Pergamon

0031-9422(94)00623-7

Phytochemistry, Vol. 38, No. 1, pp. 89–93, 1995

0031-9422/95 $9.50 + 0.00

SEPARATION AND CHARACTERIZATION OF CYTOKININ-INDUCIBLE (*S*)-TETRAHYDROBERBERINE OXIDASES CONTROLLING BERBERINE BIOSYNTHESIS IN *THALICTRUM MINUS* CELL CULTURES

MASAKAZU HARA, HIROKI MORIO, KAZUFUMI YAZAKI, SHIGEO TANAKA and MAMORU TABATA

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida, Kyoto 606, Japan

(*Received* 31 *May* 1994)

Key Word Index—*Thalictrum minus*; Ranunculaceae; plant cell culture; cytokinin; 6-benzylaminopurine; tetrahydroberberine oxidase; berberine biosynthesis.

Abstract—Three forms of cytokinin-induced tetrahydroberberine (THB) oxidase from *Thalictrum minus* cell suspension cultures were individually separated by chromatography using DEAE-TOYOPEARL. Two of them were (*S*)-THB-specific oxidases, whereas the third was found to be a non-stereospecific one. The stereospecific oxidases were different from each other not only in the K_m value, but also in the time course of activation induced by 6-benzylaminopurine (BAP). The substrate specificity of these oxidases from *T. minus* cells was more similar to that reported for the (*S*)-THB oxidase of *Coptis japonica* than that for *Berberis stolonifera* cell cultures.

INTRODUCTION

Berberine, a benzylisoquinoline alkaloid, is produced and excreted into Linsmaier–Skoog (LS) medium [1] containing both 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) in *Thalictrum minus* var. *hypoleucum* cell suspension cultures [2, 3]. Berberine biosynthesis, which is completely suppressed in LS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), can be induced fully by transferring the cells into LS medium supplemented with BAP in place of 2,4-D [4].

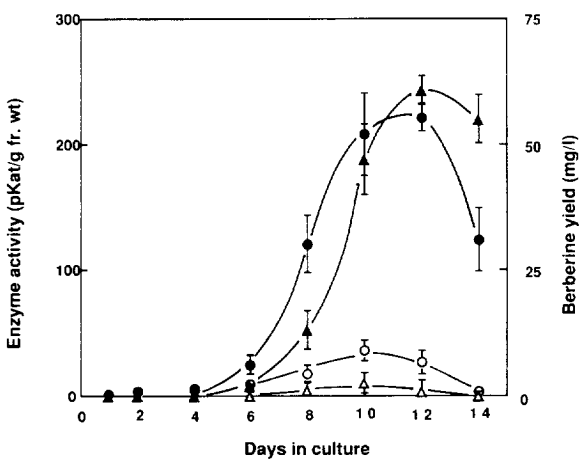

Fig. 1. Time courses of THB oxidase activity and berberine production in *T. minus* cell suspension cultures. BAP (10^{-5} M) or H_2O (0.2 ml) was added to 2,4-D medium two days after inoculation. ○: ●: THB oxidase activity, and △: ▲: berberine yield in control (H_2O) cultures (open symbols) and BAP-treated cultures (closed symbols). Three replicates.

It has been demonstrated through tracer experiments using L-[U-^{14}C] tyrosine and measurements of enzyme activities that the inhibition of berberine biosynthesis by 2,4-D is primarily due to suppression of the activities of such enzymes as norcoclaurine-*O*-methyltransferase and (*S*)-tetrahydroberberine (THB) oxidase that catalyse specific intermediary and final reaction steps leading to berberine, respectively [4, 5]. The latter enzyme, which is called either canadine oxidase (COX) [6] or (*S*)-THB oxidase in *Coptis* cells [7], has been well-characterized. This enzyme is similar to (*S*)-tetrahydroprotoberberine oxidase (STOX) in *Berberis* cells [8] with regard to the optimum pH, stereospecificity for (*S*)-substrates and hydroperoxide generation in the enzymatic reaction, but different from STOX in its K_m value for (*S*)-THB and M_r [8, 9]. However, little is known about the THB oxidase of *Thalictrum* cells. We now report the properties and specific roles of the THB oxidase of *T. minus* cell cultures.

