Radioassay of the Folate-Hydrolyzing Enzyme Activity, and the Distribution of the Enzyme in Biological Cells and Tissues

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Summary A sensitive radioassay method has been developed to quantitate the activity of the folate-hydrolyzing enzyme which catalyzes the hydrolysis of folic acid to pteroic acid and glutamic acid. The method is based on analyzing [2-14C]pteroic acid separated by a thin-layer chromatography on an Avicel SF cellulose plate using 0.1M potassium phosphate buffer, pH 7.0, as a solvent. This method was found to be more sensitive than a conventional photometric method to determine the activity of the folate-hydrolyzing enzyme. High activities of the enzyme were found in Crithidia fasciculata ATCC 12857, Neurospora crassa IFO 6979 and rat liver. Smaller activities of the enzyme were widely distributed in other microbial cells and mammalian tissues.

Key Words folate-hydrolyzing enzyme, radioassay of pteroic acid, Crithidia fasciculata, Neurospora crassa, rat liver

An enzyme which catalyzes the hydrolysis of folic acid to pteroic acid and L-glutamic acid or of methotrexate to 4-amino-4-deoxy-10-methylpteroic acid (AMPte) and L-glutamic acid has been first isolated from a soil bacteria, Pseudomonas sp. (1) and has been named as carboxypeptidase G (2). Various names of the enzyme which catalyzes a similar reaction have been proposed as follows: carboxypeptidase G1 from Pseudomonas stutzeri (3) and folate amidase (4) or carboxypeptidase (5) from Flavobacterium sp. The activities of these enzymes have been conveniently measured by a photometric method (1–5) based on change in absorbance of folic acid and methotrexate at 310 nm and 320 nm, respectively. Recently, we have found that AMPte is accumulated in the cells of Crithidia.
fasciculata ATCC 12857 grown in the medium supplemented with methotrexate, and also have found that the cell-free extract of this protozoan has the hydrolyzing activities of methotrexate and folate to AMPpt and pteroic acid, respectively (6). However, these activities could not be detected by the photometric method. So, we developed a more sensitive radioassay method for analyzing the enzyme activity using [2-14C]folic acid as a substrate, and analyzed the activity in microorganisms and mammalian tissues. In this paper, we tentatively name the hydrolyzing enzyme as the folate-hydrolyzing enzyme (FH).

MATERIALS AND METHODS

Materials. The following chemicals were obtained from the specified manufacturers: [2-14C]Folic acid potassium salt (58.2 mCi/mmol) from the Radiochemical Centre (Amersham); 2,5-diphenyloxazole (PPO) and 1,4-bis-2(5-phenyloxazoyl)benzene (POPOP) from Packard Instrument Co.; pteric acid from Lederle Ltd.; Avicel SF cellulose and its plate (20 × 20 cm) from Funakoshi Pharmaceutical Co.; Sephadex G-10 from Pharmacia Fine Chemicals; yeast extract from Oriental Yeast Co.; polypepton from Daigo-Eiyo Co.; beef extract from Mikuni Kagakusangyo Ltd.; thiglycollate medium from Nissui Pharmaceutical Co. Other chemicals were purchased from Nakarai Chemicals Ltd., Kyoto.

Yeasts and fungi were gifts from Dr. A. Kimura, Research Institute for Food Science, Kyoto University. Euglena gracilis was a gift from Dr. K. Asada, Research Institute for Food Science, Kyoto University. Azotobactor chroococcum IFO 12393 was a gift from Dr. S. Ida, Research Institute for Food Science, Kyoto University. Flammulina velutipes (enokitake) and Lentinus edodes (shiitake) were obtained from a local market.

Methods

Standard assay conditions for folate-hydrolyzing enzyme (FH). The FH activity was measured under the following conditions. The reaction mixture consists of 0.1 M Tris-HCl buffer, pH 7.1, 1 mM [2-14C]folate (75 nCi) and the enzyme solution in a final volume of 0.1 ml. The mixture was put into a small brown test tube (0.7 × 4.5 cm) with a plug and incubated at 37°C for 1 h. The reaction was terminated by adding 0.1 ml of ethanol. The mixture was centrifuged at 10,000 × g for 10 min. The resulting supernatant (20 μl) was spotted on an Avicel SF cellulose plate (20 × 20 cm). The plate was developed with 0.1 M potassium phosphate buffer, pH 7.0, for 100 min at room temperature. After the plate was well dried in a drying oven at 80°C, a 1-cm region from the origin, on which [2-14C]pteroyl acid localized, was scraped off and put into a counting vial containing 10 ml of a scintillation fluid consisting of 0.1 g of POPOP and 4 g of PPO dissolved in one liter of toluene. The radioactivity was measured with a Packard 2425 Tri-Carb Liquid Scintillation Spectrometer. The FH activity was shown as the amount of pteroic acid formed per h under the standard assay conditions.

