Purification and Some Properties of Folate-hydrolyzing Enzyme from *Crithidia fasciculata*[†]

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The folate-hydrolyzing enzyme was purified 49-fold from the crude extract of *Crithidia fasciculata* ATCC 12857 by heat treatment, column chromatographies on DEAE-cellulose and Sephadex G-200, and preparative polyacrylamide gel electrophoresis. The final preparation was electrophoretically homogeneous. The enzyme had a molecular weight of 200,000 daltons and consisted of 4 identical subunits of which the molecular weight was about 51,000 daltons. The enzyme hydrolyzed aminopterin, methotrexate, and pABG more effectively than folate. The enzyme hydrolyzed the reduced folates, dihydrofolate and 10-formyltetrahydrofolate, more weakly than folate. The enzyme did not act on pteroyl- γ,γ -diglutamylglutamate. The optimum pH for the reaction with each substrate described above was 7.0. *K_m* values for folate, methotrexate, aminopterin, and pABG were 0.13, 0.46, 0.40, and 0.43 mM, respectively. The enzyme activity was inhibited by 2-mercaptoethanol, pCMB, chelating reagents such as α,α',α'' -tripyridyl and bathophenanthroline, divalent cations such as Hg^{2+} , Cu^{2+} , Cd^{2+} , Pb^{2+} , and Zn^{2+} , and by pyrophosphate and orthophosphate.

We had developed a sensitive method for analyzing the folate-hydrolyzing enzyme (FH enzyme) which catalyzed the hydrolysis of folic acid to pteric acid and glutamic acid.¹⁾ We had also found the enzyme activity in cell-free extracts of *Crithidia fasciculata* ATCC 12857 and other cells and tissues.^{1,2)} Similar enzyme activities have been found inductively in soil bacteria such as *Alcaligenes faecalis*,³⁾ *Pseudomonas* sp.,^{4,5)} and *Flavobacterium* sp.^{6,7)} which can grow on folate compounds as the sole source of carbon and nitrogen. But *Crithidia* was constitutive the FH enzyme activity. In this paper, we purified the FH enzyme from *C. fasciculata* and compared the properties of the enzyme with those of the inductive bacterial enzymes.

MATERIALS AND METHODS

Materials. The following chemicals were obtained from the specified manufacturers: [$2\text{-}^{14}\text{C}$]Folic acid potassium salts (58.2 mCi/mmol) from the Radiochemical Centre (Amersham); bovine pancreas α -chymotrypsinogen A, pepsin, ovalbumin, bovine serum albumin, aminopterin, and DFP from the Sigma Chemical Co.; methotrexate from Lederle Ltd.; bovine serum γ -globulins from Mann Research Lab.; human serum γ -globulin from the Nutritional Biochemical Co.; 5-methyl, 5-formyl, and 10-formyltetrahydrofolates from the Eisai Chemicals Co.; 8-hydroxyquinoline, β -mercaptopropionic acid, and α,α' -dipyridyl from Wako Pure Chemical Industries Ltd.; Sephadex G-200 from Pharmacia Fine Chemicals; and Avicel SF cellulose plates (20 $\times$ 20 cm) from the Funakoshi Pharmaceutical Co. Bathophenanthroline disulfonic acid disodium salt, bathocuproine disulfonic acid disodium salt, *o*-phenanthroline, α,α',α'' -tripyridyl, pABG, and other chemicals were obtained from Nakarai

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Abbreviations: pABG, *p*-aminobenzoylglutamic acid; aminopterin, 4-amino-4-deoxypteroylglutamic acid; pCMB, *p*-chloromercuribenzoic acid; DFP, diisopropylfluorophosphate; methotrexate, 4-amino-4-deoxy-10-methylpteroylglutamic acid; PMSF, phenylmethylsulfonylfluoride.

Chemicals Ltd., Kyoto. Tryptophanase from *Proteus rettgeri* was a gift from Dr. H. Yamada, Department of Agricultural Chemistry, Kyoto University. Pepstatin A and PMSF were kind gifts from Dr. R. Hayashi, Research Institute for Food Science, Kyoto University.