RESULTS AND DISCUSSION

Activation of THB oxidase

Time courses of THB oxidase activity and berberine yield in BAP-treated and control cultures of *T. minus* are shown in Fig. 1. Cultured cells began to produce and excrete berberine into medium on day 6 of culture, when THB oxidase was also activated. The berberine yield and the enzyme activity increased up to their respective maximum values, 243 mg l^{-1} and 55.3 pKat g^{-1} fresh weight, on day 12. To investigate the properties of THB oxidase, the BAP-treated cells were harvested on day 8–10.

Separation of isoforms of THB oxidase

A crude extract prepared from frozen cells of *T. minus* in 50 mM Tris–HCl buffer (pH 8.5) containing 20 mM β-mercaptoethanol was loaded on to a DEAE-TOYOPEARL 650 M (Tosoh) column equilibrated with the same buffer. After washing the column with the loading buffer, proteins absorbed were eluted with a linear gradient of KCl (0–500 mM). Analysis of THB oxidase activity in each fraction suggested the existence of three different oxidases (Fig. 2).

The first isoform, enzyme-1, passed through the column, while enzyme-2 and -3 were eluted by 100 and 350 mM KCl, respectively.

When each enzyme separated by DEAE-TOYOPEARL chromatography was rechromatographed on the same column, a single peak of activity appeared in the corresponding fraction. Relative activities of three separate enzymes remained unchanged even when β-mercaptoethanol added to the enzyme solution was increased to a concentration of 60 mM or replaced by dithiothreitol (5 mM), suggesting that they were intact enzymes and not artifact proteins.

The existence of such isoforms in certain enzymes involved in secondary metabolism has been reported for chorismate mutase [10], phenylalanine ammonia-lyase [11], chalcone synthase [12], tyrosine aminotransferase [13] and tetrahydrobenzylisoquinoline-*N*-methyltransferase [14]. Some of the isoforms were more or less different from each other in their properties or responses to environmental factors [10, 11]. However, isoforms of (*S*)-THB oxidase have been found for the first time in the present study on *Thalictrum* cell cultures.

Properties of isoforms of (S)-*THB oxidase*

To examine the substrate stereospecificity of each enzyme, the appropriate enzyme solution was incubated with either (*S*)- or (*R*)-THB as a substrate (Fig. 3). Enzymes-2 and -3 could oxidize only (*S*)-THB to form berberine, whereas enzyme-1 converted both (*S*)- and (*R*)-THB equally to berberine (Fig. 4), indicating that enzyme-1 was quite different from the other enzymes in terms of its substrate stereospecificity. Enzymes-2 and -3 showing a (*S*)-stereospecificity seem to be similar to STOX of *Berberis stolonifera*, which stereospecifically converts (*S*)-tetrahydrocolumbamine to columbamine [6], as well as to (*S*)-THB oxidase of *Coptis japonica*, which oxidizes only (*S*)-THB [7]. On the other hand, enzyme-1 of *T. minus*, which is different in its chromatographic behaviour and substrate stereospecificity from the other enzymes, could not be detected in crude extracts

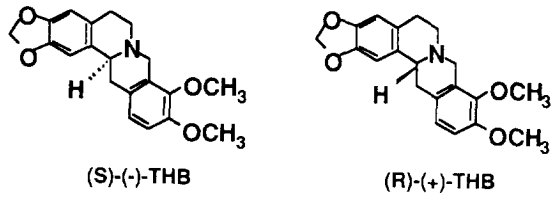

Fig. 3. Structures of (*S*)-(−)-THB and (*R*)-(+)-THB.

