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INCREASES IN ENZYME LEVELS DURING THE FORMATION OF PHENOLIC ACIDS IN CARROT CELL CULTURES

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Abstract—The levels of glutamic acid dehydrogenase (GDH), phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (CAH) and *O*-methyltransferase (OMT) were measured during the formation of phenolic acids in carrot cells in suspension culture. Caffeic, ferulic and *p*-hydroxybenzoic acids were always present as the culture proceeded. Total content of these acids increased at the early logarithmic and linear phases. GDH showed high activity at the early logarithmic and stationary phases. PAL activity was much enhanced at the linear and stationary phases. CAH activity was found in actively growing cells, especially at the early and late logarithmic phases OMT behaved similarly to PAL. The increases in GDH and CAH might be responsible for the rapid synthesis of phenolic acid at the early logarithmic phase. The increase in phenolic acid at the linear phase would certainly be due to enhancements of both PAL and OMT. On the other hand, the accumulation of vanillic acid was observed in cells which were transferred and cultured on an agar medium, but not in cells in suspension culture. This accumulation is related to increases in OMT levels and also to changes in the degree of β-oxidation.

INTRODUCTION

In our previous investigations [1, 2], the high biosynthetic activity of phenolic acid was confirmed in carrot cells in suspension culture at the early logarithmic phase, although the activity of phenylalanine ammonia-lyase (PAL) was not appreciably high. Furthermore, these results suggested that the formation of phenolic acid was affected by changes in the pool of phenylalanine or total amino acid. Such above findings led us to examine further the levels of several enzymes, glutamic acid dehydrogenase (GDH), cinnamic acid-4-hydroxylase (CAH) and caffeic or protocatechuic acid-*O*-methyltransferase (cOMT or pOMT) in carrot cells in suspension culture. Since the accumulation of vanillic acid was observed in the present cells which were transferred and cultured on the agar medium, the levels of cOMT and pOMT in these cells were also examined.

RESULTS AND DISCUSSION

Carrot cells in suspension culture multiplied and grew logarithmically for 8 days and then linearly for 3 days before the culture entered the stationary phase. Three phenolic acids, caffeic, ferulic and *p*-hydroxybenzoic acids were always present in cells at all the above growth phases. As shown in Table 1, total content of these acids was considerably high at both early logarithmic and linear phases. After the late stationary phase (17-day culture), cinnamic acid derivatives, caffeic and ferulic acids almost disappeared whereas *p*-hydroxybenzoic acid accumulated. Table 2 shows the levels of GDH, PAL, CAH and OMT as the culture proceeded. Activities of all the enzymes were found to be higher at the early logarithmic than late logarithmic phases. GDH and CAH revealed their maximum activities at the former phase. However, CAH activity was found only in actively growing cells, especially at the early and late logarithmic phases. After the linear phase, activities of both PAL and OMT were much enhanced and still high even at the late stationary phase.

Other workers have investigated the behaviour of these enzymes in relation to the formation of phenolic compounds in cultured plant cells. Hasegawa *et al.* observed a gradual increase in GDH activity and the concomitant decrease in PAL activity in a developing fruit of grapefruit [3]. Russell demonstrated that CAH

Table 1. Content of phenolic acid in carrot cells in suspension culture

Growth phase (culture day)	Phenolic acid (μg/g dry wt) Caffeic acid	Ferulic acid	*p*-Hydroxy benzoic acid	Total
Early logarithmic (3-day culture)	81	12	19	112
Late logarithmic (6-day culture)	62	11	13	86
Linear (10-day culture)	93	13	18	124
Early stationary (14-day culture)	48	9	38	95
Late stationary (17-day culture)	7	+	42	49

Table 2. Changes in activities of GDH, PAL, CAH, cOMT and pOMT in carrot cells in suspension culture

Growth phase (culture day)	Enzyme activity (pkat/mg protein)* GDH	PAL	CAH	cOMT	pOMT
Early logarithmic (3-day culture)	141.5	3.4	8.3	1.00	0.06
Late logarithmic (6-day culture)	74.0	2.3	4.1	0.45	0.04
Linear (10-day culture)	72.3	17.8	1.5	0.64	0.09
Early stationary (14-day culture)	86.8	8.1	0	2.15	0.13
Late stationary (17-day culture)	107.7	6.6	0	1.89	0.20
Very late stationary (27-day culture)	43.4	0	0	1.38	0.10

* pkat means picokatals.

activity was only restricted to young and developing tissues or organs [4]. In general, PAL activity is enhanced during an increase in phenolic acids at the linear phase. Ebel *et al.* [5] reported that CAH behaves similarly to PAL in soya bean cells and revealed its maximum activity at the late logarithmic or linear phase. However, they did not observe any change in OMT activity. In contrast, Maule *et al.* [6] reported an increase in OMT activity before lignification in wheat leaves infected with a fungus. Our present experiments, together with the previous findings [1, 2], would thus support the following conclusion. The high activity of GDH at the early logarithmic phase probably enlarges the pools of phenylalanine and total amino acid. Consequently, PAL, with the high activity of CAH, rapidly converts phenylalanine to cinnamic acid to increase the biosynthetic activity of phenolic acid at the early logarithmic phase. At the late logarithmic phase, these enzyme activities were lowered, due to the small pool of phenylalanine [2]. Such changes probably cause the decrease in phenolic acid at this phase. At the linear phase, both the phenolic acid and phenylalanine increased again [2], with the remarkable enhancements of PAL and OMT. Phenolic acid content was lower than expected, because of the rather low activity of CAH at this phase. Such a low activity in CAH explains the decrease in cinnamic acid derivatives at the stationary phase. We are now investigating the levels of *p*-coumaric acid-3-hydroxylase in this system.