Methods.

Growth conditions for *Crithidia fasciculata*. *C. fasciculata* ATCC 12857 was statically cultivated at 25°C in 200 ml Erlenmeyer flasks containing 50 ml of the culture medium described by Guttman.⁸⁾ The cell growth was measured by an increase of turbidity at 675 nm using a Coleman Model 6-20 Spectrophotometer. The cells were harvested at a defined interval of growth by centrifugation at $3,000 \times g$ for 10 min and washed twice a cold 0.9% (w/v) saline solution. The cells were suspended in 2 to 5 ml of 0.1 M Tris-HCl buffer, pH 7.1, and disrupted for 2 min on ice with a Kaijo-Denki 20 kHz Ultrasonic Oscillator. The suspension was centrifuged at $12,000 \times g$ for 30 min. The resulting supernatant solution was used for assaying the FH enzyme activity.

For a large-scale preparation of cells, *C. fasciculata* was cultivated in 120 liters of the culture medium at 25°C for 60 hr by stirring at 300 rpm and by aerating at a flow rate of one liter/min. The cells were harvested by continuous centrifugation at $10,000 \times g$ with a High Speed Centrifuge CM-60RN, (Tomy Seiki Co., Ltd.), and washed with the saline solution. The cells were stored at -20°C until use.

Standard assay conditions for the folate-hydrolyzing enzyme (FH enzyme) activity. The FH enzyme activity was measured by the radioassay described previously.¹⁾ The reaction mixture consisted of 0.1 M Tris-HCl buffer, pH 7.1, 2 mM [2-¹⁴C]folate (50 μ Ci) and the enzyme solution in a final volume of 0.1 ml. The mixture was incubated at 37°C for 1 hr. When non-radioactive compounds and pABG were used as the substrate, the FH enzyme activity was measured by a colorimetric method using a ninhydrin reagent described previously.¹⁾ The enzyme activity was defined by the amounts of pteroate formed per hr or by changes in absorbance at 570 nm per hr under standard assay conditions.

Purification of the folate-hydrolyzing enzyme.

a) Preparation of crude extract. All procedures were carried out at 0~4°C. Five hundred grams of *Crithidia* cells (wet weight) were suspended in 2.5 liters of 0.1 M Tris-HCl buffer, pH 7.1, containing 0.1 mM CoCl₂, and homogenized with glass beads (0.25~0.5 mm in diameter) using a Dyno-Mill KDL, (the Shinmura Enterprise Co.) The homogenate was centrifuged at $12,000 \times g$ for 20 min.

b) Heat treatment. The resulting supernatant solution (2.3 liters) was heated at 60°C for 5 min and centrifuged at $12,000 \times g$ for 20 min. The resulting supernatant solution (2.25 liters) was diluted with 2.25 liters of water. The recovery of the enzyme activity was almost 100%.

c) DEAE-cellulose column chromatography. The diluted solution was put on a DEAE-cellulose column (5.7 $\times$

32 cm) which had been previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.1, containing 0.1 mM CoCl₂ (hereafter this buffer will be referred to as the Buffer). The enzyme was eluted by a linear Tris gradient with 1.8 liters of the Buffer in the mixing chamber and 1.8 liters of 0.6 M Tris-HCl buffer, pH 7.1, containing 0.1 mM CoCl₂, in the reservoir. Fractions of 17 ml were collected. Tubes from #100 to #130 were pooled and the solution was dialyzed against 10 liters of the Buffer. The dialyzed solution (560 ml) was put on a DEAE-cellulose column (2.5 $\times$ 26 cm) which had been equilibrated with the Buffer. The column was eluted by a linear Tris gradient with 600 ml of the Buffer in the mixing chamber and 600 ml of 0.5 M Tris-HCl buffer, pH 7.1, containing 0.1 mM CoCl₂ in the reservoir. Fractions of 7 ml were collected. Tubes from #70 to #90 were pooled, and the solution was concentrated with a collodion bag (Ultrapur UH 100) and dialyzed against 0.1 M Tris-HCl buffer, pH 7.1, containing 0.1 mM CoCl₂. The recovery of the enzyme activity was about 14%.