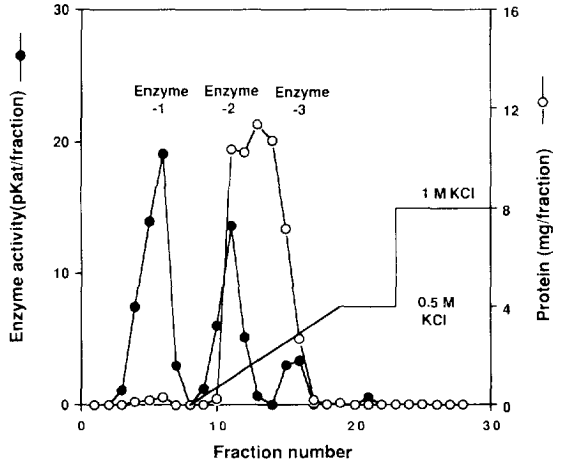

Fig. 2. Separation of enzymes-1, -2 and -3 by DEAE-TOYOPEARL column chromatography. Crude extract was prepared from BAP-treated cells harvested on day 9 of culture. ●: THB oxidase activity, ○: protein.

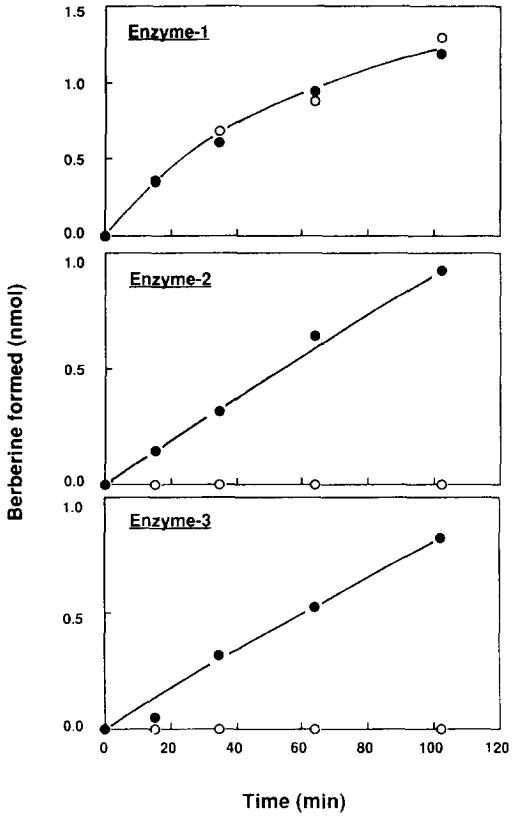

Fig. 4. Substrate stereospecificity of enzymes-1, -2 and -3. Each enzyme was incubated with 12 nmol of (*S*)- or (*R*)-THB in the standard reaction mixture described in the Experimental. ●: (*S*)-THB, ○: (*R*)-THB.

from cell cultures of *Coptis japonica*, *Thalictrum dipterocarpum*, and *T. flavum*, all of which showed a high yield of berberine and a high (*S*)-specific THB oxidase activity (data not shown). These observations suggest that enzyme-1 might be a non-specific oxidase which is not involved in alkaloid biosynthesis, in contrast to enzymes-2 and -3 which will be tentatively called (*S*)-THB oxidase-I and -II, respectively.

Significant differences in enzymic properties were found between (*S*)-THB oxidase-I and -II (Table 1). The former exhibited a much smaller K_m value for the substrate (*S*)-THB than the latter, suggesting a high affinity for (*S*)-THB. Furthermore, estimation of the native M_r of these (*S*)-THB oxidases by gel filtration chromatography using a Sepharose CL-6B column showed that enzyme-I had a greater M_r (> 200000) than enzyme-II which consisted of two molecules of M_r 145000 and 46000. The presence of enzyme-I in the void volume on gel filtration chromatography suggests that it might have been highly aggregated. As regards enzyme-II, it is likely that the high M_r form was a trimer of the low one. Interestingly, these M_r values are similar to those of the (*S*)-THB oxidase of *C. japonica* (M_r of 155000 and 58000).

