Purification and Properties of Skipjack Liver Tryptophan Hydroxylase

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(Received December 13, 1993)

Tryptophan hydroxylase (5-monooxygenase) (EC 1.14.16.4; Trp hydroxylase) in skipjack liver was extracted with Tris–acetate buffer solution and purified by acid treatment, ammonium sulfate fractionation, Sephadex G-150, DEAE-Sepharose CL-6B, Butyl-Sepharose 4B, and Toyopearl HW-55F chromatography. The enzyme was purified 1,500-fold with a 6.2% yield from skipjack liver. The apparent molecular weight was estimated to be 288,000 by gel filtration on Sephadex G-150. The enzyme gave a single protein band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis which also revealed that the enzyme was composed of identical subunits with a molecular weight of 97,000. The optimum temperature was 35°C and the enzyme was stable under 35°C. The optimum pH was 8.0 and the enzyme retained more than 80% of its original activity between pH 7.5 and 8.5 after incubation at 35°C for 30 min. The enzyme was inhibited by Co^{2+} , Mn^{2+} , and Zn^{2+} ions. However, it can be activated by adding Fe^{3+} , K^{+} , and Li^{+} ions.

Key words: tryptophan hydroxylase, skipjack, SDS–PAGE, purification, properties

Tryptophan hydroxylase[L-Tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating)] catalyzes the conversion of tryptophan to 5-hydroxytryptophan, which is the initial and rate-limiting step in the biosynthesis of the neurotransmitter serotonin (5-HT).^{1,2)} In addition, serotonin is converted to melatonin. In mammalia, serotonin is generally synthesized in the peripheral tissue and regulates the motor and perception system; sleeping, body temperature, appetite for food, sex behavior, secretion of growth hormone and prolactin, and so on.³⁾ Trp hydroxylase was first discovered in extracts of brain tissue,⁴⁾ and purified from rat brain stem and mouse mastocytoma.^{5,6)} In fish, it was found in liver⁷⁾ but has never been studied in the light of serotonin biosynthesis.

To clarify the mechanism of serotonin metabolism in fish, liver Trp hydroxylase from skipjack was purified and characterized in the present study.

Materials and Methods

Materials

Skipjack *Katsuwonus pelamis* were purchased from a fish market, and immediately transported to the laboratory. The liver was excised, and stored at –80°C until use. Phenol reagent was purchased from Wako Pure Chemicals. D,L-6-Methyl-5,6,7,8-tetrahydropterine and 5-hydroxy-L-tryptophan were obtained from Sigma Chemical Co. Other reagents used were of analytical grade.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli *et al.* using 8% gel.⁸⁾

Estimation of Molecular Weights

The molecular weight of purified Trp hydroxylase was estimated using Sephadex G-150 (2.6 × 90 cm; eluted by 10 mM sodium phosphate buffer, pH 7.0) gel filtration. Ferritin (450,000), catalase (240,000), aldolase

(158,000), and albumin (68,000) were used as standards.

Determination of Protein Concentration

Protein concentration was determined by the method of Lowry *et al.* as modified by Peterson using bovine serum albumin as standard.^{9,10)}

Assay of Enzyme Activity

The activity of Trp hydroxylase was assayed fluorometrically by measuring the formation of 5-hydroxy-L-tryptophan by the method of Friedman *et al.*¹¹⁾ The assay mixture contained 0.05 ml of 1.0 mM ferrous ammonium sulfate, 0.57 ml of 1.0 M HEPES buffer (pH 7.6), 0.025 ml of 0.2 M 2-mercaptoethanol (2-ME), 0.025 ml of 20 mg/ml catalase, 0.05 ml of 10 mM L-tryptophan, 0.05 ml of 5 mM D,L-6-methyl-5,6,7,8-tetrahydropterine in 10 mM HCl, 0.38 ml of H_2O , and 0.1 ml of the enzyme preparation. The reaction was started by the addition of D,L-6-methyl-5,6,7,8-tetrahydropterine. The reaction mixture was incubated at 35°C for 30 min with mild shaking. The reaction was stopped by the addition of 0.125 ml of 40% perchloric acid. After the precipitated protein was removed by centrifugation, a 1.0 ml aliquot of the supernatant was added to 1.5 ml of 5 N HCl. The fluorescence of the solution was measured with the excitation wavelength set at 295 nm and the emission at 530 nm. One unit of enzyme activity was defined as the activity which produces 1 nmol of 5-hydroxy-L-tryptophan per min.

