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Characterization of Thermostable Tyrosine Phenol-Lyase from an Obligatory Symbiotic Thermophile, *Symbiobacterium* sp. SC-1

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Tyrosine phenol-lyase of thermophilic *Symbiobacterium* sp. SC-1, which is obligately and symbiotically dependent on thermophilic *Bacillus* sp. SK-1, was purified and characterized. The enzyme is composed of four identical subunits and contains approximately 1 mol of pyridoxal 5′-phosphate (PLP) per mol subunit as a cofactor. The enzyme showed absorption maxima at 330 and 420 nm, and lost this absorption profile by treatment with phenylhydrazine. The apparent dissociation constsnt, K'_D, for PLP was determined with the apoenzyme to be about 1.2 μM. The isoelectric point was 4.9. The optimal temperature and pH for the α,β-elimination of L-tyrosine were found to be 80°C and pH 8.0, respectively. The substrate specificity of the enzyme was very broad: L-amino acids including L-tyrosine, 3,4-dihydroxyphenyl-L-alanine (L-DOPA), L-cysteine, L-serine, *S*-methyl-L-cysteine, β-chloro-L-alanine, and *S*-(*o*-nitrophenyl)-L-cysteine all served as substrates. D-Tyrosine and D-serine were also decomposed into pyruvic acid and ammonia at rates of 7% and 31% relative to their corresponding L-enantiomers, respectively. D-Alanine, which was inert as a substrate in α, β-elimination, was the only D-amino acid racemized by the enzyme. The K_m values for L-tyrosine, L-DOPA, *S*-(*o*-nitrophenyl)-L-cysteine, β-chloro-L-alanine, and *S*-methyl-L-cysteine were 0.19, 9.9, 0.36, 12, and 5.5 mM, respectively.

Keywords: α,β-Elimination, Racemization, *Symbiobacterium* sp. SC-1, Tyrosine phenol-lyase.

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Introduction

Tyrosine phenol-lyase (TPL, E.C.4.1.99.2) is a carbon-carbon lyase catalyzing α,β-elimination and β-replacement of L-tyrosine and its related amino acids, and racemization of alanine (Kumagai *et al.*, 1970a; 1970c). At high concentrations of ammonium pyruvate, the enzyme catalyzes the synthesis of L-tyrosine from phenol, ammonia, and pyruvic acid by the reverse reaction of α,β-elimination. The synthesis reaction has attracted a lot of attention because it is applicable to the industrial production of 3,4-dihydroxyphenyl-L-alanine (L-DOPA), which has been used as a therapeutic agent for the treatment of Parkinson's disease since the 1960s (Yamada and Kumagai, 1975; Foor *et al.*, 1993).

TPLs have been found mainly in enterobacterial species, i.e. *Escherichia intermedia* (Kumagai *et al.*, 1970a), *Erwinia herbicola* (Kumagai *et al.*, 1972), *Citrobacter freundii* (Sysuev *et al.*, 1980), and *Citrobacter intermedia* (Demidkina *et al.*, 1984), and are distributed only in a few genera of bacteria (Carman and Levin, 1977). Recently, we found that *Symbiobacterium* sp. SC-1, an obligately symbiotic thermophile, produces TPL (Lee *et al.*, 1997). The bacterium is a Gram-negative aerobe growing at high temperatures of over 65°C. It specifically grows only by co-culture with thermophilic *Bacillus* sp. SK-1. Suzuki *et al.* (1988) found a similar thermophilic symbiotic bacterium growing only in a co-culture with its symbiont. They named the symbiotic bacterium *Symbiobacterium thermophilum* and found that it produces thermostable TPL and tryptophanase.

TPL of *Symbiobacterium* sp. SC-1 was much more stable than the enzyme from the enterobacterial species against, not only heat, but also, high concentrations of phenol or pyrocatechol (Lee *et al.*, 1996a; 1996b), which readily inactivates ordinary TPLs (Para and Baratti, 1988a; 1988b). TPL of *Symbiobacterium* sp. SC-1 is useful

because of its high stability against phenol, and is applicable for the removal of phenol from industrial wastewater. When recombinant *E. coli* XL1Blue cells harboring pHLT1 that overproduced the TPL were added to industrial wastewater containing 10,000 ppm phenol and 0.3 M ammonium pyruvate, 92% of the phenol in the water was removed and converted to L-tyrosine within 24 h (Lee *et al.*, 1996a). The L-tyrosine produced was easily sedimented in the water due to the low solubility of tyrosine.

