Isolation and Characterization of Pteroylpolyglutamate Hydrolase from Rat Intestinal Mucosa*

Bernd Elsenhans†, Osman Ahmad, and Irwin H. Rosenberg‡

From the Department of Medicine, Section of Gastroenterology, the University of Chicago, Chicago, Illinois 60637

The pteroylpolyglutamate hydrolase was isolated from rat intestinal mucosa and purified with the aid of affinity chromatography. The affinity ligand was poly-γ-glutamic acid (M_r ~ 12,000) derived from Bacillus subtilis. The specific enzymatic activity was increased 2,000-fold over the 100,000 × g supernatant of the mucosal homogenate with a yield of 20%. Sephadex G-200 gel filtration yielded an estimated molecular mass of 80,000 daltons. The isoelectric point was pH 8.2. The pH optimum in acetate buffer containing 1 mM zinc was 4.5. The K_M values for pteroylheptaglutamate and pteroyltetrاغlutamate were 0.21 and 0.67 μM, respectively. Polyanionic compounds, poly-γ-glutamic acid, dextran sulfate, and heparin were noncompetitive inhibitors. Studies of the time course of hydrolysis of synthetic [³H]pteroylheptaglutamate by three separate techniques demonstrated the appearance of [³H]pteroylmonoglutamate, synchronous with substrate cleavage. Intermediate pteroyl triglutamates were not detected. An endopeptidase-like mode of hydrolysis was further established by identification of a hexaglutamyl peptide as the other reaction product.

Enzymes hydrolyzing the poly-γ-L-glutamyl side chain of pteroylpolyglutamates have been of interest in the field of folic acid metabolism and cellular transport. These enzymes are of special interest in view of their role in the bioavailability of dietary pteroylpolyglutamates (1-6). Intestinal absorption of folate from dietary pteroylpolyglutamates depends upon hydrolysis of the γ-glutamyl side chain. Although the exact anatomical site of hydrolysis is not certain, it is very likely that this step occurs at or near the mucosal border of the epithelial cell by intrinsic or, possibly, adsorbed enzymes (6). For this enzymatic hydrolysis of the γ-glutamyl peptide, several mechanisms are possible. An endopeptidase mode of action was demonstrated in the chicken intestine enzyme (7) and in the rat bile (8), whereas the hog kidney enzyme appears to be an exopeptidase (9). The pteroylpolyglutamate hydrolase of human intestine is reported to behave like a carboxypeptidase (10). Recent studies of the bovine hepatic enzyme suggested a mixed hydrolysis pattern (11). For further elucidation of this cleavage step in the rat intestine, we have isolated and partially characterized a pteroylpolyglutamate hydrolase from the 100,000 × g supernatant of rat intestinal mucosal homogenate with the aid of affinity chromatography.

EXPERIMENTAL PROCEDURES

Materials—[³H]Pteroyltetrługlutamate and [³H]pteroylheptaglutamate labeled in the pteroyl moiety were synthesized from custom labeled [³H]pteroyl acid (performed by New England Nuclear), and γ-glutamyl peptides were prepared by the solid phase method (12, 13). Unlabeled PteGlu₄ and γ-Glu₄ were prepared by the same procedure. α-Glu₄, obtained from its corresponding polymer by controlled partial hydrolysis and DEAE-cellulose separation, and poly-γ-glutamic acid, a mixture of the D- and L-polymer with approximately 80 glutamic acid residues, were isolated from the culture broth of a Bacillus subtilis strain (14) and were gifts from Professor Ephraim Katzir, The Weizmann Institute of Sciences, Rehovot, Israel. The dinitrophenyl and tritylglutamyl peptides were purchased from Fox Chemical Corp.; Sepharose 4B, Sephadex G-200 and G-15, and dextran sulfate were obtained from Pharmacia Fine Chemicals. Heparin was a product of Sigma. The other reagents were of the highest quality available commercially.

Enzyme and Protein assay—Enzyme activities were measured by an assay which is based on the short term uptake by Lactobacillus casei and Streptococcus faecalis of radioactive pteroylmonoglutamate enzymatically released from [³H]PteGlu₄ (15). The specific activities were determined as nanomoles of pteroylmonoglutamate released per h and per mg of protein with 1 unit of enzyme activity equal to 1 nmol of PteGlu released per h. Protein was assayed by the method of Lowry et al. (16) with bovine serum albumin as a standard.

