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PURIFICATION AND CHARACTERIZATION OF (*S*)-TETRAHYDROBERBERINE OXIDASE FROM CULTURED *COPTIS JAPONICA* CELLS

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Key Word Index—*Coptis japonica*, Ranunculaceae, cultured cells, berberine, (*S*)-tetrahydroberberine, (*S*)-tetrahydroberberine oxidase

Abstract—(*S*)-Tetrahydroberberine (THB) oxidase was purified to homogeniety from cultured *Coptis japonica* cells by DEAE-Sepharose chromatography, gel filtration, and HPLC The enzyme catalysed the removal of four hydrogen atoms from one mol of (*S*)-THB and produced two mol of hydrogen peroxide and one mol of berberine in the presence of molecular oxygen The purified enzyme had neither the yellow fluoresence characteristic of flavin derivatives nor an absorption spectrum showing the presence of haem. The enzyme had a M_r of 58 000 and consisted of two identical subunits of 28 000 each

INTRODUCTION

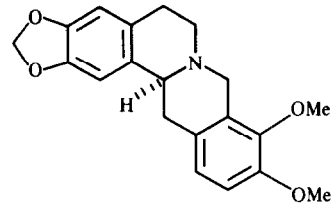
Cultered plant cell lines that produce large quantities of anthocyanins [1], alkaloids [2–5], steriods, or ubiquinone-10 [6] have been isolated by repetitive selection These cell lines provide materials suitable for analysis of biosynthetic pathways One example is the cell line of *Coptis japonica*, which produces berberine at a yield of 8 to 10% of the dry cell weight [2, 7, 8]. Berberine biosynthesis in intact plants (*Hydrastis canadensis*, [9]) has been studied by means of feeding experiments and seems to have the specific dehydrogenation of (*S*)-tetrahydroberberine (THB, **1**) as the final step (Fig 1) A cell-free extract prepared from cultured *C japonica* cells catalyses the stereospecific dehydrogenation of (*S*)-THB, but not the enantiomer [10], dihydroberberine, a hypothetical intermediate, was not detected during the reaction. These results indicate that the removal of the four electrons from the substrate is catalysed by a single enzyme or two enzymes operating in tandem.

We report here on the purification and characteristics of (*S*)-THB oxidase, an enzyme which catalyses the four-electron oxidation of (*S*)-THB to form berberine (Fig 1).

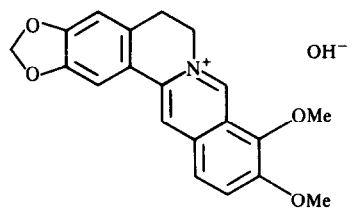
RESULTS

Purification of (S)-*THB oxidase*

The enzyme was purified from the crude enzyme preparation by CM-Sephadex and DEAE-Sepharose chromatography, gel filtration on Sephadex G-100, and size-exclusion HPLC. Purification steps 1–4 (Table 1) were completed within 60 hr because of the instability of the enzyme. The crude enzyme solution contained much berberine, which interfered with measurement of the enzyme activity. Step 1 removed the cationic yellow substances, including berberine. The enzyme sample at step 4 had increased 10 times in specific activity, at a yield of 5% from the starting sample The enzyme sample after HPLC gave a single protein band in both PAGE (Fig 2, lane 1) and SDS-PAGE (Fig 2, lane 2). The gel of the parallel run of PAGE (Fig 2, lane 1) was sliced into 5 mm lengths, crushed, and extracted with 75 mM phosphate buffer (pH 7.0) for 16 hr at 4°. The (*S*)-THB oxidase activity in the extract was measured The single slice with the enzyme activity corresponded to the slice containing the protein band The enzyme activity in both the crude and purified enzyme solution was unstable; the



(*S*)-Tetrahydroberberine (**1**)



Berberine (**2**)

Fig 1 Reaction catalysed by (*S*)-tetrahydroberberine oxidase

Table 1 Purification of (S)-THB oxidase

Step	Protein (mg)	Activity (nkat)	Sp act (nkat/mg)	Yield (%)	Enrichment (-fold)
Crude enzyme		600		100	
(1) CM-Sephadex	470	360	0 75	60	1
(2) 1st DEAE-Sepharose	36 5	210	5 8	35	7 7
(3) 2nd DEAE-Sepharose	13	72	5 5	12	7 3
(4) Sephadex G-100	3 7	30	8 1	5	10 8

Enzyme activity was measured as described in Experimental

crude enzyme solution had lost half of its activity after 20 hr at 4°

Characteristics of (S)*-THB oxidase*

The optimum pH for (*S*)-THB oxidase was 8 8 in 50 mM phosphate buffer (pH 5 0 to 8 0) and borate buffer (pH 8 0 to 10 0) The enzyme had an optimum temperature at 37° for a one hr reaction period It lost 40 and 90% of its activity in one hr at 45 and 50°, respectively Only (*S*)-THB was converted to berberine by this enzyme The K_m value with (*S*)-THB, 6 5 μM, was obtained from double-reciprocal plots of the substrate concentration and the initial reaction velocity The solution of purified enzyme was colourless and had absorption maxima at 215 and 280 nm.