Another reaction product from folate, i.e., glutamic acid, was determined by a
colorimetric method described by Moore (7). The reaction mixture was diluted to 0.5 ml with water and mixed with 0.5 ml of 2% ninhydrin solution, pH 5.2, containing 0.0625% hydridantin dissolved in dimethylsulfoxide and 4 M lithium acetate (3:1, v/v). The mixture was heated at 100°C for 20 min. The resulting blue-colored solution was diluted with 2.5 ml of 50% ethanol and the absorbance at 570 nm was measured with a Hitachi 124 Spectrophotometer. The activity of the enzyme which catalyzed the hydrolysis of methotrexate was also measured by the colorimetric determination of the glutamic acid released.

Preparation of the partially purified FH from Crithidia fasciculata. The cells (10 g) of Crithidia fasciculata ATCC 12857 were suspended in 50 ml of 0.1 M Tris-HCl buffer, pH 7.1, and disrupted for 5 min on ice with a Kojio-Denki 20 kHz Ultrasonic Oscillator. The crude extract was centrifuged at 12,000 × g for 30 min, and the resulting supernatant was heated at 60°C for 5 min. After the insoluble materials were removed by centrifugation at 12,000 × g for 10 min, the resulting supernatant was made to 55% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation at 10,000 × g for 10 min and dialyzed overnight against a liter of 0.1 M Tris-HCl buffer, pH 7.1. The specific activity of the final solution was 8-fold higher than that of the crude extract.

Isolation of the reaction products. The crude extract of C. fasciculata (29 mg of protein) was incubated with 1 mM folate in 0.1 M Tris-HCl buffer, pH 7.1, at 37°C for 2 h. The reaction was terminated by adding ethanol. After the mixture was centrifuged at 10,000 × g for 10 min, the reaction products, i.e., pteroic acid and glutamic acid, were isolated from the resulting supernatant as follows: Pteroic acid was isolated by a thin-layer chromatography on an Avicel SF cellulose plate and purified by desalting with a Sephadex G-10 column as described previously (6). The fractions showing an absorbance at 280 nm were collected and lyophilized. For isolating glutamic acid, a half of the resulting supernatant described above was concentrated with an evaporator in vacuo and was applied to an Avicel SF cellulose column (1.1 × 39 cm) which was previously equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. Two milliliter fractions were collected. Fractions from #14 to #17 were pooled and concentrated under vacuum. The residue was suspended in a small amount of 0.2 M sodium citrate-HCl buffer, pH 2.2. After filtering the solution through a Millipore filter (0.45 μm in pore diameter), glutamic acid was analyzed with a Hitachi KLA-5 Automatic Amino Acid Analyzer.

Growth conditions for microorganisms. Lactobacillus casei ATCC 7469, L. arabinosus ATCC 8014, L. fermenti ATCC 9338, Streptococcus faecalis R ATCC 8043, Pediococcus cerevisiae ATCC 8081 and Pseudomonas riboflavina IFO 3140 were grown at 37°C in the medium described by Iwai et al. (8). Bacillus cereus IFO 3131, Serratia indica IFO 3759, Serratia marcescens IFO 3048 and Escherichia coli B were grown at 30°C by vigorously shaking in the medium described by Iwai et al. (9). Saccharomyces cerevisiae FKU 1451, Candida utilis IFO 0396 and Hansenula jadinii IFO 0987 were grown at 30°C in the medium, pH 6.5, containing 1% yeast extract, 2% polypeptone and 2% glucose. Aspergillus niger M-62, Penicillium

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**RESULTS AND DISCUSSION**

Reaction products of folate-hydrolyzing enzyme

UV spectra of the reaction product were in good agreement with those of an authentic pteroic acid as shown in Fig. 1. The ninhydrin-positive product was identified as glutamic acid by a thin-layer chromatography as shown in Table 1.