Preparation of Column for Affinity Chromatography—Heparin was immobilized according to a previously described method (17); for this purpose, Sepharose 4B was activated with cyanogen bromide in acetonitrile (18). A similar procedure was used for the preparation of poly-γ-glutamyl-Sepharose. Poly-γ-glutamic acid (1.2 g) was suspended in 15 ml of water and brought into solution by adjusting the pH to 8.0 with 4 M NaOH. After adding 2 ml of 1 M NaHCO₃, 10 ml of wet packed CNBr-activated Sepharose 4B were added, and the mixture was stirred at 4 °C for 36 h. The product was then packed into a column (2 × 3 cm) and subsequently washed with 200 ml of 1 M aqueous ethanolamine (pH 9.0), 200 ml of 0.1 M NaHCO₃ containing 0.5 M NaCl, 200 ml of 4 M NaCl, and 100 ml of water. Prior to use, the column was washed with 0.1 M sodium acetate buffer (pH 4.5) containing 3 mM mercaptoethanol and 1 mM zinc acetate.

Mucosal Homogenates and Ammonium Sulfate Fractionation—Mucosal scrapings from rat proximal intestine (usually from 5 to 7 animals, Sprague-Dawley, 150-200 g) were homogenized in a Sorvall Omni-Mixer at full speed for 3 min, in about 30 ml of 0.2 M sodium acetate buffer (pH 4.5) containing 5 mM mercaptoethanol and 1 mM zinc acetate. These additives were present at these concentrations in all buffer systems containing the enzyme. The homogenate was centrifuged at 100,000 × g for 1 h, and the supernatant was used for ammonium sulfate fractionation. The precipitate between 55 and 85% saturation was used for Sephadex gel filtration in the next purification step. For this purpose, it was dissolved in 8 ml of Sephadex buffer (see below) and, in order to remove insoluble precipitates, the solution was centrifuged at 10,000 × g for 30 min. The resulting supernatant was used for further studies.

1 The abbreviations used are: PteGlu₄, pteroylglutamyl peptides, Glu₄, L-glutamyl peptides, n, in each case is the number of glutamyl residues; PPH, pteroylpolyglutamate hydrolase.
ite centrifuged at 20,000 × g for 10 min.

Analytical and preparative gel filtration was performed on Sephadex G-200 columns (2.5 × 100 cm) with 0.1 M sodium acetate buffer (pH 4.5) containing 0.25 M NaCl. For molecular weight estimation, the same column was calibrated with the following proteins (molecular weights and the obtained Kav values are given in parentheses): aldolase (150,000; 0.255), human transcobalamin I (120,000; 0.255), bovine serum albumin (67,000; 0.37), and myoglobin (17,900; 0.67); the void volume was determined with blue dextran 2000.

Affinity Chromatography.—The pooled fractions from Sephadex gel filtration (fractions 84–110, when collecting 3-ml fractions) were either diluted with an equal volume of water or dialyzed against 0.1 M sodium acetate buffer (pH 4.5), depending on the enzyme. This solution was passed through the affinity column (bed volume about 10 ml) at a flow rate of 1 ml/min. The column was washed subsequently with 50 ml of 0.1 M sodium acetate buffer (pH 4.5) and 30 ml of 0.1 M acetic acid. The enzyme was eluted with 0.1 M sodium acetate buffer (pH 4.5) containing 1 M NaCl. The final enzyme preparation, obtained by dialysis against 0.1 M sodium acetate buffer (pH 4.5), could be stored frozen for 6 weeks at −20 °C without significant loss of activity.

The affinity chromatography on poly-γ-glutamyl-Sepharose resulted in a 95% recovery of PPH activity. Two times the column bed volume of 1.0 M NaCl were employed to elute the enzyme. The column was reequilibrated by passing through a solution of urea with subsequent washing by 0.1 M sodium acetate buffer (pH 4.5). Repeated use of the affinity columns with crude enzyme preparations (e.g., first supernatant) gradually reduced their capacity.

Isoelectric Focusing and Sodium Dodecyl Sulfate-Gel Electrophoresis.—Isoelectric focusing was carried out on LKB 8101 column with 110-ml capacity. Procedural details were essentially the same as outlined in the manufacturer’s manual. Ampholine carrier (2%) with pH range of 3–10 were used. Sodium dodecyl sulfate-gel electrophoresis was performed as described by Weber and Osborn (19).