The M_r of the denatured (*S*)-THB oxidase protein was estimated by SDS-PAGE to be 28 000 (Fig 2, lane 2) By size-exclusion HPLC on a TSK G-3000 sw column (Fig 3), the M_r of the native (*S*)-THB oxidase was found to be 58 000 We concluded that the enzyme consisted of two identical subunits each with an M_r of 28 000

The enzyme reaction required oxygen (Table 2), and produced hydrogen peroxide (H_2O_2) Quantitative analysis of the reaction products showed that 2 0 mol of H_2O_2 were produced for each mol of berberine formed The kinetic relationship between the reaction products showed that berberine and H_2O_2 were formed simultaneously and stoichiometricallly We concluded that four hydrogen atoms from the substrate were transferred to two mol of oxygen to produce two mol of H_2O_2 and one mol of berberine

Enzyme inhibitors

Table 2 shows the effect of enzyme inhibitors on (*S*)-THB oxidase Some metal chelators (EDTA, 2,2'-bipyridie, and bathocuproine sulphonic acid, a specific chelator for Cu^{2+} ion) did not cause inhibition However, the enzyme activity was inhibited 45% by 2 mM bathophenanthroline sulphonic acid, a specific chelator for Fe^{2+} ion The activity was not restored by the addition of 0 1 mM ferrous sulphate after the removal of the chelator by dialysis against 60 mM phosphate buffer (pH 7 0) These results indicate that ferrous iron firmly bound to the enzyme protein was involved in the oxidase activity

DISCUSSION

(*S*)-THB oxidase was purified to homogeneity from cultured *C japonica* cells The enzyme catalyses the conversion of (*S*)-THB to berberine by removal of four hydrogen atoms No product other than berberine was detected by HPLC analysis of the enzyme reaction mixture [10] The enzyme reaction requires oxygen, and produces two mol of H_2O_2 per mol of berberine formed Thus, we concluded that (*S*)-THB oxidase either catalyses the simultaneous removal of four hydrogen atoms from (*S*)-THB, or catalyses two sequential dehydrogenations of the substrate without dissociation of the intermediate from the enzyme molecule All of the oxidases examined so far catalyse the removal of two hydrogen atoms, so (*S*)-

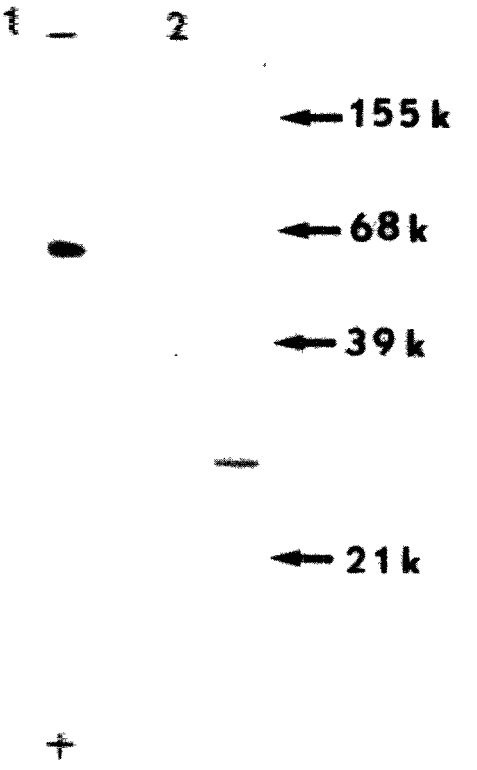


Fig 2 PAGE of the (*S*)-THB oxidase from cultured *C japonica* cells Lane 1 Electrophoretic analysis of the (*S*)-THB oxidase at step 5 (6 μg of protein) was done as reported elsewhere [14] on a 7 5% polyacrylamide slab gel Lane 2 SDS-PAGE of the same sample as lane 1(12 μg of protein) was done, on a 10% polyacrylamide slab gel in the presence of 0 1% SDS [15] Arrows indicate the position of marker proteins 21K for trypsin inhibitor (M_r 21 000), 39K for RNA polymerase α-subunit (39 000), 68K for bovine serum albumin (68 000), 155K for RNA polymerase β-subunit (155 000)