![Fig. 1. Ultraviolet absorption spectra of the reaction product from folic acid in 0.1 M NaOH (-- -- --), 0.1 M HCl (-----) and 0.1 M potassium phosphate buffer, pH 7.0 (----). Spectra were measured with a Hitachi 124 Spectrophotometer.](image)
Table 1. $R_f$ values on thin-layer chromatography of the amino acid product.

Folic acid (1 mM) was incubated at 37°C for 2 h with the crude extract of C. fasciculata (29 mg of protein) in 0.1 M Tris-HCl buffer, pH 7.1. The amino acid product was isolated by chromatography on an Avicel cellulose column (see the text in details). The product and authentic L-glutamic acid were spotted on an Avicel SF cellulose plate (20 x 20 cm) and developed by various solvent systems.

| Solvent system & | Product | L-Glutamic acid |
|------------------|---------|-----------------|
| a                | 0.02    | 0.05            |
| b                | 0.40    | 0.40            |
| c                | 0.22    | 0.24            |
| d                | 0.28    | 0.28            |
| e                | 0.31    | 0.30            |
| f                | 0.22    | 0.22            |
| g                | 0.12    | 0.11            |
| h                | 0.95    | 0.94            |

* a, ethanol–28% ammonium hydroxide–water, 18:1:1 (by volume); b, methanol–pyridine–water, 20:1:5; c, n-butanol–pyridine–water, 1:1:1; d, n-butanol–acetic acid–water, 12:3:5; e, phenol solution (160 g in 40 ml of water); f, phenol solution–28% ammonium hydroxide, 200:1; g, phenol solution–ethanol–28% ammonium hydroxide–water, 150:40:1:10; h, 0.1 M potassium phosphate buffer, pH 7.0.

The product was also confirmed as glutamic acid by its elution time on an amino acid analyzer (data not shown).

**Stoichiometry of the FH reaction**

The stoichiometry for FH reaction was shown in Table 2. The amount of $[^{14}C]$pteroic acid determined by the radioassay was similar to that of glutamic acid determined by the amino acid analyzer, although a slightly higher amount of glutamic acid was estimated by the colorimetric assay. These results indicate that FH hydrolyzes 1 mol of folate to each 1 mol of pteroic acid and glutamic acid. The amount of $[^{14}C]$pteroic acid increased linearly for a period of 3 h-incubation (Fig. 2), and the amount formed per h was proportional to the concentration of the enzyme (Fig. 3). The above 0.2 nmol of pteroic acid could be detected by this radioassay. The sensitivity of this method was several times higher than that of the conventional photometric method (1–5) for assaying the FH activity.

**The FH activity in microorganisms and mushrooms**

As shown in Table 3, higher activities of FH were found in C. fasciculata and N. crassa. The partially purified FH from C. fasciculata catalyzed the hydrolysis of methotrexate to AMPte. This indicates that FH in C. fasciculata may catalyze both hydrolyzing reactions of folate and methotrexate as shown by bacterial enzymes (1–5).
Table 2. Stoichiometry for the reaction of folate-hydrolyzing enzyme.
The reaction mixture (0.3 ml) containing 0.1 M Tris-HCl buffer, pH 7.1, 1 mM [2-14C]folate and the crude extract of *C. fasciculata* was incubated at 37°C for 1 h. The amounts of protein used in experiments I and II were 450 and 900 μg, respectively. An aliquot (0.1 ml) of the reaction mixture was used for the radioassay of folic acid and pterotic acid, and another aliquot for the colorimetric analysis and the amino acid analysis of glutamic acid (see the text in details).

| Experiment | Radioassay | Colorimetric analysis |
|------------|------------|----------------------|
|            | Folic acid consumed* | Pterotic acid formed* | Glutamic acid formed* |
|            | (nmol)      | (nmol)               | (nmol)               |
| I          | 30.5        | 31.4                 | 36.0                 |
| II         | 51.2        | 52.3                 | 55.7 (51.6)*         |

The amount per 0.1 ml of the reaction mixture. *Parenthesis indicates the value determined by an amino acid analyzer.*

Fig. 2. Formation of pteroic acid as a function of incubation time. The reaction mixture (0.1 ml) containing 1 mM [2-14C]folate (75 nCi), 0.1 M Tris-HCl buffer, pH 7.1, and the partially purified enzyme (30 μg of protein) from *C. fasciculata* was incubated at 37°C for 4 h. After a defined interval of incubation, [2-14C]pterotic acid formed was separated and measured by the method described in the text.