d) Sephadex G-200 column chromatography. The dialyzed solution (ca. 2 ml) was subjected to chromatography on a Sephadex G-200 column (1.5 $\times$ 90 cm) which had been equilibrated with 0.1 M Tris-HCl buffer, pH 7.1, containing 0.1 mM CoCl₂. Fractions of 1.8 ml were collected. Tubes from #28 to #38 were pooled and the solution was concentrated to a small volume with a collodion bag. The recovery of the enzyme activity was 95%.

e) Preparative polyacrylamide gel electrophoresis. An aliquot (0.2 ml) of the concentrated solution was put on a 7.5% polyacrylamide gel column (2 $\times$ 5 cm) which was prepared by the method of Williams and Reisfeld.⁹⁾ Preparative gel electrophoresis was performed at a current of 8 mA at 4°C for 10 hr with a Fuji-Riken Furicoverer II. Overrunning protein fractions were continuously washed out from the gel into the running elution buffer of 10 mM Tris-HCl, pH 7.1, at a flow rate of 0.4 ml/min. Fractions of 1.86 ml were collected. Tubes from #27 to #30 were pooled and the solution was concentrated with a collodion bag. The recovery of the enzyme activity was 16%.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out at pH 8.9 in a 7.5% gel for 90 min with a current of 2 mA per tube by the method of Davis.¹⁰⁾ Polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulfate (SDS) was carried out in a 10% gel for 5 hr with a current of 8 mA per tube by the method of Weber and Osborn.¹¹⁾ Protein was stained with Coomassie brilliant blue G-250.

Estimation of the molecular weight. The molecular weight of the enzyme was estimated by gel filtration on a Sephadex G-200 column (1.5 $\times$ 90 cm) which had been equilibrated with 0.1 M Tris-HCl buffer, pH 7.1, containing 0.1 mM CoCl₂. Tryptophanase (220,000 daltons), bovine γ -globulin (160,000 daltons), human γ -globulin

(153,000 daltons), bovine serum albumin (63,000 daltons) and ovalbumin (43,000 daltons) were used as molecular weight markers.

Determination of protein. Protein was determined by the method of Lowry *et al.*¹²⁾ using bovine serum albumin as a standard and by absorbance at 280 nm using a Hitachi 124 Spectrophotometer.

RESULTS

Course of folate-hydrolyzing enzyme formation

The formation of the FH enzyme activity during growth is shown in Fig. 1. The maximum formation was observed at 80 hr after cultivation. After that time, total enzyme activity decreased rapidly. The cells cultivated until their concentration reached $T=50\sim60\%$ were used for the purification of the enzyme.

Purification of folate-hydrolyzing enzyme (FH enzyme)

The elution profile of FH enzyme activity on preparative polyacrylamide gel electrophoresis is shown in Fig. 2. Table I summarizes the purification procedure for the FH enzyme. The enzyme was purified 49-fold from the crude extract with a yield of 2.2%. The final preparation was electrophoretically homogeneous (Fig. 3). The specific activity was 1985 nmol of

FIG. 1. Course of Folate-hydrolyzing Enzyme Formation and Growth of *Crithidia fasciculata*.

C. fasciculata was statically cultivated at 25°C in a 200 ml Erlenmeyer flask containing 50 ml of the culture medium. The enzyme activity in the cells obtained at defined intervals of growth was assayed under standard assay conditions described in the text. The activity is shown as amounts of pterate formed per cells at 37°C for 1 hr (O---O). The growth is shown as transmittance ($T\%$) at 675 nm (●—●).

FIG. 2. Preparative Polyacrylamide Gel Electrophoresis of the Folate-hydrolyzing Enzyme.

The enzyme fraction (0.2 ml) obtained from the chromatography on Sephadex G-200 column was put on a 7.5% polyacrylamide gel column. The protein was eluted as described in METHODS. (●—●), absorbance at 280 nm; (O---O), total activity per tube.