Separation of Enzymatic Reaction Products—Ascending chromatography on Whatman No. 1 paper was used for detecting the enzymatic reaction products of tritium-labeled or unlabeled pteroylglutamates. The solvents for compounds containing the pteroyl moiety was 5% NH4HCO3, whereas for glutamic acid and glutamyl peptides a solvent system consisting of 105:1 (v/v) n-propyl alcoholwater:concentrated ammonia was used. For radioactive assay, strips were cut in 1-cm segments and counted by liquid scintillation spectrometry. Glutaminyl chain length was determined by comparison to authentic pteroylglutamates. Glutamic acid and peptides were visualized by ninhydrin reagent. Pteroylglutamates were also analyzed by Sephadex G-15 chromatography, using a method adapted from Shin et al. (20). Sephadex G-15 columns (0.9 × 100 cm) correlated with a molecular weight of 8101 column with 100 cm) was obtained when the reaction mixtures were analyzed by Sephadex G-200 columns (2.5 × 100 cm) correlated with a molecular weight of 78,000. This value is in good agreement with the result obtained by gel filtration where the Kav value of 0.34 on Sephadex G-200 columns (2.5 × 100 cm) correlated with a molecular weight of 80,000. An isoelectric point of pH 8.2 was determined by isoelectric focusing in 2% ampholine carriers. The heat stability of the enzyme was demonstrated by the fact that exposure of the crude supernatant to 60 °C for 40 min resulted in retention of 96% of control activity. The enzyme was completely inhibited by 100 μM p-hydroxymercuribenzoate.

pH Profile—The pH optimum in an acetate buffer containing 1 mM zirconium acetate was 4.5. This value was determined with [3H]PteGlu7 as substrate using the bacterial uptake assay with either L. casei or S. faecalis. Because S. faecalis uptake is specific for pteroylmonoglutamate and L. casei is able to incorporate pteroyl- and triglutamate as well as the monoglutamate, the almost identical pH curves are significant. First, there is no apparent alteration in the mode of hydrolysis over the pH range studied, and second, the product at each pH is pteroylmonoglutamate.

Enzyme Kinetics—The purified enzyme exhibits typical Michaelis-Menten kinetics. The hydrolysis of [3H]PteGlu7 was studied by comparing the results of L. casei uptake assay with the measurement of the released tritiated pteroylmonoglutamate by paper chromatography. The close agreement between these two methods confirms the identity of the product pteroylmonoglutamate. Addition of unlabeled PteGlu7 results in a competitive mode of inhibition; here too, agreement between bacterial uptake assay and paper chromatography was observed. Experiments were also performed with the shorter chained substrate [3H]PteGlu6 with and without addition of unlabeled pteroylheptaglutamate. The amount of radioactive pteroylmonoglutamate formed was determined by paper chromatography. The results clearly demonstrated competitive behavior between both substrates PteGlu7 and PteGlu6. The kinetic parameters are presented in Table II. Whereas Vmax is quite independent of the length of the glutamyl side chain, reducing the number of glutamic acid residues leads to an increase in the Km. In confirmation, the Km for unlabeled PteGlu7 was essentially unchanged.

Hydrolysis Mechanism.—In order to determine whether the mechanism of hydrolysis was that of an exo- or endopeptidase, the cleavage products of the enzymatic reaction were analyzed with respect to reaction time. As shown in Fig. 1, hydrolysis was followed by means of the two microbiological uptake assays and paper chromatography. Almost identical curves were obtained by all three systems, thus emphasizing again that the only enzymatic cleavage product is pteroylmonoglutamate. No intermediate product was observed. The paper chromatographic analysis is given by Fig. 2. The time course of hydrolysis of pteroylheptaglutamate from 2 through 64 min shows accumulation of pteroylmonoglutamate synchronous with the disappearance of the substrate. The same pattern was obtained when the reaction mixtures were analyzed by Sephadex G-15 column chromatography (Fig. 3a). Furthermore, the enzymatic hydrolysis of [3H]PteGlu7 did not show any trace of intermediate pteroyldiglutamate as analyzed by means of Sephadex chromatography (Fig. 3b) or paper chromatography.

To determine that this direct, endopeptidase-like mechanism observed in vivo was operative in vitro, experiments were performed using loops of rat intestine in vivo. The hydrolysis...
The released folic acid. Incubation was at products were determined. Fig. 1. Time course of enzymatic hydrolysis. With \[^{3}H\]PteGlu as substrate, the released pteroylmonoglutamate was determined by paper chromatography and \(L.\) casei uptake assay. The same determinations were carried out when unlabeled PteGlu (0.96 \(\mu\)M) was added to the enzyme incubation mixture. With \[^{3}H\]PteGlu as substrate, only paper chromatography was used to measure the released folic acid. Incubation was at 37°C for 10 min with an enzyme concentration of 2.6 units/ml.

