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5-EPI-ARISTOLOCHENE 3-HYDROXYLASE FROM GREEN PEPPER

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Abstract—5-epi-Aristolochene 3-hydroxylase, one of the key enzymes of the biosynthetic pathway leading to capsidiol, a major phytoalexin of green pepper (*Capsicum annuum*), is localized in the microsomes of elicitor treated green pepper fruit. The enzyme was found to be a P-450-dependent enzyme with an estimated molecular mass of 59 kDa. The total microsomal P-450 content increased and the λ_{max} of the CO-binding spectrum changed in response to elicitation. Immunoblot analysis of fruit microsomal proteins was performed with anti-tulip constitutive P-450 antibody during the elicitation period. A 48 kDa protein strongly immunoreacted with the P-450 antibody, and a decrease of this P-450-like 48 kDa protein was observed with time. These results suggest that elicitation induces the production of P-450 isoforms.

INTRODUCTION

The biosynthesis of phytoalexins is one of the major defensive systems of higher plants. We have focused on the induction mechanism and the biosynthetic pathway for phytoalexin production in green pepper (*Capsicum annuum*) [1]. Capsidiol is the major phytoalexin produced by inoculation of green pepper and tobacco with pathogenic fungi [2]. The biosynthetic pathway for capsidiol formation has been partially elucidated using tobacco cultured cells [3-6]. On elicitation, farnesyl pyrophosphate is converted to 5-epi-aristolochene by farnesyl pyrophosphate cyclase [7]. There are only few reports on the enzymatic regulation of the 1- and 3-hydroxylation of 5-epi-aristolochene to form capsidiol. In cell-free capsidiol biosynthesis by homogenates of cultured tobacco cells, this reaction is dependent on NADPH and molecular O_2 [5] and, thus, seemed to be a typical cytochrome P-450 (P-450) mediated process. In our investigations, ancymidol and ketoconazole, which are P-450 inhibitors, have been found to suppress phytoalexin accumulation in green pepper cell cultures [8]. However, the enzymatic regulation of 1- and 3-hydroxylation of 5-epi-aristolochene is still obscure. Our aim is to elucidate the enzymatic hydroxylation pattern of 5-*epi*-aristolochene and to characterize the hydroxylases which catalyse the reactions.

RESULTS AND DISCUSSION

Enzymatic characters of 5-*epi-aristolochene hydroxylase*

In tobacco cultured cells, the enzymatic hydroxylation of 5-epi-aristolochene can be induced by elicitor treatment [5]. Subcellular fractions were prepared from elicited fruits and etiolated seedlings of green pepper by differential centrifugation. In the elicited microsomal fraction, 5-epi-aristolochene was hydroxylated only at the 3-position, to form 1-deoxycapsidiol as the enzymatic product. Another hypothetical intermediate, 3-deoxycapsidiol was not detected. The monohydroxy derivative of 5-epi-aristolochene, 1-deoxycapsidiol was previously isolated from elicited fruits of green pepper [9]. This enzymatic activity is localized only in the microsomal fraction, and is induced by elicitor treatment.

Microsomal 5-epi-aristolochene 3-hydroxylase activity required both NADPH and molecular O_2 for activity (Table 1). Moreover, ancymidol, ketocanazole and CO gas, which are known P-450 inhibitors, suppressed 5-epi-aristolochene 3-hydroxylase activity, and the inhibition by CO gas was reversed by visible light irradiation (450 nm). These enzymatic characters are typical of P-450-dependent enzymes. Therefore, 5-epi-aristolochene 3-hydroxylase is a new member of the P-450s.

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Table 1. Characteristics of 5-epi-aristolochene 3-hydroxylase

Conditions	Relative activity (%)
NADPH + O_2	100
O_2 alone	34
NADPH + Ar	24
− NADPH + Ar	1
Complete system (NADPH + O_2)	
+ 100 μM ancymidol	21
+ 100 μM ketoconazole	64
+ CO	41
+ CO + 450 nm light	85

Change of P-450 content and isoforms

Figure 1 shows the difference spectrum of the constitutive P-450 from unelicited fruits of green pepper. The fruit microsomal fraction contained a greater amount of P-450 than those of other tissues (data not shown). The λ_{max} of CO-binding spectrum of the constitutive P-450 was 450.7 nm.

Fruits and etiolated seedlings were treated with elicitor, and microsomal fractions were prepared at various times after elicitation. The enzymatic activity of 5-epi-aristolochene 3-hydroxylase in elicited microsome increased after arachidonic acid treatment. During the elicitation period, the total P-450 level initially dropped, and then rose towards the end of the elicitation period (Table 2). CO-binding spectra showed changes at λ_{max} (450–488 nm) during elicitation. These results suggested that inducible P-450 isoforms might be synthesized *de novo*. Moreover, an increase in P-420 content during the early elicitation period was also detected in the microsomes. This was probably due to the degradation of consitutive P-450s by the elicited stress. The following results support this hypothesis.

