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Recombinant zebrafish γ–glutamyl hydrolase exhibits comparable properties and catalytic activities to mammalian enzyme

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Abbreviations: γGH, γ-glutamyl hydrolase; NH-zγGH, Nus-His-tag fusion γ-glutamyl hydrolase; THF, tetrahydrofolate; 5-CHO-THF-Glu_1, 5-formyltetrahydrofolate monoglutamate; 5-CHO-THF-Glu_3, 5-formyltetrahydrofolate triglutamate; MTX, methotrexate; IPTG, isopropyl-beta-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Abstract

A cDNA encoding for zebrafish γ–glutamyl hydrolase (γGH) was cloned and inserted into a pET43.1a vector via SmaI and EcoRI sites and expressed in Rosetta (DE3) cells as a Nus-His-tag fusion enzyme (NH-zγGH). After induction with isopropyl thiogalactoside, the enzyme was purified with a Ni-Sepharose column and approximately 8 mg of pure enzyme was obtained per liter of culture. The primary sequence of the recombinant zγGH was similar to mammalian γGH. Thrombin digestion of this NH-zγGH fusion protein resulted in zγGH with approximately 2-fold higher catalytic activity as compared with the NH-zγGH fusion enzyme. This recombinant zγGH is active and exhibits comparable endopeptidase activity with folate substrate and antifolate drug methotrexate. Use of this recombinant zγGH significantly increased efficiency in folylpolyglutamate hydrolysis for folate analysis compared to current protocols.

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Introduction

Folate is an essential B vitamin and participates in the biosynthesis and metabolism of nucleic acids, proteins, several amino acids, methyl groups, many neurotransmitters, and some vitamins. Mammalian cells are unable to synthesize folates *de novo* and therefore depend on their food for the supply of folates. Naturally occurring folates are synthesized as poly-γ-glutamate forms (folylpolyglutamate) but are absorbed and transported most efficiently as folylmonoglutamates. The conversion of folylpolyglutamates in dietary food to folylmonoglutamates is catalyzed by carboxypeptidase II (EC 3.4.22.12.) in mammals. Recently, γ-glutamyl hydrolase (γGH, EC 3.4.19.9), a lysosomal cysteine peptidase, was reported to be the enzyme responsible for hydrolyzing dietary folate in rat small intestine (Shafizadeh and Halsted, 2007). After entering cells, these folylmonoglutamates are elongated to folylpolyglutamates, by folylpoly-γ-glutamate synthetase (EC 6.3.2.17), for more effective retention and cofactor activity. As part of a salvage pathway, γGH catalyzes the hydrolysis of Glu-γ-Glu bonds to form folylmonoglutamates, enabling folate cofactors to be exported from cells and enter circulation again (Suh, et al., 2001). Therefore, folylpoly-γ-glutamate synthetase and γGH are crucial for the maintenance and regulation of intracellular folate homeostasis. Nevertheless, the mechanism of turnover and control of cellular folylpolyglutamate levels by γGH remains unclear. A better understanding to this enzyme and an efficient assay system is prerequisite to answer this question.

Consistent with this notion, the activity of γGH to hydrolyze the γ-glutamyl peptide bonds of folylpolyglutamates has rendered this enzyme a potential target of antifolate chemotherapy and, at

the same time, a primary component in regulating the intracellular levels of some antifolate drugs. Antifolate drugs, such as methotrexate, owe much of their effectiveness to being substrates for both folylpoly-γ-glutamate synthetase and γGH. Removal of γ–linked glutamate residues decreases the retention and activity of these drugs. A polymorphism resulting in reduced catalytic activity of γGH was observed to be associated with greater accumulation of long-chain methotrexate polyglutamate forms (Cheng, et al., 2004). Conversely, higher γGH activity has been connected to the development of drug resistance (Rhee, et al., 1993). Therefore, alteration to γGH availability or activity appears to have functional and pharmacological consequences and even to be a potential tumor marker (Schneider and Ryan, 2006). Having pure and active γGH is essential to structural and kinetic studies of γGH and to additional investigation for its role in affecting antifolate drug efficacy.

The determination of individual folate derivatives in serum of patients receiving antifolate chemotherapy and in foods is an important current protocol. The first step in these determinations is converting folylpolyglutamates to folylmonoglutamates by γGH. Currently, hog kidney, chicken pancreas and rat serum/plasma are the main sources used to provide γ-glutamyl hydrolase activity (Quinlivan, et al., 2006). However, large amounts of plasma or extract and long incubation times are often required for an acceptable degree of conversion, resulting in significant dilution, higher background and loss of folate cofactors in samples. Having a pure and concentrated γGH will improve the accuracy of folate determinations.

