BIOLOGIA PLANTARUM 45 (2): 311-313, 2002

BRIEF COMMUNICATION

Optimum assay conditions of the activity of phytochelatin synthase from tobacco cells

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Abstract

We determined the characteristics of phytochelatin synthase from tobacco (*Nicotiana tabacum* cv. Bright Yellow-2) cells, especially the conditions for the enzyme stability. From the results, we proposed the optimum assay conditions of the enzyme activity.

Additional key words: metal stress, *Nicotiana tabacum*.

When plant cells are exposed to heavy metals, they induce the biosynthesis of heavy metal-binding peptides called phytochelatins (PCs) (Grill *et al.* 1987). PCs protect plant enzymes from Cd^{2+} by chelation with SH-groups (Kneer and Zenk 1992). PCs' synthesis uses glutathione (GSH) as a substrate and is catalyzed by PC synthase (Grill *et al.* 1989). The enzyme is continuously present in plant cells, and the catalytic activity depends on heavy metals (Loeffler *et al.* 1989). The involvement of PCs in tolerance to several heavy metals has been documented by several investigators (Reese and Wagner 1987, Mendum *et al.* 1990, Howden *et al.* 1995a,b, Nakazawa *et al.* 2000). Therefore, it is important to analyze relationship between heavy metal-tolerance and PC synthase activity, so we have been investigating PC synthase activity in plants (Nakazawa and Takenaga 1998a,b). Whereas the catalytic activity of this enzyme is unstable (Grill *et al.* 1989). In this study, we proposed the optimum assay conditions of PC synthase activity in tobacco cell extracts.

Suspension cultures of tobacco (*Nicotiana tabacum* cv. Bright Yellow-2) cells were grown as described previously (Nakazawa *et al.* 2000). The 7-d-old cells were homogenized in an equal volume of 20 mM Hepes-NaOH (pH 7.5) containing 0 - 40 mM mercaptoethanol, and then the homogenate was centrifuged at 15 000 *g* for 5 min. The supernatant (0.08 cm^3) was added to the mixture (0.02 cm^3) containing 100 mM GSH, 250 μM $Cd(NO_3)_2$, and 250 mM Hepes-NaOH (pH 7.5). After incubation at 35 °C for 15 min, the reaction was stopped by the addition of 0.02 cm^3 of 30 % (m/v) 5-sulfosalicylic acid (SSA), and centrifuged at 15 000 *g* for 1 min. Synthesized PCs were measured using with C_{18}-HPLC (*Shimadzu Shim-Pack CLC-ODS* , 0.6 × 15 cm, Kyoto, Japan) as described previously (Nakazawa *et al.* 2000). The metal salts tested in this study were: silver nitrate, cadmium nitrate, cupric nitrate, zinc nitrate, lead nitrate, mercuric chloride, nickel nitrate, manganese chloride, cobalt chloride, sodium arsenite[As(III)], and sodium arsenate[As(V)].

The highest activities were detected in the extracts prepared with the buffer containing 10 mM mercapto-ethanol, and that activities with 10 mM mercaptoethanol were 26-fold of those without mercaptoethanol. In addition, the activities in the extracts prepared with the buffer containing 2 mM phenylmethanesulfonyl fluoride (PMSF), were 79 % of those without PMSF. From these results and the finding that PMSF is serine-protease inhibitor (Gold 1967), it is indicated that the catalytic activity may be regulated by serine residue.

Received 2 February 2001, *accepted* 7 September 2001.

Abbreviations: GSH - glutathione; PCs - phytochelatins; PMSF - phenylmethanesulfonyl fluoride; SSA - 5-sulfosalicylic acid.

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The stability of PC synthase activities at 4 and 35 °C in cell extracts was determined (Table 1). The half-life was 0.5 h at 4 °C and 6 h at 35 °C. PC synthase activity from tobacco cells was unstable: The enzyme activities were most stable at pH 7.5, and half-maximal stability was at pH 6.7 and pH 8.5 (Table 2). This result indicates the necessity of the use of the buffer which has strong buffer capacity (such as Hepes-NaOH).

Table 1. Effect of temperature on the stability of PC synthase activity in cell extracts. After the mixtures containing 50 mM Hepes-NaOH (pH 7.5) and enzyme solution were kept at 4 or 35 °C for various times, PC synthase activities in these mixtures were measured. The activities at zero-time are expressed as 100 %. Means ± SD, $n = 9$.

Treatment	Residual activities [%]
0 h	100.0 ± 0.4
4 °C for 1 h	91.0 ± 0.1
4 °C for 2 h	76.9 ± 0.2
4 °C for 4 h	75.1 ± 0.5
35 °C for 0.5 h	51.2 ± 5.1
35 °C for 1 h	9.6 ± 1.0

Table 2. Effect of pH on the stability of PC synthase. After the mixtures containing 50 mM Hepes-NaOH (pH 6.5 - 9.0) and enzyme solution were kept at 35 °C for 0.5 h, PC synthase activities in these mixtures were measured. Stability is expressed as the residual activities after treatments at 35 °C for 0.5 h. Means ± SD, $n = 9$.

pH in enzyme solution	Residual activities [%]
6.5	8.6 ± 0.2
7.0	40.1 ± 0.4
7.5	59.3 ± 0.7
8.0	38.9 ± 0.3
8.5	29.8 ± 0.1
9.0	4.6 ± 0.1

Next, we examined the effects of several chemicals on the stability of PC synthase activities in cell extracts. The results were that the enzyme was stabilized by the addition of 50 μM $Cd(NO_3)_2$, or glycerol [more than 10 % (m/v)] also stabilized this enzyme (Table 3). The other metal ions besides Cd(II) also stabilized the enzyme activities (Table 4). However, Cd was optimum stabilizer, followed by Cu(II), Zn(II), Ag(I), As (III), Hg(II), Pb(II), Mn(II), Ni(II), and As (V).