The preparation of anti-tulip constitutive P-450 antibody had been described [10, 11]. This antibody formed a single precipitin line with solubilized microsomal proteins of green pepper fruits by Ouchterlony double diffusion method (data not shown). The unelicited microsomal 48 kDa protein strongly immunoreacted with the anti-tulip P-450 antibody (Fig. 2). The 48 kDa protein was distributed in microsomal fractions of all organs, with fruit microsomes being the richest source of this protein. Immunoreacted protein had a similar molecular mass to tulip bulb P-450 (allene oxide synthase, 48.5 kDa) [12]. Previous studies had shown that the anti-tulip P-450 antibody reacted only with tulip microsomes, but not

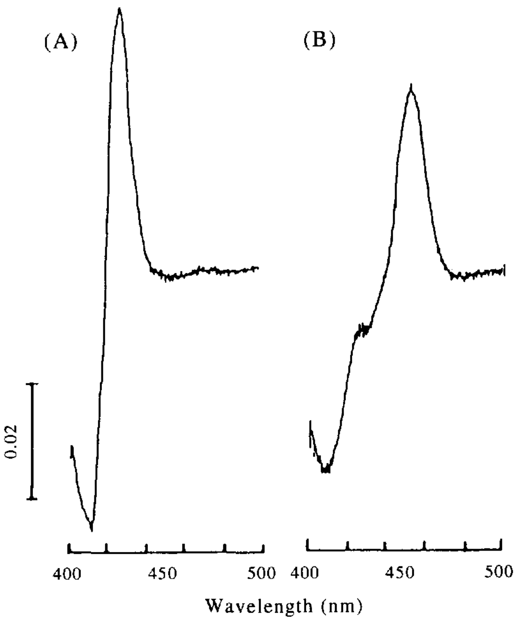

Fig. 1. Difference spectra of unelicited microsomes from green pepper fruits. (A) Oxidized vs. reduced microsomes. The peak at 425.7 nm corresponds to cytochrome b_5. (B) Reduced vs. reduced microsome plus CO gas. The peak at 450.7 nm corresponds to P-450. Total P-450 content was 0.2 nmol mg^{-1} protein.

Table 2. Cytochrome P-450 changes during elicitation

Elicitation period (hr)	5-epi-Aristolochene 3-hydroxylase activity (pKat mg^{-1} protein)	P-450 content (nmol mg^{-1} protein)	λ_{max} of CO-difference spectrum (nm)	P-420 content (nmol mg^{-1} protein)
Fruits				
0	0.14	0.217	450.7	trace
6	0.85	0.167	450.3	0.015
12	1.27	0.144	450.3	0.022
18	2.76	0.257	448.4	0.044
24	1.98	0.253	448.3	0.032
Etiolated seedlings				
0	0.11	0.155	450.6	ND*
6	1.15	0.217	449.3	0.035
12	2.62	0.289	448.6	0.021
18	3.85	0.737	449.1	trace
24	3.23	0.613	448.0	trance

*ND: Not detected.

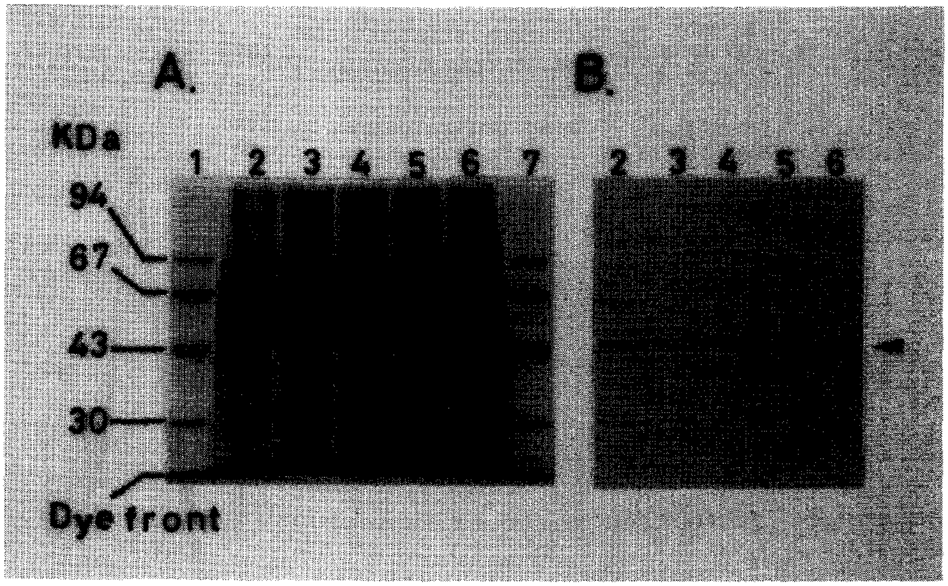

Fig. 2. Changes of constitutive P-450 in green pepper fruit microsomes following elicitation. (A) CBB staining. (B) Immunoblotting (anti-tulip P-450 antibody). Lanes 1 and 7, molecular marker; 2, green pepper fruit microsome (0th hr, before elicitor treatment); 3–6, 6, 12, 18 and 24th hr after elicitor treatment.