**TABLE I**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Specific activity (nmol PteGlu/(\mu)g protein)</th>
<th>Total units (nmol PteGlu/h)</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>100</td>
<td>2030</td>
<td>0.058</td>
<td>118.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant, 100,000 x 5 x h</td>
<td>96</td>
<td>453</td>
<td>0.262</td>
<td>118.8</td>
<td>4.5</td>
<td>100</td>
</tr>
<tr>
<td>(NH(_4))SO(_4) fraction, 55–85% saturation</td>
<td>20</td>
<td>63.8</td>
<td>0.96</td>
<td>61.2</td>
<td>16.5</td>
<td>51.5</td>
</tr>
<tr>
<td>Gel filtration, Sephadex G-200</td>
<td>250</td>
<td>5.3</td>
<td>4.77</td>
<td>25.2</td>
<td>82</td>
<td>21.5</td>
</tr>
<tr>
<td>Affinity chromatography, (\gamma)-poly-Glu-Sepharose</td>
<td>10</td>
<td>0.2</td>
<td>113.5</td>
<td>22.8</td>
<td>1950</td>
<td>19.5</td>
</tr>
</tbody>
</table>

**TABLE II**

Kinetic parameters of the purified enzyme

With \[^{3}H\]PteGlu as substrate, the released pteroylmonoglutamate was determined by paper chromatography and \(L.\) casei uptake assay. The same determinations were carried out when unlabeled PteGlu (0.96 \(\mu\)M) was added to the enzyme incubation mixture. With \[^{3}H\]PteGlu as substrate, only paper chromatography was used to measure the released folic acid. Incubation was at 37°C for 10 min with an enzyme concentration of 2.6 units/ml.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) ((\mu)M)</th>
<th>(V_{max}) (nmol/min/(\mu)g protein)</th>
<th>(K_I) (PteGlu) ((\mu)M)</th>
<th>(K_I) (PteGlu) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[^{3}H]PteGlu</td>
<td>0.67</td>
<td>0.045</td>
<td>0.20</td>
<td>1</td>
</tr>
<tr>
<td>[^{3}H]PteGlu</td>
<td>0.21</td>
<td>0.051</td>
<td>0.25</td>
<td>1</td>
</tr>
</tbody>
</table>

**Fig. 2.** Paper chromatograms of the hydrolysis of \[^{3}H\]PteGlu. At indicated time intervals (0–64 min), the enzymatic reaction was stopped by adding perchloric acid. After neutralizing with KOH and centrifuging from precipitated KClO\(_4\), the supernatant was spotted on Whatman No. 1 paper strips and developed in 5% NH\(_4\)HCO\(_3\). Radioactivity of 1-cm segments was determined and plotted here relative to the \(R_f\). For unlabeled standards, the following \(R_f\) values were obtained: PteGlu, 0.84; PteGlu, 0.63; and PteGlu, 0.44.

by paper chromatography. After the first 15 min of enzymatic hydrolysis, the main product found was a hexaglutamyl peptide. There was a trace of a more rapidly migrating ninhydrin product. Incubation for another 15 min resulted in a further degradation of the hexapeptide into smaller glutamyl peptides.

Enzyme Inhibition—The enzyme is inhibited by polyanionic compounds. At a PteGlu substrate concentration of 0.5 \(\mu\)M, 95% inhibition of enzyme activity was achieved with 2 \(\mu\)M poly-\(\gamma\)-glutamic acid, or 0.3 \(\mu\)M dextran sulfate 500, or 5 \(\mu\)M heparin. Other oligocarboxylic compounds were chosen according to a structural relationship to the substrate and tested for their ability to inhibit the enzyme. As shown by Fig. 5, the inhibitory effect clearly depends on the number of carboxyl groups in the molecule. The linkage between the glutamyl residues seems to have only moderate influence on the inhibition of enzyme activity as seen by the differences between hexa-\(\alpha\)- and hexa-\(\gamma\)-glutamyl acid. Citrate in concentrations usually used for buffer systems (\(\sim\)0.1 M) reduced the enzymatic activity to about 50% of control. Even glutamic acid exhibited an inhibitory effect at high concentration; a 50% decrease of enzyme activity was achieved by a concentration of 0.3 \(M\).

From kinetic experiments with \[^{3}H\]PteGlu as substrate, the influence of Glu and pteroylmonoglutamate (folic acid) on enzyme activity was determined. The \(\gamma\)-L-glutamyl tripep-
Rat Intestinal Pteroylpolyglutamate Hydrolase

Current concepts of the function of pteroylpolyglutamate hydrolases emphasize their regulatory effects on folate absorption and metabolism. Since pteroylpolyglutamates are probably not able to cross cellular membranes, the enzymatic breakdown to pteroylmonoo- or diglutamates may regulate the intestinal absorption and the amounts of circulating and intracellular folates.