Despite the marked differences in K_m and M_r between enzyme-I and -II, the optimum pH was found to be 9 for both. Addition of either NAD^+ or $NADP^+$ (1 mM each) to enzyme reaction mixtures had no effect on reaction rates, as described for the corresponding enzymes of *Berberis* and *Coptis* [8, 7].

In marked contrast to the oxidases of *Berberis* [8] and *Coptis* [6] shown in Table 1, none of the (*S*)-THB oxidases of *Thalictrum* was capable of oxidizing (*S*)- or (*R*)-tetrahydrojatrorrhizine. However, (*S*)-THB oxidases prepared from cell cultures (12-day-old) of *T. flavum* and *T. dipterocarpum* and which gave single peaks on chromatography with DEAE-TOYOPEARL, did convert (*S*,*R*)-tetrahydrojatrorrhizine to jatrorrhizine at conversion rates of 18 and 40%, respectively. These results seem to suggest that THB-oxidases of *Thalictrum* species are more closely related to that of *Coptis* of the same family (Ranunculaceae) than to that of *Berberis* (Berberidaceae).

Activation of (S)-*THB oxidases by BAP*

Although the activities of both (*S*)-THB oxidases-I and -II could be induced by adding 10 μM BAP to *T. minus* cell cultures on day 2, there was a characteristic difference between the two in their time courses (Fig. 5). The activity of enzyme-I reached a maximum on day 8 of culture when berberine began to be synthesized, maintaining a relatively constant level until day 14 before it declined. On the other hand, the activity of enzyme-II increased rapidly after day 8 to attain a peak on day 11, in parallel with the increase of berberine. This relationship is suggestive that a high activity of enzyme-II may be required for an efficient production of berberine, in addition to the activation of enzyme-I having a low K_m value at an earlier stage. Such early induction of an isoform with a lower K_m has also been reported for phenylalanine ammonia-lyase in *Phaseolus vulgaris* cell suspension cultures exposed to a *Colletotrichum* elicitor [11]. These phenomena seem to imply that the selective activation of particular isoforms with different K_m values at different stages might be important in order to ensure an orderly regulation of biosynthesis.

EXPERIMENTAL

Cell culture. Cell suspension cultures of *Thalictrum minus* L. var. *hypoleucum* Miq. native to Japan [15] were maintained in 100 ml of Linsmaier–Skoog (LS) medium [1] containing 10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D) in 300-ml conical flasks, agitated on a reciprocal shaker at a speed of 100 strokes min^{-1} at 25° in the dark with subculturing every 2 weeks. For the induction of berberine production, 10^{-5} M BAP was added to the cultures in the 2,4-D medium (30 ml) on day 2 after

Table 1. Comparison of tetrahydroberberine oxidases from different cell cultures

	Thalictrum minus		*Berberis stolonifera**	*Coptis japonica*†
	(*S*)-THB oxidase-I	(*S*)-THB oxidase-II		
K_m for (*S*)-THB (μM)	2.1	10.6	26.7	6.5
Native M_r	> 200000	145000	100000	155000
		46000		58000
Optimum pH	9	9	8.9	8.7
Effect of cofactors				
NAD^+ (1 mM)	—	—	—	—
$NADP^+$ (1 mM)	—	—	—	—
Oxidation of tetrahydrojatrorrhizine	0%‡	0%‡	K_m: 1.3 μM	15%‡

*Data cited from ref. [8] for (*S*)-tetrahydroberberine oxidase (STOX).

†Data cited from refs [6, 9] for (*S*)-tetrahydroberberine oxidase.

‡The oxidation capability of each (*S*)-THB oxidase from (*S*)-THB to berberine is 100%.