TABLE I. SUMMARY OF PURIFICATION OF THE FOLATE-HYDROLYZING ENZYME FROM *Crithidia fasciculata*

Purification step	Protein (mg)	Activity* (nmol/hr)	Specific activity* (nmol/hr/mg)	Yield (%)
Crude extract	47,190	1,920,700	40.70	100.0
Heat treatment	7,750	1,919,900	247.7	100.0
1st DEAE-cellulose	2,966	870,070	293.3	45.3
2nd DEAE-cellulose	709	273,500	385.8	14.2
Sephadex G-200	270	260,540	965.0	13.6
Electrophoresis	21.3	42,281	1985	2.2

* The enzyme activity was estimated as amounts of pteroate formed under the standard assay conditions.

FIG. 3. Polyacrylamide Gel Electrophoresis of the Purified Folate-hydrolyzing Enzyme.

Electrophoresis was performed using a 7.5% gel as described in METHODS. The protein was stained with Coomassie brilliant blue G-250.

pteroate formed per mg of protein per hr at 37°C.

Molecular weight and subunit structure

The molecular weight of the enzyme was estimated to be 200,000 daltons by gel filtration on a Sephadex G-200 column. The SDS-treated enzyme showed a single polypeptide band whose molecular weight was 51,000 daltons. These results indicate that the enzyme consists of 4 subunits with the same molecular size.

Effect of pH on the enzyme activity and stability

The pH-activity profile of the enzyme is shown in Fig. 4A. The optimum pH was 7.0.

FIG. 4. Effects of pH on the Folate-hydrolyzing Enzyme Activity (A) and pH Stability of the Enzyme (B).

(A): The enzyme (14 μ g of protein) was incubated with each 0.1 M buffer under standard assay conditions. The buffers used were: $\circ$ — $\circ$, sodium citrate; $\bullet$ — $\bullet$, Tris-maleate; $\oplus$ — $\oplus$, potassium phosphate; $\square$ — $\square$, Tris-HCl. (B): The enzyme was preincubated with the buffer described at (A) at 37°C for 2 hr, and the residual activity was measured under standard assay conditions.

At pH 6.8, the enzyme activity was the lowest in potassium phosphate buffer. The enzyme was stable below pH 8, but unstable over pH 8.5 (Fig. 4B).

Effect of temperature on the enzyme activity and stability

As shown in Fig. 5A, the optimum temperature for the reaction was 50°C. The enzyme rapidly lost its activity by heat treatment over 60°C. The enzyme was stable at pH 7.0 below 50°C as shown in Fig. 5B. Unlike the crude enzyme, the purified enzyme lost 9 and 19% of

FIG. 5. Effects of Temperature on the Folate-hydrolyzing Enzyme Activity (A) and on Stability of the Enzyme (B).

(A): The enzyme (8 μ g of protein) was incubated at the temperature indicated, and the enzyme activity was measured under standard assay conditions.

(B): After the enzyme incubated at a defined temperature for 10 min, the remaining activity was measured under standard assay conditions.

the activity by heat treatment at 60°C for 5 and 10 min, respectively.

Substrate specificity

As shown in Table II, the purified FH enzyme hydrolyzed various folate compounds. Methotrexate and aminopterin were more effectively used as substrate than folate. But the reduced forms of folate compounds such as dihydrofolate, 10-formyl, 5-formyl and 5-methyltetrahydrofolates were less effectively used than folate. The pABG was used actively as folate. The enzyme did not have any conjugase activity, which catalyzes the hydrolysis of the γ -linkage of pteroyl- γ , γ -diglutamyl-glutamate (teropterin). K_m values for folate, methotrexate, aminopterin, and pABG were calculated to be 0.13, 0.46, 0.40 and 0.43 mM, respectively, from double-reciprocal plots of reaction rate *versus* substrate concentration.