One approach to elucidation of the function of an enzyme is to establish the mode of action and its inhibition. Several pteroylpolyglutamate hydrolases have been partially purified or enriched, and characterized. Usually, hydrolis products containing the pteroyl moiety have been analyzed either by microbiological assay (21) or chromatographic procedures (9, 22, 23). Even with purified enzymes, usually only one of the cleavage products was determined. To date, only the hydrolisis mechanism of the chicken intestinal enzyme and that of rat bile has been established by determining both cleavage products (7, 8).

The findings of this study, particularly those which determined no difference in the short term uptake of the final pteroyl derivative by L. casei or S. faecalis, demonstrate that pteroylmonoglutamate is the final product of rat intestinal PPH hydrolysis of PteGlu7. As determined by chromatographic methods, the cleavage pattern may be interpreted as a single step, i.e. the disappearance of substrate, PteGlu7 or PteGlu6, is accompanied by a directly proportional increase in the amount of final product. The existence of intermediates such as PteGlu5, PteGlu4, or PteGlu9, is obligatory for a carboxypeptidase-like mode of hydrolysis, can be ruled out on the basis of the following findings. First, the time course of the release of pteroylmonoglutamate does not show a lag period, which would be required to accumulate sufficient intermediates for further hydrolysis. And second, shorter chained pteroyl oligoglutamates, as generalized from the results with PteGlu6, exhibit a greater $K_M$ than the substrate. A reaction mixture containing both substrate and intermediates would lead to a competitive displacement of the intermediates from the enzyme by the substrate. This would result in an accumulation of medium chain length pteroyl oligoglutamates (22) rather than in the exclusive formation of pteroylmonoglutamate observed in the present studies.

Only a progressive enzyme which cleaves one glutamic acid residue after another, at such a fast rate that diffusion of the enzyme to another substrate molecule would become the rate-limiting step, could explain these findings and a carboxypeptidase-like mode of cleavage. In this case, however, only glutamic acid should be detected as a reaction product together with pteroylmonoglutamate acid. Instead, after the first 15 min of enzymatic hydrolysis, the main product found is hexaglutamic acid, which clearly confirms the presence of a pteroyloligo-γ-L-glutamate endopeptidase in the mucosa of rat intestine.
The further degradation of the released hexaglutamyl peptide could be due either to the action of PPH itself or to a contamination by a similar peptidase (a result, perhaps, of the purification procedure using poly-γ-glutamyl Sepharose). Therefore, hydrolase activity was tested with substrates α-Glu₉, γ-Glu₉, and glycylglycylglycine. There was virtually no cleavage of the glycyl peptide, whereas there was preferential hydrolysis of the γ-glutamyl peptide.

That the purified enzyme really represents a pteroylpolyglutamate endopeptidase and not a nonspecific peptidase is further confirmed by the results from the inhibition experiments. In view of the enzymatic release of pteroylmonoglutamic acid from PteGlu₇ and the inhibition of this release by PteGlu₃, Glu₁₀, Glu₁₀, and even PteGlu is 100-fold more effective than Glu and 3-fold more than γ-Glu₁₀.

Thus, we have presented evidence for a pteroylpolyglutamate endopeptidase in rat intestine. This is reminiscent of the chick intestinal enzyme, which is also an endopeptidase but whose initial site of cleavage is at pteroylglutamate so that PteGlu₉ avian PPH → PteGlu₃ + Glu₁₀. By contrast, the endopeptidase from rat intestine cleaves directly at pteroylmonoglutamate so that PteGlu₉ rat PPH → PteGlu₃ + Glu₁₀.

Pteroylpolyglutamate hydrolase is reported in rat pancreatic fluid (24), but the mode of action of this enzyme has not been identified. Evidence that the enzyme in rat intestine is a separate enzyme derives from experiments showing the presence of the intestinal enzyme even days after pancreatic duct ligation (2). The intracellular origin of the intestine enzyme in the rat is not firmly established. The enzyme reported here was purified from the 100,000 × g supernatant of the intestinal homogenate. Although it has been proposed that there are separate membrane-bound and soluble enzymes in human intestine (25), our studies (6, 26) and those of others (3) have failed to identify more than one pteroylpolyglutamate hydrolase in the rodent intestine. Further studies are required to elucidate these apparent species differences.

REFERENCES