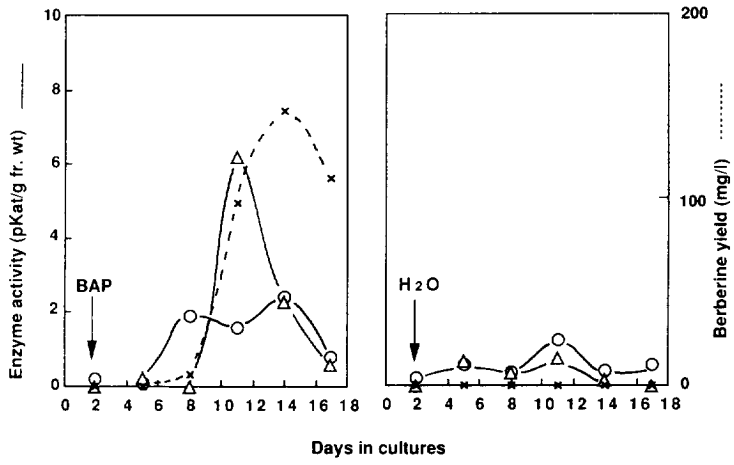

Fig. 5. Changes in the activities of (*S*)-THB oxidases and berberine production in *T. minus* cell cultures. BAP (10^{-5} M) or H_2O (0.2 ml) was administered to 2,4-D medium two days after inoculation. The value of each plot in the graph represents an average of three replicated cultures. ○: (*S*)-THB oxidase-I, △: (*S*)-THB oxidase-II, ×: berberine production.

inoculation with cells. Cell suspension cultures of *T. flavum* L. ssp. *glaucum* Batt. and *T. dipterocarpum* Franch., and those of *Coptis japonica* Makino var. *dissecta* Nakai were maintained under conditions described elsewhere [16, 17]. The quantitative analysis of berberine produced by cultures was carried out by HPLC as described in ref. [2].

Chemicals. 2,4-D and BAP were purchased from Sigma and berberine hydrochloride from Wako Pure Chemicals. Jatrorrhizine was isolated and purified from dry roots of *Coptis japonica*, and identified with an authentic sample (MS and NMR).

(*S,R*)-Tetrahydroberberine (THB) and (*S,R*)-tetrahydrojatrorrhizine were synthesized according to the method of Mirza [18] and shown to be identical to authentic samples (MS and NMR). (*S*)- and (*R*)-THB were separately prepd from racemic THB recrystallization using (+)-10-camphorsulphonate [19]. Each sample was identified by reference to the published data [7]. (*S*)-(−)-THB: mp 133–135° (lit. 133–134°), $[\alpha]_D^{28}$ − 298 ($CHCl_3$; *c* 1.6) [lit. − 299], (*R*)-(+)-THB: mp 133° (lit. 132°), $[\alpha]_D^{28}$ + 293 ($CHCl_3$; *c* 1.5) [lit. + 299].

Enzyme assay. The reaction mixt. (30 μl) consisted of 25 μl enzyme prepn in 50 mM Tris–HCl buffer (pH 8.5) and 5 μl 6.3 mM (*S,R*)-THB in *N,N*-dimethylformamide. It was incubated at 30° for 30 min. The reaction was stopped by the addition of 5 μl 50% TCA. After centrifugation at 12000 *g* for 5 min, the berberine concn in the supernatant was determined by HPLC analysis, as described above. For kinetic studies, incubations were performed for 20 min; the substrate specifity was tested by using 0.4 mM (*S*)-, (*R*)-THB or (*S,R*)-tetrahydrojatrorrhizine as the substrate. The reaction products, berberine and jatrorrhizine, were sepd by TLC (silica gel, $CHCl_3$–MeOH, 5:2) and identified by direct comparison with authentic samples (HPLC, UV and MS).