with microsomal proteins from other plants such as artichoke, cauliflower, avocado, potato, lily, allium and narcissus when tested by the double diffusion method [11]. Our results indicated that anti-tulip P-450 antibody recognized the heterogeneous plant P-450 isoforms. Similar results were obtained for pea epicotyls when it was found that 47, 48, 52 and 58 kDa proteins cross-reacted with anti-*Pseudomonas putida* camphor hydroxylase (P-450_{cam}) antibody [13]. These results suggested that the microsomal 48 kDa protein was one of the constitutive P-450s. In the elicitor treated fruits, the microsomal content of 48 kDa protein (consitutive P-450) was found to decrease following elicitation. This response is similar to the decrease in allene oxide synthase in tulips during aging or on wounding [14].

Identification of 5-*epi-aristolochene* 3-*hydroxylase*

In the etiolated seedlings, 50–60 kDa proteins were induced in the microsomes by the elicitation process (data not shown). The molecular masses of these inducible proteins are closer to those reported for P-450s from other plants.

5-epi-Aristolochene 3-hydroxylase was partially purified from elicited etiolated seedlings (Fig. 3). The partially purified enzyme, in keeping with its P-450 character, was inhibited by CO gas and 100 μM ancymidol. The molecular mass of 5-epi-aristolochene 3-hydroxylase, inducible P-450, was 59 kDa. The inducible P-450 showed a similar λ_{max} in its CO-binding spectrum as the elicited (448 nm) microsomes.

EXPERIMENTAL

Plant materials. Green pepper (*Capsicum annuum*) seeds were obtained from Sapporo Konoen Seed Co. (Sapporo, Japan). Etiolated seedlings were grown in vermiculite in the dark for 10 days at 24 – 26°. Fruits of green pepper were purchased locally. Fruits and etiolated seedlings were elicited with arachidonic acid [1].

Preparation of plant microsomes. Fruits and etiolated seedlings of green pepper were homogenized, respectively, for 2 min in a blender with 2 vol (w/v) of 0.1 M Hepes–NaOH buffer (pH 7.2) containing 25 mM KCl, 5 mM EDTA, 2 mM EGTA, 5 mM DTT, 0.2 mM PMSF, leupeptin (10 mg l^{-1}), 20% (v/v) glycerol and 10% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was centrifuged (12 000 *g*, 10 min) and then passed through 2 layers of Miracloth (Calbiochem, CA, U.S.A.). The filtrate was centrifuged (12 000 *g*, 60 min), and solid $MgCl_2$ was added to the supernatant to a final concentration of 10 mM. The aggregated microsomal fraction was collected by centrifugation at 4300 *g* for 60 min, and resuspended in 2 vol (w/v) of the same buffer. It could be stored at – 80° without loss of activity.

Purification of 5-*epi-aristolochene* 3-*hydroxylase from etiolated seedlings.* The purification of this enzyme was monitored by means of its enzymatic activity and P-450 CO-binding spectrum. Emulgen 911 (Kawo Chemicals, Tokyo, Japan) was added to the microsomal fraction prepared from elicitor-treated (18 hr) etiolated seedlings to a final concentration of 1% (w/v) and the P-450s in the mixture were solubilized by 4 strokes in a Potter–Elvehjem homogenizer. The mixture was then centrifuged at 100 000 *g* for 60 min and the supernatant fractionated with 7 to 14% (w/v) PEG 6000. The 100 000 *g* pellet, which contained P-450s and 5-epi-aristolochene 3-hydroxylase activity, was dissolved in 25 mM Hepes–NaOH buffer (pH 7.2) containing 1.0% (w/v) Emulgen 911, 25 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 0.2 PMSF, leupeptin (10 mg l^{-1}) and 20% (v/v) glycerol (buffer A).