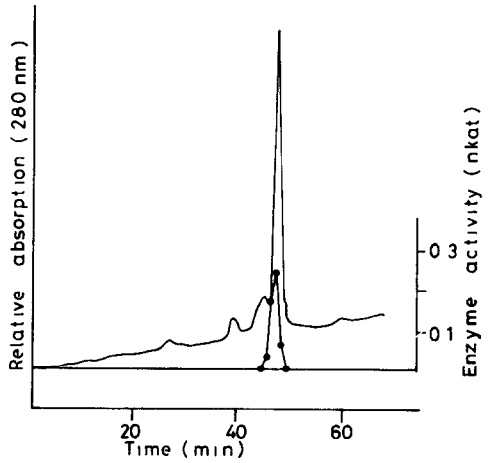


Fig 3 HPLC chromatogram of the enzyme sample from step 4. ——, A_{280}, ●——●, (*S*)-THB oxidase activity The elution times (min) of marker proteins were as follows glutamate dehydrogenase (290 000) 34; lactate dehydrogenase (142 000) 39, enolase (67 000) 46, adenylate kinase (32 000) 51; cytochrome *c* (12 400) 58

Table 2. Effect of inhibitors on (*S*)-THB oxidase

Inhibitor	Conc (mM)	Activity (%)
None	0	100
EDTA	2 0	96
2,2′-Bipyridyl	2.0	100
Bathocuproine sulphonic acid	2 0	90
Bathophenanthroline sulphonic acid	0 1	83
	2 0	55
	10	20
Anaerobic conditions		10

THB oxidase should be classified as a new kind of oxidase.

The absorption spectrum of the enzyme showed that it contains no haem-iron and no flavin derivatives such as FAD or FMN. (*S*)-THB oxidase is not inhibited by bathocuproine sulphonic acid. This indicates that the oxidase is not a Cu-oxidase such as ascorbate oxidase. A specific chelator for Fe^{2+} ion, bathophenanthroline sulphonic acid, weakly inhibits the enzyme.

More than 1000 μM of the chelator is required for 50% inhibition of (*S*)-THB oxidase activity, when the concentration of the enzyme is 0.3 μM The molecular ratio of the enzyme to the chelator is 1:3000 From these results, we suspect that a non-haem iron such as an iron acid labile sulphur cluster is bound firmly to the enzyme molecule. One example of an oxidase that does not contain haem iron and does produce H_2O_2 is the secondary alcohol oxidase of *Pseudomonas* This enzyme is resistant to specific chelators for iron atoms [11]

An (*S*)-THB oxidase isolated from cultured *Berberis wilsonae* var *subcaulialata* Schneid cells has been reported [12] This enzyme differs from the (*S*)-THB oxidase from cultured *C. japonica* cells in the following ways The M_r of the (*S*)-THB oxidase from *B wilsonae* is 100 000, while that of the enzyme from *C. japonica* is 58 000. The (*S*)-THB oxidase from *B wilsonae* contains flavin, as judged from its yellow-green fluoresence, but flavin was not detected in the enzyme from *C. japonica* The oxidase from *B. wilsonae* produces one mol of H_2O_2 and water per mol of substrate consumed, but the oxidase from *C. japonica* produces two mol of H_2O_2.

EXPERIMENTAL

Cultured cells The original cultured *C japonica* cells were induced from small fragments of rootlets of *C japonica* Makino var dissecta (Yatabe) Nakai (in Japanese, *seribaworen*) [7]. Small aggregates were used for the cloning [2, 8] Fine cell aggregates were collected by filtration and plated in Petri dishes with Linsmaier-Skoog suspension medium [13] After cloning, cell lines producing large amounts of berberine were selected [2]. These cell lines were cultured on Linsmaier-Skoog suspension medium containing 10^{-5} M 1-naphthaleneacetic acid and 10^{-8} M 6-benzyladenine at 26° on a rotary shaker at 100 rpm in the dark

Assay of enzyme activity The assay was carried out in 60 mM Pi buffer (pH 7 0) containing 0 423 mM (*R, S*)-THB, 10% *N,N*-dimethylformamide (DMF), and a suitably diluted enzyme soln at 30° for 1 hr The reaction was stopped by immersion of the reaction tube in a boiling water bath for 3 min. The increase in the amount of berberine was assayed by the increase in absorbance at 345 and 425 nm [10]. The enzyme reaction was linear up to 2 hr, and consumed up to 40% of the substrate The amount of berberine formed increased linearly with increase in the amount of enzyme added to the reaction mixture

When the enzyme reaction was carried out under anaerobic conditions, the assay mixture (0 5 ml) was placed in a stoppered vial (14 × 35 mm) N_2 was bubbled through the soln for 5 min, then was passed through the vial for another 15 min The enzyme soln (0 1 ml) was injected into the vial The reaction was stopped by putting the vial in a boiling water bath for 3 min, then the increase in absorbance at 425 nm was measured The protein was assayed from the absorption at 280 nm It was assumed that 1 absorbance unit corresponded to 0 5 mg/ml protein