Thus, thermostable TPL from *Symbiobacterium* sp. SC-1 is highly useful, and its application could be expanded if it could be engineered in a substantially more stable form. It is then necessary to clarify the fine characteristics of this enzyme, and to this end, we here describe the enzymological properties of TPL from *Symbiobacterium* sp. SC-1.

Materials and Methods

Materials L-DOPA and lactate dehydrogenase were purchased from Boehringer Mannheim GmbH (Germany). Sodium pyruvate and pyrocatechol were from Musashino Shoji (Tokyo, Japan). Amino acids, pyridoxal 5′-phosphate (PLP) and all other chemicals, except *S*-(*o*-nitrophenyl)-L-cysteine, which was synthesized from 1-fluoro-2-nitrobenzene and L-cysteine (Phillips *et al.*, 1989), were from Sigma (St. Louis, USA).

Purification and preparation of apoenzyme TPL of *Symbiobacterium* sp. SC-1 was purified from recombinant *E. coli* cells as described previously (Lee *et al.*, 1997). The homogeneous preparation of TPL was obtained in an overall yield of about 50%, and stored in 20 mM Tris·HCl buffer (pH 8.0) containing 0.1 mM PLP at −20°C until use. The purified enzyme catalyzes α, β-elimination of L-tyrosine at a specific activity of 1.9 units/mg protein at 60°C and pH 7.2. The apoenzyme was prepared as follows: TPL (2.9 mg) was kept for 30 min on ice in 0.1 M Tris·HCl buffer (1.0 ml, pH 8.2) containing 5 mM phenylhydrazine hydrochloride and then chromatographed with a Sephadex G25 column equilibriated with 0.1 M Tris·HCl buffer (pH 8.2). The enzyme was dialyzed further against the same buffer to completely remove phenylhydrazine and its adduct with PLP.

Assays Unless otherwise specified, TPL activity was measured with a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.2), 2.5 mM L-tyrosine, 0.05 mM PLP, and an appropriate amount (about 0.02 unit) of TPL in a total volume of 2 ml. The mixture was incubated at 60°C for 20 min, and the amount of pyruvate formed was determined colorimetrically (Friedemann and Haugen, 1943). One unit of TPL was defined as the amount of enzyme that catalyzes the formation of 1 μmol of pyruvate per min. Specific activity was expressed as units per mg protein.

Protein concentration was measured with Coomassie Brilliant Blue G250 (BioRad) using bovine serum albumin as a standard.

PLP content of TPL was measured by the method of Wada and Snell (1961). Samples were incubated with 0.1% (w/v) phenylhydrazine in 0.5 M sulfuric acid at room temperature (about 20°C) for 10 min. The molar absorption coefficient of the PLP adduct at 410 nm was 17.1 $mM^{-1} cm^{-1}$.

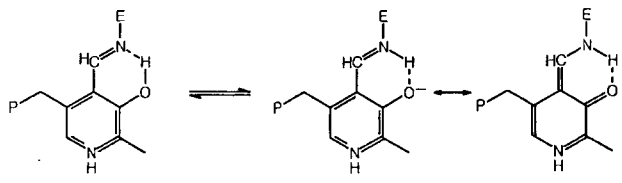
HPLC analysis Amino acid enantiomers were derivatized with *o*-phthalaldehyde and *N*-acetyl-L-cysteine in borate buffer (pH 9.5), and analyzed by HPLC. A μBondapak C18 column was used with a methanol gradient of 5 to 50% in 50 mM sodium acetate buffer (pH 6.8), at a flow rate of 1.0 ml/min. Enantiomers were detected by fluorescence with excitation and emission at 342 nm and 452 nm, respectively.

Results and Discussion

Physicochemical properties The molecular weight of TPL from *Symbiobacterium* sp. SC-1 was determined to be about 202,000 by gel filtration column chromatography in the absence of denaturants. Analytical gel filtration chromatography was performed using Superose 12 (Pharmacia, Sweden) after the column was equilibrated with 20 mM Tris·HCl buffer (pH 8.0) containing 0.15 M NaCl. Elution volumes for marker proteins and purified TPL were determined and correlated with molecular mass on a semi-logarithmic plot.