Reaction products

The reaction products formed from folate were identified as pteroate and L-glutamate by the same methods as described previously.¹⁾ The enzyme stoichiometrically hydrolyzed folate to pteroate and L-glutamate. Thus, one mol of the enzyme hydrolyzed 397 mol of

TABLE II. SUBSTRATE SPECIFICITY OF FOLATE-HYDROLYZING ENZYME FROM *C. fasciculata*

The enzyme (8.2 μ g of protein) was incubated with each substrate (2 mM) under standard assay conditions. The enzyme activity was assayed by the colorimetric method using a ninhydrin reagent described previously.¹⁾

Substrate	Enzyme activity* ($\Delta A_{570\text{nm}}/\text{hr}$)	Relative activity
Folate	0.103	1.00
Aminopterin	0.309	3.00
Methotrexate	0.287	2.79
Dihydrofolate	0.047	0.46
10-Formyltetrahydrofolate	0.057	0.55
5-Formyltetrahydrofolate	0.035	0.34
5-Methyltetrahydrofolate	0.023	0.22
Pteroyl- γ , γ -diglutamyl-glutamic acid (teropterin)	0	—
p-Aminobenzoylglutamic acid	0.123	1.19

* The enzyme activity was defined as a change in absorbance at 570 nm per hr.

folate to equal mol of pteroate and L-glutamate under our standard assay conditions. The reaction products from methotrexate or aminopterin were also identified as 4-amino-4-deoxy-10-methylpteroate or 4-amino-4-deoxypteroate and L-glutamate, respectively, from the similar results of their behavior on thin-layer chromatography and their ultra-

TABLE III. EFFECT OF VARIOUS INORGANIC AND ORGANIC SALTS ON THE FOLATE-HYDROLYZING ENZYME ACTIVITY

The enzyme (8 μ g of protein) was preincubated at 37°C for 10 min with each salt in 0.1 M Tris-HCl buffer, pH 7.1, and incubated with 2 mM [2-¹⁴C]folate (50 μ Ci) under standard assay conditions.

Addition	Concentration (mM)	Relative activity (%)
None	—	100.0
NiCl ₂	0.1	92.8
CuCl ₂	0.1	10.4
ZnCl ₂	0.1	40.2
SrCl ₂	0.1	92.7
PbCl ₂	0.1	30.2
CdCl ₂	0.1	24.0
HgCl ₂	0.1	0.4
CrCl ₃	0.1	70.1
YbCl ₃	0.1	88.7
NaNO ₃	20	88.3
Na ₂ SO ₄	20	89.3
Na ₂ CO ₃	20	95.4
Na ₂ B ₄ O ₇	20	67.5
Na ₃ PO ₄	20	73.5
Na ₄ P ₂ O ₇	20	10.1
Na-Acetate	20	88.3
Na ₂ -Maleate	20	89.2
Na ₂ -Malate	20	86.4
Na ₂ -Tartarate	20	95.4
Na ₂ -Glutamate	20	90.1
Na ₂ -Aspartate	20	88.8
Na ₃ -Citrate	20	78.9

violet and infrared absorption spectra⁴⁾ (data not shown).

Effects of metal ions and salts

As shown in Table III, Hg²⁺, Cu²⁺, Cd²⁺, Pb²⁺ and Zn²⁺ strongly inhibited the enzyme activity in this order. Cr³⁺ and Yb³⁺ were also inhibitory. But the following salts did not affect the enzyme activity: MgCl₂, CaCl₂, SnCl₂, BaCl₂, MnCl₂, FeCl₂, FeCl₃ and AlCl₃ at the concentration of 0.1 mM, and LiCl, NaCl and KCl at the concentration of 0.1 M. Inorganic and organic sodium salts such as pyrophosphate, borate, orthophosphate and citrate at the concentration of 20 mM also inhibited the enzyme activity (Table III).

TABLE IV. EFFECT OF CHELATING REAGENTS ON THE FOLATE-HYDROLYZING ENZYME ACTIVITY

The enzyme (8 μ g of protein) was preincubated at 37°C for 10 min with 0.1 mM of each reagent in 0.1 M Tris-HCl buffer, pH 7.1, and incubated under standard assay conditions.