The highest activity of PC synthase was observed at pH 8.0 (Fig. 1*A*) and at 35 °C (Fig. 1*B*). The K_m for GSH was 8.6 mM (Fig. 1*C*). The K_m value is similar to that of *Silene cucubalus* cells (6.7 mM; Grill *et al.* 1989), but not to *Pisum sativum* L. (51 mM; Klapheck *et al.* 1995). These differences of catalytic properties may affect to the physiological response to heavy metals.

Table 3. Effects of Cd(II) and glycerol on the stability of PC synthase activities in cell extracts. After the mixtures containing 50 mM Hepes-NaOH (pH 7.5) and enzyme solution with Cd(II) or glycerol, were kept at 35 °C for 0.5 h, PC synthase activities in these mixtures were measured. Stability is expressed as the residual activities after treatment at 35 °C for 0.5 h. Means ± SD, $n = 9$.

Treatment	Residual activities [%]
None	53.1 ± 5.6
1 μM Cd(II)	69.0 ± 7.2
10 μM Cd(II)	82.4 ± 2.1
50 μM Cd(II)	101.1 ± 1.1
100 μM Cd(II)	88.1 ± 5.1
10 % (m/v) glycerol	74.7 ± 6.0
20 % (m/v) glycerol	82.1 ± 8.6
30 % (m/v) glycerol	83.0 ± 5.3

Table 4. Effects of several metal salts on the stability of PC synthase activities in cell extracts. After the mixtures containing 50 mM Hepes-NaOH (pH 7.5) and enzyme solution with several metal salts, were kept at 35 °C for 1 h, PC synthase activities in these mixtures were measured. Stability is expressed as the residual activities after treatment at 35 °C for 1 h. Heavy metal salt concentration is that which stabilized most effectively in each salt. Means ± SD, $n = 9$.

Metal salts	Concentration	Residual activities [%]
$Cd(NO_3)_2$	50	102.0 ± 3.2
$Cu(NO_3)_2$	10	90.1 ± 2.2
$Zn(NO_3)_2$	50	48.6 ± 1.4
$AgNO_3$	50	37.2 ± 1.2
Na-arsenite	500	37.0 ± 5.6
$HgCl_2$	100	36.7 ± 1.6
$Pb(NO_3)_2$	10	31.0 ± 0.2
$MnCl_2$	100	24.3 ± 0.1
$Ni(NO_3)_2$	10	18.8 ± 0.3
Na-arsenate	500	16.2 ± 6.3
$CoCl_2$	10	10.3 ± 0.0
None		13.1 ± 1.8

Proposal of optimum assay condition of PC synthase activity in tobacco cell extracts: The cells were homogenized in an equal volume of 20 mM Hepes-NaOH (pH 7.5) containing 10 mM mercaptoethanol, 100 μM $Cd(NO_3)_2$, and 20 % (m/v) glycerol, and then the homogenate was centrifuged at 15 000 *g* for 5 min. The enzyme solution (0.08 cm^3) was added to the mixture (0.02 cm^3) containing 100 mM GSH, 50 μM $Cd(NO_3)_2$, 10 % (m/v) glycerol and 250 mM Hepes-NaOH (pH 8.0).

After incubation at 35 °C for 15 min, the reaction was stopped by the addition of 0.02 cm^3 of 30 % (m/v) SSA, and centrifuged at 15 000 *g* for 1 min. Synthesized PCs were measured by the method described above.

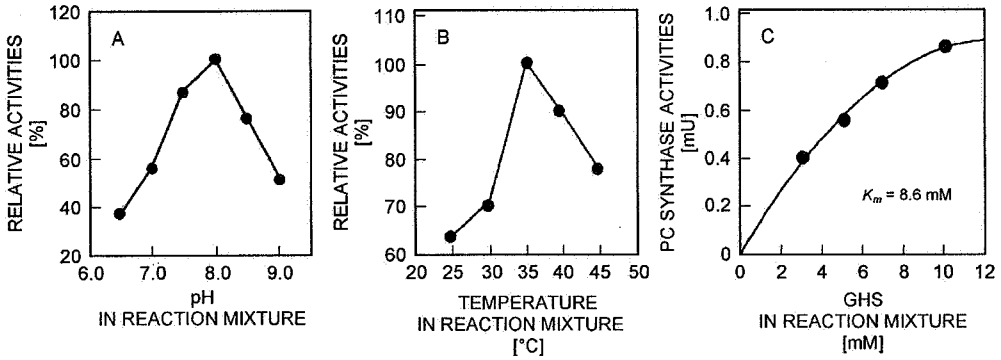

Fig. 1. Determination of optimum pH (*A*) and temperature (*B*) for the catalytic activity of PC synthase from tobacco cells (the activities at pH 8.0 and 35 °C were expressed as 100 %, respectively), and K_m for GSH of the enzyme (*C*) ($n = 9$).

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