The molecular weight of one TPL subunit has been estimated to be 52,196 from the deduced amino acids sequence (Lee *et al.*, 1997). Therefore, the native enzyme is believed to be composed of four identical subunits. The isoelectric pH of the enzyme was determined to be 4.87 with a Phast System isoelectric focusing gel (Pharmacia, Upsala, Sweden).

Cofactor PLP, a derivative of vitamin B6 (Choi *et al.*, 1992), is absolutely required for the enzymatic function of TPL. The apoenzyme prepared by the resolution of enzyme-bound PLP was virtually inactive in the absence of added PLP, but was activated by the addition of PLP. The apparent dissociation constant, K'_D, for PLP was estimated to be about 1.2 μM by the addition of various concentrations of PLP to the apoenzyme (Fig. 1). The apoenzyme showed no appreciable absorption around 420 nm. However, by addition of PLP, the holoenzyme with absorption maxima at 330 and 420 nm was reconstituted (Fig. 2). When the pH of the holoenzyme solution was shifted to 6.5, the absorbance at 420 nm was increased with concurrent decrease in absorbance at 340 nm, probably due to an equilibrium-shift towards formation of the ketoenamine structure of PLP (Kumagai *et al.*, 1970b; Kallen *et al.*, 1985):



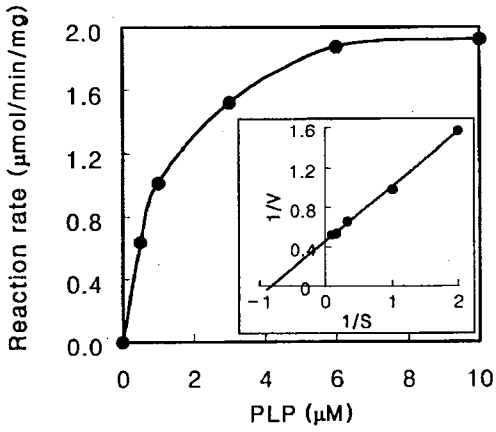


Fig. 1. Dependence of TPL activity on PLP concentration. Inset is the double-reciprocal plot of the original figure.

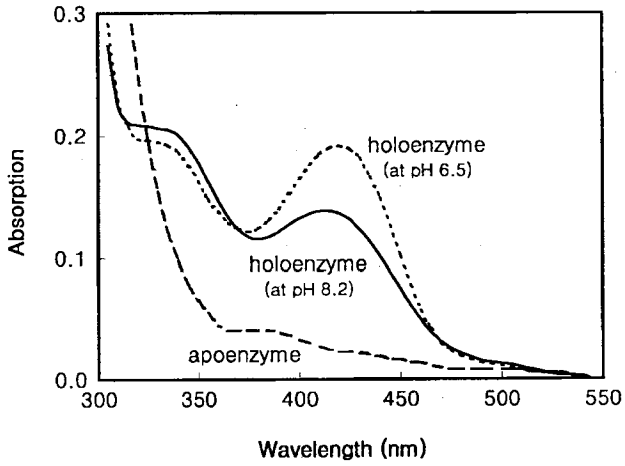


Fig. 2. Absorption spectra of TPL of *Symbiobacterium* sp. SC-1 in the presence and absence of PLP; holoenzyme at pH 8.2 (——); holoenzyme at pH 6.5 (-----); apoenzyme at pH 8.2 (– –).

Since PLP could reversibly form a Schiff base with the active-site lysyl residue (Bang and Kim, 1990; Lee and Kim, 1993), the apoenzyme was dialyzed in the presence of 10 μM PLP to reconstitute the holoenzyme. The amount of enzyme-bound PLP was determined to be about 3.4 mol per mol of tetrameric enzyme with phenylhydrazine, each subunit containing approximately 1 molecule of PLP.

Effect of temperature and pH The activity of α,β-elimination of L-tyrosine increased with temperature and reached a maximum at 80°C (Fig. 3A). A linear relationship between the logarithm of activity and the reciprocal of temperatures (Arrhenius plot) was obtained at temperatures below 75°C. (Fig. 3B). The activation energy (E_a) of α, β-elimination of L-tyrosine was determined to be 10.6 kcal/mol from the slope of Fig. 3B.