An efficient animal model for drug safety and efficacy screening is indispensable, despite the historic progress in the past few decades in pharmaceutical manufacturing. Zebrafish, a vertebrate

is becoming a prominent animal model, providing a platform for fast drug screening. The advantages are its similarity to mammals in many biological pathways and pathogenesis, abundant offspring, rapid development, transparent embryo and easy growth and breeding. Especially important for drug discovery is that zebrafish embryos are permeable to small molecules and drugs during organogenesis, providing easy access for drug administration and vital dye staining (Kari, et al., 2007). In addition, the external fertilization and development of embryos prevent the often occurring mis-interpretation of experimental results due to maternal supply of folates. Well-established tools of molecular biology for gene manipulation have also significantly assisted progress with zebrafish. We have started an extensive study of folate-mediated one-carbon metabolism in zebrafish (Chang, et al., 2006;Chang, et al., 2007;Kao, et al., 2008). In this study, we cloned the coding sequence of zebrafish γGH (zγGH) from a zebrafish cDNA library and developed an efficient protocol for expression and purification of the recombinant zγGH. To our knowledge, this is the first report for the cloning, expression and purification of zebrafish γGH. We showed that this recombinant zγGH catalyzed the hydrolysis of natural folate substrates and an antifolate drug with similar efficiency. In addition, data showing improved efficiency of converting folate-polyglutamates to monoglutamates in a folate analytical protocol with HPLC by replacing conventional rat serum with the recombinant zγGH is discussed.

Material and Methods

Materials. PCR primers were ordered from MDBio, Inc. (Taipei, Taiwan). The SMART™ RACE amplification kit was purchased from Clontech,/Takara Bio Co. (Mountain View, California,

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US). PCR Master Mix was purchased from ABgene House (Surrey, UK). Enzymes used for cloning were purchased from Invitrogen (Carlsbad, California, US) and New England BioLabs, Inc (Ipswich, Maryland, US). The HPLC Aquasil C_{18} column and guard columns were purchased from ThermoFisher Scientific (Waltham, MA, US). Nickel-Sepharose (Ni-Sepharose) resin slurry was purchased from Amersham Bioscience (Piscataway, New Jersey, US). Rat serum was prepared as described previously (Wilson and Horne, 1982). All fully reduced monoglutamated folates were generous gifts from Dr. Moser (Merck Eprova AG), Switzerland. (6S) N^5-CHO-tetrahydrofolate (Leucovorin) triglutamate was a gift from Dr. Schirch (Virginia Commonwealth University, VA, US). Methotrexate pentaglutate was purchased from Schircks Laboratories (Jona, Switzerland). All other chemicals, including folic acid, dihydrofolate, buffers and amino acids were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, Wisconsin, US).

Fish care and preparation of cDNA libraries. Zebrafishes (*Danio rerio*, AB strain) were bred and maintained in a 10 h-14 h light-dark diurnal cycle following the standard procedure described by Westerfield (Westerfield, 1995). Embryos were staged according to Kimmel et al.(Kimmel, et al., 1995). Total RNA isolation and cDNA library construction from zebrafish embryos and tissues were prepared with RNAzol B reagent (Tel-Test. Inc.) and the SMART-RACE cDNA Amplification Kit (Clonetech, Inc.) as described previously (Kao, et al., 2008).

Bacterial strains, plasmids and general cloning procedures. The *E. coli* strain XL1 Blue (*rec*A1, *end*A1, *gyr*A96, *thi-1*, *hsd*R17(r_K^-,m_K^+), *sup*E44, *rel*A1, *lac*$^-$) was used for the construction of clones. The *E. coli* strains Rosseta (DE3) (F^- recA r_{k12}^- m_{k12}^+) containing the T7 RNA polymerase gene

was used for protein expression. The pET43.1a plasmid and all the *E. coli* strains for cloning and expression were obtained from Novagen (Madison, WI, US). The materials and methods for the general cloning procedures were as previously described (Kao, et al., 2008).