Separation of THB oxidase isoforms. All the following procedures were performed at 4°. Cells frozen by liquid N_2 (20 g) were thawed by stirring in 40 ml 100 mM Tris–HCl buffer (pH 7.5) containing 60 mM β-mercaptoethanol (buffer A). The homogenate was centrifuged at 12000 *g* for 20 min, then $(NH_4)_2SO_4$ was added to the supernatant to 70% satn. The ppt. collected by centrifugation (10 min, 10000 *g*) was taken up in buffer A. The protein soln was applied to a PD-10 (Pharmacia) column equilibrated with 50 mM Tris–HCl buffer (pH 8.5) containing 20 mM β-mercaptoethanol (buffer B). A fr. (10 ml) containing the protein was loaded on a DEAE-TOYOPEARL column (10 × 40 mm), equilibrated with buffer B. After washing the column, the protein was eluted with 35 ml buffer B containing KCl in a linear gradient from 0 to 500 mM at a flow rate of 40 ml hr^{-1}. Frs (3 ml each) were collected and checked for THB oxidase activity. For time course studies on the activity of isoforms, enzyme extracts from 2.0 g (fr. wt) cells were subjected to chromatography on a DEAE-TOYOPEARL column (10 × 12 mm).

Protein content. Protein contents were measured by the Bradford method [20]. Bovine serum albumin (Sigma) was used as a ref.

REFERENCES

1. Linsmaier, E. M. and Skoog, F. (1965) *Physiol. Plant.* **18**, 100.
2. Nakagawa, K., Konagai, A., Fukui, H. and Tabata, M. (1984) *Plant Cell Rep.* **3**, 254.
3. Yamamoto, H., Suzuki, M., Suga, Y., Fukui, H. and Tabata, M. (1987) *Plant Cell Rep.* **6**, 356.
4. Hara, M., Kitamura, T., Fukui, H. and Tabata, M. (1993) *Plant Cell Rep.* **12**, 70.
5. Hara, M., Tanaka, S. and Tabata, M. (1994) *Phytochemistry* **36**, 327.
6. Galneder, E., Rüffer, M., Wanner, G., Tabata, M. and Zenk, M. H. (1988) *Plant Cell Rep.* **7**, 1.

7. Yamada, Y. and Okada, N. (1985) *Phytochemistry* **24**, 63.
8. Amann, M., Nagakura, N. and Zenk, M. H. (1984) *Tetrahedron Letters* **25**, 953.
9. Okada, N., Shinmyo, A., Okada, H. and Yamada, Y. (1988) *Phytochemistry* **27**, 979.
10. Singh, B. K., Lonergan, S. G. and Conn, E. E. (1986) *Plant Physiol.* **81**, 717.
11. Bolwell, G. P., Bell, J. N., Cramer, C. L., Schuch, W., Lamb, C. J. and Dixon, R. A. (1985) *Eur. J. Biochem.* **149**, 411.
12. Beerhues, L. and Wiermann, R. (1985) *Z. Naturforsch.* **40c**, 160.
13. De-Eknamkul, W. and Ellis, B. E. (1987) *Arch. Biochem. Biophys.* **257**, 430.
14. Frenzel, T. and Zenk, M. H. (1990) *Phytochemistry* **29**, 3491.
15. Nakagawa, K., Fukui, H. and Tabata, M. (1986) *Plant Cell Rep.* **5**, 69.
16. Suzuki, M., Nakagawa, K., Fukui, H. and Tabata, M. (1988) *Plant Cell Rep.* **7**, 26.
17. Fukui, H., Nakagawa, K., Tsuda, S. and Tabata, M. (1982) in *Plant Tissue Culture* 1982 (Fujiwara, A., ed.), p. 313. Maruzen, Tokyo.
18. Mirza, R. (1957) *J. Chem. Soc.* 4400.
19. Takao, N., Kamigauchi, M. and Okada, M. (1983) *Helv. Chim. Acta* **66**, 473.
20. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.