Results and Discussion

Purification of Tryptophan Hydroxylase

Liver samples were weighed and homogenized with 3 volumes of 50 mM Tris–acetate buffer (pH 7.6) containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and 4 mM 2-ME in a Potter–Elvehjem homogenizer with a motor-driven Teflon pestle. The homogenate was then centrifuged at $100,000 \times g$ for 30 min. The pH of the supernatant was brought to 4.8 by adding 50% acetic acid. After gentle stirring for 10 min, the solution was centrifuged at $12,000 \times g$ for 10 min. The pellet was dissolved in a minimal volume of 30 mM Tris–HCl buffer (pH 8.5). The enzyme solution was treated with solid ammonium sulfate and the precipitate

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formed between 30–60% saturation was collected. The precipitate was dissolved in a minimal volume of 50 mM Tris-acetate (pH 7.6) containing 2 mM PMSF and 4 mM 2-ME and dialyzed overnight against 10 mM sodium phosphate buffer (pH 7.0) containing 10% glycerol and 50 μ M EDTA.

Sephadex G-150 Column Chromatography

The dialyzed enzyme was applied to a Sephadex G-150 column (2.6 $\times$ 90 cm) and the dialysate was eluted with 10 mM sodium phosphate buffer (pH 7.0) containing 10% glycerol and 50 μ M EDTA at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected.

DEAE-Sephacrose CL-6B Column Chromatography

Fractions containing Trp hydroxylase activity were applied to a DEAE-Sephacrose CL-6B column (1.0 $\times$ 5.0 cm) which was pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 10% glycerol and 50 μ M EDTA. The column was washed with the same buffer and the enzyme was eluted with a linear gradient of 0–1.0 M NaCl in the same buffer at a flow rate of 1.0 ml/min. Fractions of 5 ml were collected. After the sample was dialyzed against the same buffer, the enzyme solution was applied to a DEAE-Sephacrose CL-6B (1.0 $\times$ 5.0 cm) which was previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 10% glycerol and 50 μ M EDTA. The column was washed with the same buffer and the enzyme was eluted with a linear gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 1.0 ml/min. Fractions of 5 ml were collected (Fig. 1).

Butyl-Sepharose 4B Column Chromatography

The sample collected from DEAE-Sephacrose CL-6B column chromatography was loaded onto a Butyl-Sepharose 4B column (1.0 $\times$ 4.0 cm) previously equilibrated with 10 mM sodium phosphate (pH 7.0) containing 1.3 M ammonium sulfate, 10% glycerol and 50 μ M EDTA. The column was washed with the same buffer, and the enzyme was eluted with a linear gradient of 1.3–0 M ammonium sulfate in the same buffer at a flow rate of 1.0 ml/min. Fractions of 3 ml were collected (Fig. 1).

Toyopearl HW-55F Column Chromatography

After the sample was dialyzed against the same buffer, the enzyme solution was concentrated by ultrafiltration. The enzyme was further purified by applying it to a Toyopearl HW-55F column (1.9 $\times$ 90 cm) which was pre-equilibrated with 10 mM sodium phosphate (pH 7.0) containing 10%

glycerol and 50 μ M EDTA. The enzyme was eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 2.5 ml were collected (Fig. 1). A 1463-fold purification was achieved at this step. The purification of Trp hydroxylase

Fig. 1. Purification of tryptophan hydroxylase.