Chelating reagent	Relative activity (%)
None	100.0
α,α',α'' -Tripyridyl	19.0
<i>o</i> -Phenanthroline	52.7
Bathophenanthroline disulfonate	45.8
Bathocuproine disulfonate	50.3

Effect of chemical reagents

The enzyme activity was inhibited by chelating reagents such as α,α',α'' -tripyridyl, bathophenanthroline disulfonate, bathocuproine disulfonate, and *o*-phenanthroline (Table IV). EDTA, rubeanic acid, α,α' -dipyridyl, 8-hydroxyquinoline, and β -mercaptopropionic acid at the concentration of 0.1 mM inhibited by 15, 11, 7, 2, and 1% of the enzyme activity, respectively. When the enzyme was preincubated with 0.8 mM bathophenanthroline disulfonate at 37°C for 30 min and dialyzed against 5 mM Tris-HCl buffer, pH 7.0, for 6 hr, the enzyme lost 81% of its activity. However, the enzyme activity recovered to 44% upon the addition of 0.1 mM CoCl₂. The addition of other metal ions did not restore the enzyme activity.

pCMB at the concentration of 0.02 mM inhibited by 50% of the enzyme activity. 2-Mercaptoethanol at 10 mM also inhibited by 45% of the activity. However, 0.2 mM PMSF, 1 mM DFP or 15.2 μ M pepstatin A did not affect the enzyme activity.

DISCUSSION

The present study was undertaken to characterize the folate-hydrolyzing enzyme which was purified homogeneously from the crude extract of *C. fasciculata* ATCC 12857. The enzyme hydrolyzes various folate compounds at a neutral pH (Table II and Fig. 4A) as do

soil bacterial peptidases such as carboxypeptidase (CPase) G⁴⁾ and G₁⁵⁾ of *Pseudomonas*, and folate amidase⁶⁾ and CPase⁷⁾ of *Flavobacteria*. The enzyme activity of *C. fasciculata* depends on the structure of the pteridine moiety of folate compounds. The reduced forms were hydrolyzed only 0.2 to 0.5-fold of folate, and diaminopteridine derivatives such as aminopterin and methotrexate were more effectively hydrolyzed than folate. But the *K_m* values for folate and methotrexate of the *Crithidia* enzyme were 2 to 130 times higher than those of the three bacterial CPases.^{4,5,7)} Like the *Pseudomonas* CPase G₁⁵⁾ and the *Flavobacterium* CPase,⁷⁾ the *Crithidia* enzyme also hydrolyzed pABG, but did not hydrolyze the γ -glutamyl linkage of pteroyl- γ , γ -diglutamylglutamate. This indicates that the *Crithidia* enzyme may have a specificity for splitting the α -glutamyl linkage of folate compounds and pABG.

The two *Pseudomonas* CPases require Zn²⁺ for their activities.^{4,13)} The *Crithidia* enzyme activity was inhibited by various chelating reagents (Table IV), heavy metal ions, and organic and inorganic anions (Table III). But the activity of the bathopehnanthroline-treatment enzyme was partially restored by adding CoCl₂. These evidences suggest that the *Crithidia* enzyme may be a kind of metallo-protein and require Co²⁺ for its activity.

Like the two *Flavobacterium* enzymes, folate amidase⁶⁾ and CPase,⁷⁾ the *Crithidia* enzyme was inhibited by 2-mercaptoethanol. Although folate amidase is only sensitive to phosphate buffer and bacterial CPase is also to citrate buffer, the *Crithidia* enzyme activity was affected by inorganic and organic salts including pyrophosphate, borate, phosphate and citrate. There is no information about inhibition of bacterial enzymes by pCMB and peptidase inhibitors such as DFP, PMSF and pepstatin A. The *Crithidia* enzyme was sensitive to pCMB.

Activities of proteinases and aminopeptidases have been found in crude homogenates of protozoa, *Trypanosomatidae*, such as *Crithidia*,^{14~17)} *Trypanosoma*^{14,17~21)} and

Leishmania.¹⁴⁾ However, there are no reports on whether these proteases of protozoa may hydrolyze folate compounds. It is now under study whether the purified folate-hydrolyzing enzyme of *C. fasciculata* may have such substrate specificity as carboxypeptidases of soil bacteria have.^{4,13)}

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