Electrophoresis Polyacrylamide gel (7 5%) electrophoresis (PAGE) was performed at pH 8 0 [14] Electrophoresis under denaturated conditions was performed by the procedure of ref [15]. A polyacrylamide gel (10%) was used for the estimation of the M_r of the enzyme

Preparation of crude enzyme All procedures were carried out at 0–5° Cultured *C japonica* cells (200 g fr wt) were crushed with quartz sand in 150 ml 100 mM Pi buffer (pH 7 0) containing 10 mM dithiothreitol DTT) in a chilled mortar The brei was sonicated (× 4, 1 min at 20 kHz) and after removal of debris in the resulting suspension, the protein that pptd at 0 7 saturation of $(NH_4)_2SO_4$ was collected by centrifugation The pellet was suspended in a minimal vol of 20 mM Pi buffer (pH 7 0) containing 2 mM DTT The soln was dialysed against the same buffer and centrifuged at 30 000 *g* for 15 min The supernatant was used as the crude enzyme soln

Enzyme purification All steps were performed at 0–5°. Step 1 CM-Sephadex CC The crude enzyme soln (50 ml) was put on a CM-Sephadex C-50 column (4 × 60 cm), equilibrated with 20 mM Pi buffer (pH 7 0) containing 2 mM DTT The column was washed with the same buffer Column fractions of 20 ml each were collected and checked for (*S*)-THB oxidase activity The fractions with the activity were free from yellow pigments, including berberine alkaloids

Step 2 First DEAE-Sepharose CC The combined fractions with enzyme activity (120 ml) were adjusted to the concentration of 30 mM NaCl The resulting soln was applied to a DEAE-Sepharose CL-6B column (2 5 × 10 cm) equilibrated with 20 mM Pi buffer (pH 7 0) containing 2 mM DTT and 50 mM NaCl, and eluted stepwise with the same buffer soln containing 50 mM (150 ml), 60 mM (60 ml), 75 mM (100 ml), or 100 mM (200 ml) NaCl at a flow rate of 75 ml/hr The enzyme was eluted by 100 mM NaCl The fractions with the enzyme activity were pooled, desalted, and concentrated by ultrafiltration with a UK-10 filter to a volume of 40 ml

Step 3 Second DEAE-Sepharose CC The concentrated enzyme from step 2 was applied to a DEAE-Sepharose CL-6B column (1 8 × 10 cm) equilibrated with 20 mM Pi buffer (pH 7 0) containing 2 mM DTT and 50 mM NaCl The enzyme was eluted with 360 ml of the same buffer containing NaCl in a linear gradient from 60 to 120 mM at a flow rate of 45 ml/hr Fractions of 6 ml each were collected The active fractions were pooled and concentrated by ultrafiltration to 3 5 ml

Step 4 Sephadex G-100 CC The enzyme soln from step 3 was applied to a Sephadex G-100 column (2 × 7 5 cm) equilibrated with 50 mM Pi buffer (pH 7 0) containing 2 mM DTT, and eluted with the same buffer at a flow rate of 40 ml/hr Fractions of 2 5 ml each were collected The active fractions were pooled and concentrated by ultrafiltration to 2 ml

Step 5 HPLC The concentrated enzyme soln from step 4 was put on a TSK G-3000 sw column (7 5 × 600 mm) equilibrated with 50 mM Pi buffer (pH 7 0), and eluted with the same buffer at a flow rate of 0 4 ml/min The absorption at 280 nm was monitored by a UV-spectrophotometer The eluent with absorption at 280 nm was fractionated (0 5 to 1 5 ml) and checked for enzyme activity The active fractions were pooled and concentrated by ultrafiltration to 2 5 ml

Assay of H_2O_2 The concentration of H_2O_2 in the reaction mixture was assayed by a modification of the method of ref [16] The reaction mixture (0 6 ml) in 100 mM Pi buffer (pH 6 8) contained 0 423 mM (*R, S*)-THB, 10% *N,N*-dimethylformamide, and a suitably diluted enzyme soln After 1 hr at 30°, 0 1 ml of 4 mM 4-aminoantipiline, 0 1 ml of 5 mM 2,4-dichlorophenol, and 0 2 ml of horseradish peroxidase (26 U/ml) were added to the reaction mixture The mixture was incubated at 30° for 3 min, and 1 ml EtOH was added to stop the reaction The absorption was measured at 505 nm

Chemicals (*R, S*)-THB was synthesized as described previously [10] Bathophenanthroline sulphonic acid and bathocuproine sulphonic acid were purchased from Dojin Laboratories (Kumamoto, Japan) Horseradish peroxidase were purchased from Toyobo Co (Osaka Japan)

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