Table 3. Content of phenolic acid in carrot cells on agar

Growth phase (culture day)	Phenolic acid (μg/dry wt) Caffeic acid	Ferulic acid	*p*-Hydroxybenzoic acid	Vanillic acid	Total
Linear (10-day culture)	15	22	22	64	123
Linear (20-day culture)	142	32	46	78	298
Early stationary (30-day culture)	140	38	97	108	383
Late stationary (40-day culture)	18	33	174	104	329

Table 4. Changes in activities of CAH, cOMT and pOMT in carrot cells on agar

Growth phase (culture day)	Enzyme activity (pkat/mg protein)* CAH	cOMT	pOMT
Linear (10-day culture)	5.30	0.32	0.01
Linear (20-day culture)	5.05	1.31	0.07
Early stationary (30-day culture)	0	0.67	0.04
Late stationary (40-day culture)	0	0.26	0.02

* values are $\times 10^3$ in the cases of cOMT and pOMT

Carrot cells on the agar medium multiply and grow linearly for about 20 days and then gradually become stationary. Vanillic acid accumulates in these cells, but not in cells in suspension culture (Tables 1 and 3). As shown in Table 4, CAH activity was found in linearly growing cells (10- and 20-day cultures). The cOMT activity was indeed much enhanced during the increase in vanillic acid from 20- to 30-day cultures. However, cells in suspension culture also showed considerable activity of cOMT (Table 2). The presence of vanillic acid in their cells is presumably due to increased β-oxidation of ferulic acid.

EXPERIMENTAL

Cultured cells. Carrot cell line GD-2 used in this expt was derived from a storage root of red carrot cv Kintoki [7, 8] and maintained in the liquid medium of Murashige and Skoog [9] containing 1 ppm 2,4-D in place of IAA and kinetin. The detailed procedure of suspension culture was as described previously [10]. About 2×10^5 cells at the linear phase (12-day culture) was transferred on the 1% agar medium (160 cm^2) containing the same components as above liquid medium and the agar culture was proceeded at 27°. Cultured cells were collected and washed with H_2O on a Buchner funnel. Qualitative and quantitative assays of phenolic acids were performed as described previously [1].

Assay of GDH. Cultured cells were disrupted with quartz sand in 50 mM Tris-50 mM β-mercaptoethanol (pH 7.7) and the cell homogenate was centrifuged at 500 *g*. The supernatant was centrifuged at 20000 *g* for 60 min and the resultant ppt was suspended and frozen in the above buffer. The frozen suspension was thowed and centrifuged at 105000 *g* for 60 min. The supernatant was referred to the enzyme solution. The reaction mixture contained 280 nmol NADH, 2 μmol α-ketoglutaric acid, 10 μmol NH_4Cl and the enzyme (*ca* 300 μg protein) in 3 ml of the above buffer (pH 7.7). The reaction proceeded for 5 min at 25° and the enzyme activity was estimated by reduction of A_{340}.

Assay of CAH. Cell homogenate in 50 mM Tris containing 0.7 M sorbitol (pH 7.5) was centrifuged at 500 *g*. The supernatant was directly used as the enzyme solution because CAH was very unstable. The reaction mixture contained 0.1 μCi cinnamic acid-[3-^{14}C], 2 μmol of cinnamic acid, 5 μmol glucose-6-phosphate, 0.4 U glucose-6-phosphate dehydrogenase, 2 μmol NADP, 3 μmol of β-mercaptoethanol, 75 μmol Tris and the enzyme (500 μg protein) in total volume of 2.0 ml at pH 7.5. The reaction proceeded for 1 hr at 30° with shaking and was stopped by the addition of HCl. Enzyme activity was determined by the method of Russell [6].

Assay of OMT. Cell homogenate in 100 mM potassium phosphate containing 5 mM β-mercaptoethanol (pH 7.5) was

centrifuged at 10000 *g*. The ppt by 30–70% saturation with $(NH_4)_2SO_4$ was dissolved in 20 mM potassium phosphate (pH 7.5) and then ultrafiltered through a membrane of Amicon UM-10 with excess of the buffer. The ultrafiltrate was referred to the enzyme solution, which was assayed by the method of Kuroda *et al.* [11]. The reaction mixture contained 10 μmol cysteine, 0.5 μmol caffeic or protocatechuic acid, 1 μmol $MgCl_2$, 0.1 μCi *S*-adenosylmethionine-[^3H_3C] and the enzyme (300 μg protein) in 1 ml 100 mM potassium phosphate containing 5 mM β-mercaptoethanol (pH 7.5). The reaction proceeded for 1 hr at 30° and was stopped by the additions of HCl and ferulic or vanillic acid. After the extraction of mixture with EtOAc, ferulic or vanillic acid was separated by PC with C_6H_6–HOAc–H_2O (6:7:3). The radioactivity was counted in a solution of toluene–EtOH–ppo–popop with a liquid scintillation counter. Protein was assayed by the method of Lowry *et al.* [12].

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