(A): 2nd DEAE-Sephacrose CL-6B column chromatography. The enzyme solution (49.4 mg of protein) was put on a column (1.0 $\times$ 5.0 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 10% glycerol and 50 μ M EDTA, and the enzyme was eluted with a linear gradient of 0–500 mM NaCl in the same buffer. Fraction volume, 5 ml. (B): Butyl-Sepharose 4B column chromatography of the DEAE-Sephacrose CL-6B fraction. The enzyme solution (5.1 mg of protein) was put on a column (1.0 $\times$ 4.0 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 1.3 M ammonium sulfate, 10% glycerol and 50 μ M EDTA, and the enzyme was eluted with a linear gradient of 1.3–0 M ammonium sulfate in the same buffer. Fraction volume, 3 ml. (C): Gel-filtration of the Butyl-Sepharose 4B fraction by a Toyopearl HW-55F column. The enzyme solution (1.4 mg of protein) was put on a column (1.9 $\times$ 90 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 10% glycerol and 50 μ M EDTA, and the enzyme was eluted with the same buffer. Fraction volume, 2.5 ml. (---), Absorbance at 280 nm; (●), enzyme activity; (—), NaCl concentration; (----), ammonium sulfate concentration. Enzyme activity was measured by the method described in "Materials and Methods".

Table 1. Summary of the purification of skipjack liver tryptophan hydroxylase

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	13,802	202	0.0146	100	1
Acid treatment	3,025	50.1	0.0166	25	1.1
30–60% (NH ₄) ₂ SO ₄	935	58.7	0.0628	29	4.3
Sephadex G-150	261	20.1	0.0770	10	5.3
DEAE-Sephacrose CL-6B (1st)	49.4	11.5	0.233	5.7	16.0
DEAE-Sephacrose CL-6B (2nd)	5.1	11.4	2.235	5.6	153
Butyl-Sepharose 4B	1.4	8.9	6.357	4.4	435
Toyopearl HW-55F	0.59	12.6	21.4	6.2	1,463

is summarized in Table 1.

Estimation of Molecular Weight

As shown in Fig. 2, the molecular weight of the purified enzyme was estimated to be about 288,000 by gel filtration on Sephadex G-150. By SDS-PAGE, the purified enzyme appeared as a single protein band of molecular weight of about 97,000 in the presence or absence of a reducing reagent (Fig. 3). This value for skipjack liver Trp hydroxylase is similar to that for the enzymes from rat brain stem⁵⁾ and mouse mastocytoma P815.⁶⁾ For comparison, the purified rabbit hindbrain enzyme is a tetramer composed of two subunits (MW 57,500 and MW 60,000).¹²⁾ This value for skipjack liver Trp hydroxylase is different from that for the

Fig. 2. Estimation of molecular weight of skipjack tryptophan hydroxylase by gel filtration on Sephadex G-150 column (2.6 × 90 cm). The enzyme was eluted with 10 mM sodium phosphate buffer (pH 7.0) containing 10% glycerol and 50 μ M EDTA. The standard proteins used are indicated.

Fig. 3. SDS-PAGE analysis of the final preparation of skipjack liver tryptophan hydroxylase.

About 15 μ g of the purified enzyme was mixed with 20 μ l of 125 mM Tris-HCl buffer, pH 6.8, containing 4% SDS and 20% glycerol, and the mixture was heated at 100°C for 5 min in the presence of 10% 2-mercaptoethanol (A) or absence (B). The samples were applied to SDS-PAGE of 8% gel, and electrophoresis was done by the method of Laemmli *et al.*

enzyme from rabbit hindbrain.

Effect of pH

The stability of purified skipjack liver Trp hydroxylase was investigated by using a buffer of various pH values. It is clear that the enzyme retained more than 80% of its original activity between pH 7.5 and 8.5 after incubation at 35°C for 30 min, but was unstable below pH 6.0 or above pH 9.5 (Fig. 4).