5). *N. crassa* has several intracellular peptidases such as carboxypeptidase and aminopeptidase (12–14). But, there is no report whether these peptidases catalyze the hydrolysis of folate. *L. casei* had the highest FH activity in *Lactobacteriaceae* tested. But, the enzyme activity was slight in *L. arabinosus* ATCC 8014 or *P.*

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Fig. 3. Formation of pteroic acid as a function of protein concentration. Components of the reaction mixture were the same as those in Fig. 2, except for the amount of the enzyme. The mixture was incubated at 37°C for 1 h. The pteroic acid formed was separated and measured by the method described in the text.

Table 3. Distribution of folate-hydrolyzing enzyme activity in microorganisms and mushrooms.

| Strain                        | Specific activitya (nmol/h/mg of protein) | Total activitya (nmol/h/g of wet cells or tissue) |
|-------------------------------|------------------------------------------|------------------------------------------------|
| *Crithidia fasciculata* ATCC 12857 | 72.8                                     | 4,870                                           |
| *Aspergillus niger* M-62       | 1.7                                      | 28                                              |
| *Pencillium chrysogenum* IFO 4879 | 1.4                                      | 19                                              |
| *Neurospora crassa* IFO 6979   | 24.2                                     | 1,020                                           |
| *Serratia indica* IFO 3759     | 1.0                                      | 24                                              |
| *Serratia marcescens* IFO 3048 | 0.4                                      | 6                                               |
| *Pseudomonas riboflava* IFO 3140 | 0.8                                      | 9                                               |
| *Lactobacillus casei* ATCC 7469 | 1.0                                      | 25                                              |
| *Lactobacillus fermenti* ATCC 9338 | 1.1                                      | 9                                               |
| *Streptococcus faecalis* R ATCC 8043 | 0.5                                      | 3                                               |
| *Bacillus cereus* IFO 3131    | 0.4                                      | 13                                              |
| *Lentinus edodes* (Shiitake)   | 1.6                                      | 18                                              |
| *Flammulina velutipes* (Enokitake) | 0.4                                      | 4                                               |

*The enzyme activity was defined as the amount of pteroic acid formed under the standard assay conditions.

cerevisiae ATCC 8081. *L. casei* metabolizes methotrexate to AMPte in vivo (15), but *P. cerevisiae* does not (16). The evidence indicates that FH of *L. casei* may take part in the metabolism of methotrexate as well as that of *C. fasciculata* (6).
Table 4. Distribution of folate-hydrolyzing enzyme activity in mammalian tissues.

| Tissue              | Specific activitya (nmol/h/mg of protein) | Total activitya (nmol/h/g of wet tissue) |
|---------------------|-------------------------------------------|------------------------------------------|
| Hog                 |                                           |                                          |
| Liver               | 0.2                                       | 27                                       |
| Kidney              | 0.3                                       | 21                                       |
| Rabbit              |                                           |                                          |
| Brain               | 0.2                                       | 6                                        |
| Liver, Kidney, Spleen | trace                                  | <1                                       |
| Rat                 |                                           |                                          |
| Brain               | 0.3                                       | 9                                        |
| Liver               | 2.7                                       | 388                                      |
| Kidney              | trace                                     | <1                                       |
| Spleen              | 0.2                                       | 17                                       |

a The enzyme activity was defined as the amount of pteroic acid formed under the standard assay conditions.

The FH activities were slight in *E. coli* B and *A. chroococcum* IFO 12393. Mushrooms had the FH activity. The FH activities were not detected in yeasts such as *S. cerevisiae* FKU 1451, *C. utilis* IFO 0396 and *H. jadinii* IFO 0987. A proteolytic enzyme from *S. cerevisiae*, i.e., carboxypeptidase Y (17), could not hydrolyze folate and methotrexate at any pH range between 4 and 9. The FH activity was not detected in *Euglena gracilis*.

The FH activity in mammalian tissues.

As shown in Table 4, the highest FH activity was found in the crude homogenate of rat liver. The homogenate also had the hydrolyzing activity of methotrexate to AMPte (data not shown). AMPte, excreted in rat urine and feces as a metabolite of methotrexate, is supposed to be formed by bacterial enzymes in rat intestine (18, 19). However, the present result suggests the possibility that rat liver may produce AMPte from methotrexate. The FH activity was also found in hog liver, but it was slight in rabbit liver.

By using the sensitive radioassay, we have found that the FH activity is widely distributed in biological cells and tissues.

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