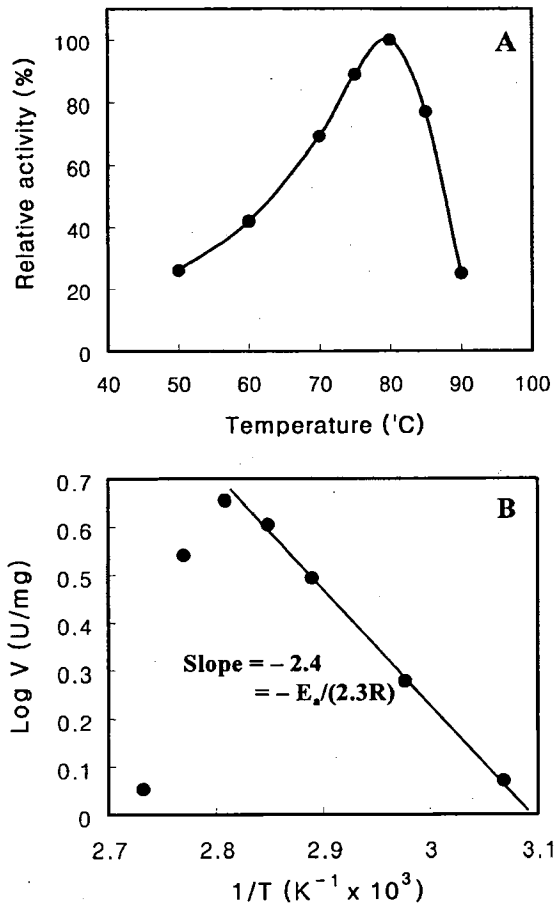


Fig. 3. Effect of temperature on activity. **A.** The TPL reaction was performed at various temperatures. **B.** The logarithm of specific activity (μmol/mg/min) was plotted against the reciprocal of absolute temperature.

The optimum pH for the reaction catalyzed by TPL from *Symbiobacterium* sp. SC-1 was around 8.0 (Fig. 4). This value is similar to those of other TPLs (pH 8.2) (Kumagai *et al.*,1970a; 1972; Sysuev *et al.*, 1980; Demidkina *et al.*, 1984), but differs markedly from that of the *Symbiobacterium thermophilum* TPL (pH 7.2) (Suzuki *et al.*, 1992).

α,β-Elimination of various amino acids L-Tyrosine was the best substrate of TPL among the natural L-amino acids investigated (Table 1), while synthetic amino acids such as *S*-(*o*-nitrophenyl)-L-cysteine, *S*-methyl-L-cysteine, and β-chloro-L-alanine were much better as substrates than L-tyrosine. D-Tyrosine and D-serine also served as substrates of the enzyme; the substrate specificity of the enzyme was as broad as that of enterobacterial TPLs. However, its high activity with L-amino acids of small side chains such as L-serine and L-cysteine and the synthetic amino acids characterizes TPL of *Symbiobacterium* sp. SC-1.

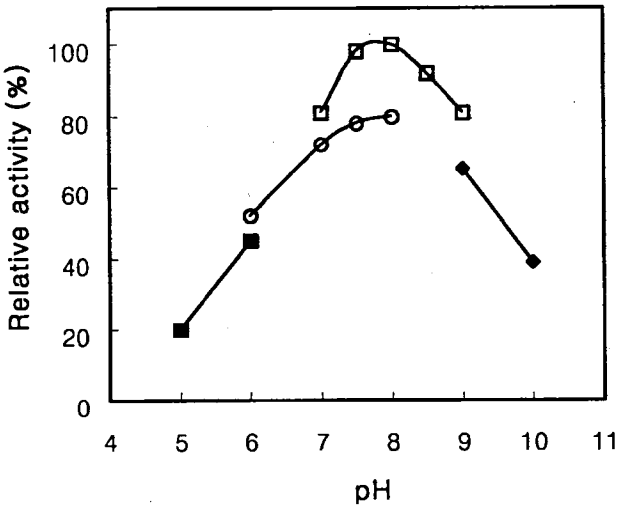


Fig. 4. Effect of pH on activity. The reaction was carried out in 50 mM concentrations of the following buffers: citrate-sodium phosphate buffer (■), potassium phosphate buffer (○), Tris·HCl buffer (□), and glycine-NaOH buffer (◆).