The activity of the purified enzyme was determined for various pH conditions. The optimum pH of this enzyme was around 8.0, which is similar to that for rat brain stem and mouse mastocytoma enzymes.^{5,6)}

Effect of Temperature

The purified skipjack liver Trp hydroxylase in 0.456 M HEPES buffer (pH 7.6) was heated at 35, 40, 50, 60, and 70°C for 10, 20, 30, and 60 min. After cooling, the residual catalytic activity was assayed (Fig. 4). It is clear that the enzyme was unstable beyond 35°C, but was stable when stored at -80°C, and lost almost none of its activity within 2 days at 4°C. By comparison, the purified mastocytoma Trp hydroxylase lost almost all its activity within 20 h at 4°C without stabilizing agents but became stable with the addition of a nonionic detergent, EDTA and ethylene

Fig. 4. Effect of pH and temperature on skipjack liver tryptophan hydroxylase activity.

(A): The enzyme activity was measured over a pH range from 4.0–12.0 in 20 mM Britton–Robinson buffer. (B): The enzyme was incubated in 20 mM Britton–Robinson buffer of various pH values at 4°C for 60 min, then the pH was adjusted to the optimum, and the residual activity was assayed under the standard conditions. (C): The enzyme activity was assayed under the standard conditions except that the temperature was varied as indicated. (D): The enzyme was incubated for 30 min at various temperatures as indicated. After incubation, the tubes were placed in ice and then assayed for residual activity under the standard conditions.

Fig. 5. Lineweaver-Burk plot of skipjack liver tryptophan hydroxylase activity for L-tryptophan.
(●), non PCPA; (O), in PCPA.

Table 2. Effect of various reagents on the activity of tryptophan hydroxylase from skipjack liver

Reagents (1 mM)	Relative activity (%)
None	100
KCl	101
NaCl	97
LiCl	104
HgCl ₂	59
MgCl ₂	97
BaCl ₂	90
ZnSO ₄	2
MnCl ₂	0
CuSO ₄	49
CaCl ₂	17
CoCl ₂	0
FeCl ₃	105
CH ₂ ICOOH	116
PCMB* ¹	118
DTNB* ²	95
Glutathione	88

*¹: PCMB: *p*-chloromercuribenzoic acid.

*²: DTNB: 5,5'-dithiobis(2-nitrobenzoic acid).

glycol.⁶⁾ The optimum temperature of this enzyme was 35°C (Fig. 4).

K_m for L-Tryptophan

From the Lineweaver-Burk plot of the enzyme, the *K_m* value for L-tryptophan was estimated to be 7.9×10^{-5} M (Fig. 5). This value agrees with that obtained for rat brain stem and mouse mastocytoma.^{5,6)}

K_i for D,L-*p*-Chlorophenylalanine (PCPA)

D,L-*p*-Chlorophenylalanine is a competitive inhibitor and

the *K_i* for this was computed to be 6.7×10^{-5} M from the Lineweaver-Burk plot of the enzyme (Fig. 5). This value is similar to that of mouse mastocytoma enzyme, 2.6×10^{-5} M,⁶⁾ and pineal enzyme, 2.0×10^{-5} M, but different from that of the brain enzyme, 30.0×10^{-5} M.

Effect of Metal Ions and SH-Blocking Reagents

The effects of various metal ions (1 mM) on enzyme activity are shown in Table 2. The enzyme was strongly inhibited by Co²⁺, Mn²⁺, and Zn²⁺, but was slightly activated by Fe³⁺, K⁺, and Li⁺. The activity of the enzyme was not affected by the SH-blocking reagents. These results suggest that the skipjack liver enzyme was not SH-enzyme. It has been reported that the activity of the mouse mastocytoma enzyme increased approximately five-fold by preincubation with dithiothreitol and Fe²⁺,⁶⁾ but the skipjack liver enzyme did not increase by preincubation with dithiothreitol.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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