not observed. The activity of benzphetamine N-demethylase in microsomes of cauliflower buds was comparable to other plant sources. Potato microsomes did not show such a predominance of activity as they did with *trans*-cinnamate 4-monooxygenase. A significant difference of enzymatic activity was absent between avocado and tulip microsomes. The specific activity of benzphetamine N-demethylase was rather decreased after the wounding of Jerusalem artichoke tuber tissues.

Discussion

The enzymatic activity of *trans*-cinnamate 4-monooxygenase in a variety of plant systems have been studied independently by many laboratories and accumulated informations indicated the involvement of cytochrome P-450 (Benveniste *et al.*, 1977; Reichhart *et al.*, 1980). Rich & Lamb (1977), suggested that only a fraction of the cytochrome P-450 is involved in *trans*-cinnamate 4-monooxygenase activity. Since then, little information on the multiplicity of plant cytochrome P-450 has been reported because of the difficulty of the purification of cytochrome P-450 from plant sources and low levels of this hemoprotein in a variety of higher plants. In the present study, we have observed the possibility of multiplicity of plant cytochrome P-450 with simultaneous determinations of contents, two enzymatic activities and kinetic studies of induction of this isozyme.

There are two possibilities regarding the multiplicity cytochrome P-450: first, each

Table 3. Activities of benzphetamine N-demethylase in the microsomes of various plant tissues

Source	HCHO formed/min	
	nmol / mg protein	nmol / nmol P-450
Cauliflower bud		(%)
Uninduced	21.6	423 (58.0) ^b
Induced ^a	26.8	315 (43.2)
Potato tuber		
Induced ^a	40.1	729 (100)
Jerusalem artichoke tuber		
Uninduced	12.6	840 (115.2)
Induced ^a	12.1	86 (11.8)
Symplocarpus spadix	13.1	226 (31.0)
Avocado mesocarp	34.3	108 (14.8)
Tulip bulb	23.2	73 (10.0)

^a Induced for 14–18 h after slicing the tissues.^b The value for potato is designated as 100 per cent.

individual plant tissue contains a single form of cytochrome P-450 and each has a different affinity for cinnamate; secondly, individual tissues have multiple forms of cytochrome P-450 and the distributions of each form are different among various plants. So far, no purified form of cytochrome P-450 has been obtained from plant tissues and there is no direct evidence to distinguish these two possibilities. However, the presence of multiple forms of cytochrome P-450 in higher plants is more likely as follows: a) *trans*-cinnamate 4-monooxygenase increased in proportion to the cytochrome P-450 content in the microsomes of Jerusalem artichoke after wounding the tuber tissues, but, decreased earlier than the level of cytochrome P-450 (Reichhart *et al.*, 1980; Higashi *et al.*, 1981), b) *trans*-cinnamate 4-monooxygenase, in the microsomes of other than Jerusalem artichoke, does not correlate with the content of cytochrome P-450, c) both *trans*-cinnamate 4-monooxygenase and enzyme metabolizing benzo(a)pyrene (Higashi *et al.*, 1981) were induced in the microsomes of Jerusalem artichoke by wounding, but few inductions were observed for benzphetamine (Table 3) and enzyme for aflatoxin B₁ (data not shown), although the metabolism of xenobiotics other than cinnamate by plant microsomes might be the result of side-reaction of a certain specific form of cytochrome P-450.

Our previous study of microsomal proteins of tulip bulbs with SDS-polyacrylamide gel electrophoresis suggested that there might be less heterogeneous forms of cytochrome P-450 in one plant tissue than in animal cells (Higashi *et al.*, 1982). The presence of multiple forms of cytochrome P-450 even in uninduced liver microsomes have been reported in the case of animals (Koop *et al.*, 1981; Agosin *et al.*, 1979). It is likely that only a few forms of this hemeprotein might be used for a particular substrate of plant

tissue under physiological conditions and we intend to study this theory further using cloned DNA for plant cytochrome P-450.

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種々植物組織におけるトランス桂皮酸モノオキシゲナーゼの高度に異なる分布

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要 旨: アボカド果実, チューリップ球根, ジャガイモ塊茎, カリフラワー芽, キクイモ塊茎, ザゼンソウ肉穂花序などよりミクロソームを調製し, チトクローム P-450 含量とその酵素活性を調べた. 多くの植物に生理的に存在するトランス桂皮酸の水酸化反応の活性は, 必ずしもチトクローム P-450 の含量とは比例せず, ジャガイモのように, この反応の P-450 当りの非常に高いものから, カリフラワーのように殆ど活性が認められないものまで, 調べた植物種のミクロソーム中では高度に分布の差が観察された. 植物のミクロソーム中には, トランス桂皮酸の水酸化に関与するチトクローム P-450 以外の分子種も存在することを示唆している.

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