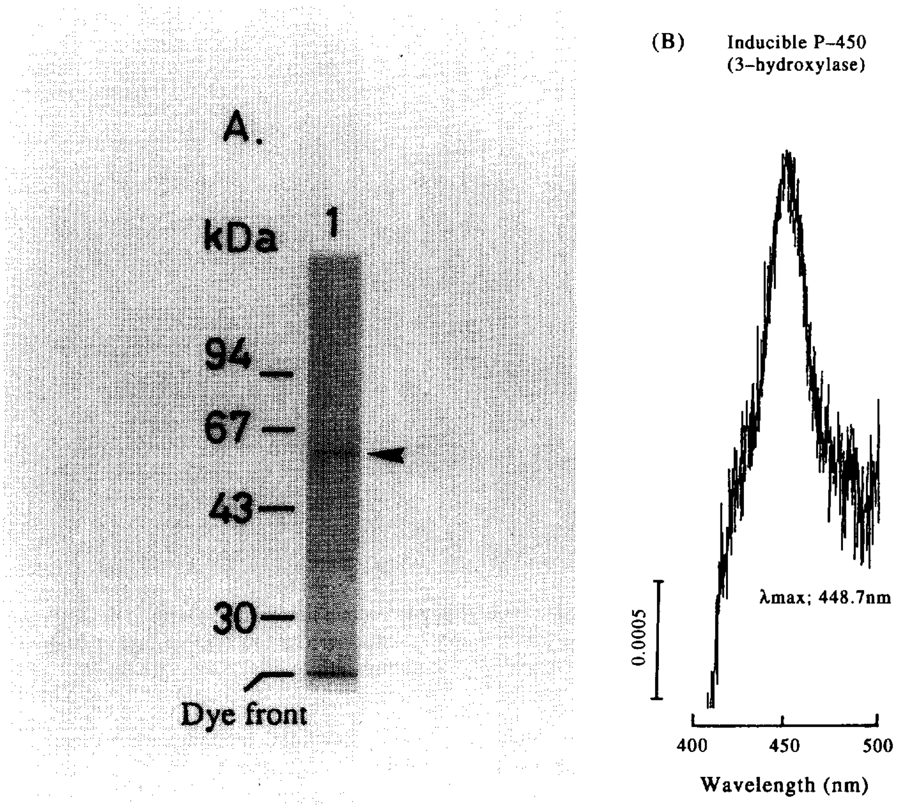

Fig. 3. Partial purification of 5-epi-aristolochene 3-hydroxylase. (A) SDS–PAGE. (B) CO-binding spectrum.

The enzymatic active fraction was loaded on to a DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column (10 × 2 cm) equilibrated with buffer A. The fraction which passed through the column was applied to a hydroxyapatite (Seikegakukogyo, Tokyo, Japan) column (10 × 1 cm), and 5-epi-aristolochene 3-hydroxylase eluted with buffer A containing 0.1 M KCl.

Analytical and assay methods. Capsidiol was obtained from green pepper fruits elicited with 3 mM arachidonic acid [1]. 1-Deoxycapsidiol and 5-epi-aristolochene were synthesized from capsidiol by the procedure of ref. [6]. 3-Deoxycapsidiol was a gift from Mr Fukushi (Hokkaido University). These substrates and authentic standard products were identified by NMR and MS.

The enzymatic assay of 5-epi-aristolochene 3-hydroxylase was conducted at 25° for 60 min. The assay system used was composed of the following; 100 μl enzyme soln, 100 μl yeast microsome [15] as a source of NADPH cytochrome P-450 reductase (10 U ml^{-1} and 750 μl 0.1 M K-Pi buffer (pH 7.5) containing 500 mmol 5-epi-aristolochene, 0.5 mM 2-mercaptoethanol, 5 mM EDTA, and 0.1 mM PMSF. The reaction was started by the addition of 50 μl 20 mM NADPH. The product of the enzymatic reaction was extracted with 2 ml EtOAc. The enzymatic product was quantified routinely by TLC on silica gel G (0.5 mm) developed with *n*-hexane–EtOAc (2:1). The estimation of 1-deoxycapsidiol, 3-deoxycapsidiol or capsidiol by GC was carried out as follows: after development of the TLC (see above), the area corresponding to 1-deoxycapsidiol (R_f 0.43), 3-deoxycapsidiol (R_f 0.52) or capsidiol (R_f 0.06) was scraped out from silica gel plate and re-extracted with 200 μl EtOAc overnight at room temp. An aliquot was then analysed by GC (Shimadzu GC-14A): column 2% Silicone OV-225 on Chromosorb WAW DMCS (3 mm × 3 m, glass column), carrier gas N_2 40 ml min^{-1}, column temp. 100–200° (4° min^{-1}), injection temp. 235°, detection FID. The amount of product present was calculated on the basis of the peak area of an authentic sample. Quantitative determinations of P-450 and P-420 were carried out according to ref. [16]. Gel electrophoresis with 10% polyacrylamide containing 0.1% SDS as performed as described in ref. [17]. Constitutive P-450s of green pepper were detected by immunoblotting using anti-tulip bulb P-450 polyclonal antibody [10, 11], as described in ref. [18]. The microsomal protein concentration was measured by means of a BCA protein kit (Pierce, IL, U.S.A.).

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