Table 1. Substrate specificity of TPL from *Symbiobacterium* sp. SC-1.[a]

Substrate	Relative activity[b] (%)
L-Tyrosine	100
3,4-Dihydroxyphenyl-L-alanine	35
L-Cysteine	42
L-Serine	35
S-(*o*-Nitrophenyl)-L-cysteine	250
S-Methyl-L-cysteine	385
β-Chloro-L-alanine	500
D-Tyrosine	7
D-Serine	11

[a] Inert: L-alanine, L-aspartic acid, L-histidine, L-methionine, L-phenylalanine, L-tryptophan, L-valine, L-threonine, D-alanine, and D-tryptophan.
[b] Relative activity means the enzyme activity for each substrate compared to that for L-tyrosine. The activities were measured with reaction mixtures containing 2.5 mM of each substrates. Other conditions were the same as the assay conditions described in the text.

The enzyme showed the smallest Michaelis constant (K_m) for L-tyrosine, probably because L-tyrosine is its natural substrate (Table 2). On the other hand, the k_{cat}/V_{max} for L-DOPA was approximately one-fiftieth of that for L-tyrosine, while V_{max} values were almost the same between the two enzymes. This result shows that L-DOPA is not a preferred substrate of the thermostable TPL. Synthetic substrates, including *S*-(*o*-nitrophenyl)-L-cysteine, served as good substrates.

Racemization of amino acids Enei *et al.* (1972) proposed that D-tyrosine undergoes α,β-elimination catalyzed by TPL of *Erwinia herbicola* via L-tyrosine. When D-tyrosine, D-phenylalanine, D-serine, and D-alanine were incubated with TPL of *Symbiobacterium* sp. SC-1 in 0.1 M potassium phosphate buffer (pH 7.2) at 60°C for 1 h, only D-alanine was racemized into its L-enantiomer (data not shown). Faleev *et al.* (1979) reported that partially-purified TPL of *Escherichia intermedia* also racemizes only D-alanine among the various D-amino acids examined. The racemization of D-alanine proceeded at a rate of 0.0017 units/mg protein with TPL of *Symbiobacterium* sp. SC-1, a value about 30 times lower than that catalyzed by enterobacterial TPLs (Kumagai *et al.*, 1970c; Chen and Phillips, 1993).

Effect of reducing agents TPL of *Escherichia intermedia* was inactivated by modification of its sulfhydryl groups with 5,5′-dinitro-2-nitrobenzoate, and restored to its initial activity by subsequent treatment with β-mercaptoethanol (Fukui *et al.*, 1975). TPL of *Symbiobacterium* sp. SC-1 was half-inactivated upon standing in 20 mM Tris·HCl buffer (pH 8.0) at 4°C for one

Table 2. Kinetic parameters for TPL[a]

Substrate	K_m (mM)	V_{max} (μmol/min/mg)	k_{cat}/K_m ($s^{-1}.mM^{-1}$)
L-Tyrosine	0.19	2.1	9.5
3,4-Dihydroxyphenyl-L-alanine	9.9	2.2	0.19
S-(*o*-Nitrophenyl)-L-cysteine	0.36	4.6	11.1
S-Methyl-L-cysteine	5.5	19.0	2.9
β-Chloro-L-alanine	12.0	53.0	3.8

[a] TPL was assayed at 60°C by measuring the amount of the produced pyruvate using lactate dehydrogenase with a molar absorption coefficient of 6.22 $mM^{-1}\,cm^{-1}$ for NADH at 340 nm. The reaction mixture (0.5 ml) contained 50 mM potassium phosphate buffer (pH 7.2), 0.05 mM PLP, 0.2 mM NADH, 10 units of lactate dehydrogenase, and various concentrations of amino acids.

wk. However, its original activity was recovered upon incubation with 2 mM dithiothreitol or 10 mM β-mercaptoethanol at 37°C for 1 h. Thus, sulfhydryl groups of TPL from *Symbiobacterium* sp. SC-1 probably suffer from reversible oxidation in the same manner as those of TLP of *Escherichia intermedia.*

Stability When TPL of *Symbiobacterium* sp. SC-1 was incubated with 200 mM pyrocatechol for 2 h, it retained 50% of its initial activity. However, TPL of *Citrobacter freundii* lost all of its initial activity under the same conditions (data not shown). *Symbiobacterium* TPL was also stable against 0.2% SDS, while *Citrobacter freundii* TPL was inactivated almost entirely in the presence of 0.1% SDS (data not shown). TPL of *Symbiobacterium* sp. SC-1 kept its activity in wastewater containing 10,000 ppm of phenol, as described previously (Lee *et al.*, 1996a).

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