Cloning of zebrafish γ–glutamyl hydrolase coding sequences. Primers were designed based on the zebrafish γGH cDNAs available in GenBank (GenBank™ accession number BC066746) to PCR amplify complete γGH coding sequence from zebrafish 5'-RACE-Ready cDNA libraries. The primer sequences were: 5'-GGGGCATATGATTCACATCTTTCTTTTG -3' (forward) and 5'-GGGGCTCGAGATTGAAAAAATATGTCTGTTC-3' (reverse) with introduced NdeI and XhoI restriction enzyme sites (underlined) to simplify the cloning procedures. The PCR fragments close to 1 Kb were cloned into the expression vector pET43.1a between NdeI and XhoI sites, generating zγGH/pET43.1a. For constructing Nus-His-fusion zebrafish γGH (NH-zγGH), PCR with another pair of primers: 5'- GACCCGGGGACCTTTAATAAAAACTAATGAAAG-3' (forward) and 5'-GGGAATTCCTAATTGAAAAAATATGTCTG-3' (reverse) was performed using the previously obtained zγGH/pET43.1a plasmid as template. The restriction enzyme sites for SmaI and EcoRI (underlined), respectively, were introduced for the convenience of subsequent cloning. The resulting 900-bp fragment was cloned into the expression vector pET43.1a between SmaI and EcoRI, resulting in an open reading frame encompassing coding sequences of Nus-Tag, His-Tag and Gly22 to Asn312 of zγGH. Successful cloning was confirmed by restriction enzyme digestion and DNA sequencing. The resulting constructs were transformed into Rosetta (DE3) cells for zγGH expression and purification.

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Expression and Purification of recombinant zγGH. *E. coli* containing the desired plasmid, either zγGH/pET43.1a or NH-zγGH /pET43.1a, was grown overnight at 37 ^{o}C in 50 ml of enriched Luria broth (2-YT) containing 100 μg/ml ampicillin. This culture was used to inoculate 1 L of the same broth and the inoculum continuously grown at 37 ^{o}C. Recombinant zebrafish γGH was induced by adding IPTG to a final concentration of 0.4 mM when the inoculum reached log phase. After 4 hrs incubation with vigorous shaking at 27 ^{o}C, bacteria cultures were centrifuged and cell pellets were subjected to protein purification.

For the purification of NH-zγGH, cells were re-suspended in 20 ml of lysis buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 5 mM 2-mercaptoethanol, 1 mM $MgCl_2$ and 10 % glycerol) and subjected to cell disruption with a French Press. The cell lysate was treated with DNase I (30 μg/ml) at 25^{o}C for 20 mins and pelleted again by centrifugation at 4 ^{o}C. The supernatant was mixed with an equal volume of binding buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl and 20 mM imidazole) before adding to 5 ml of Ni-Sepharose resin and incubating at 4 ^{o}C for 2 hrs. The slurry of cell lysate and Ni-Sepharose resin was briefly centrifuged, the supernatant containing unbound protein discarded and the Ni-Sepharose resin removed to a column (1.5 x 5.0 cm). After a thorough wash with 5 to 10 resin volumes of binding buffer, NH-zγGH was eluted with binding buffer containing 200 mM imidazole. Fractions containing protein were combined and NH-zγGH was precipitated by 50 % ammonium sulfate, dialyzed in phosphate buffer containing 10 % glycerol and stored at -20 or -80 ^{o}C.

To remove the N-terminal Nus-His tag, ammonium sulfate precipitated NH-zγGH was

subjected to a 2-hr dialysis in 1 l of thrombin cleavage buffer (50 mM Tris, pH7.4, 150 mM NaCl, 2.5 mM $CaCl_2$ and 0.1 % 2-mercaptoethanol). The desalted NH-zγGH was digested with thrombin in a 1:500 ratio at room temperature for 20 mins followed by a further incubation at 4 ^{o}C for 6 hrs. The digested sample was loaded on an equilibrated Ni-Sepharose column again as previously described. The zγGH in the fraction of flow-through was collected, ammonium sulfate precipitated and dialyzed. Both purified NH-zγGH and zγGH were stored at -80 ^{o}C in the presence of 5 mM 2-mecaptolethanol, 20 % ammonium sulfate and 10 % glycerol without significant loss of activity for at least 6 months.

Determination of stoichiometry for NH-zγGH and zγGH. Recombinant NH-zγGH and zγGH were chromatographed on a Superdex 200 size exclusion column (0.46 x 30.0 cm) equilibrated with 20 mM potassium phosphate, pH 7.0, containing 100 mM NaCl and 5 mM 2-mercaptoethanol on an Agilent 1100 HPLC. The retention times of analyzed proteins were compared to the protein standards of known molecular weight and molecular weights obtained by standard curve interpolation.

Measurements of γ-glutamyl hydrolase activity. A modified reversed-phase HPLC method was used to detect γ-glutamyl hydrolase activity (Patring, et al., 2005). γGH catalyzes the hydrolysis of the γ-glutamate bond, resulting in folate-mono-Glu. The progression and rate of reaction were monitored by comparing the peak retention times and peak areas of 5-CHO-THF-Glu_1 and $–Glu_3$ with those of pure corresponding folates of known concentrations. NH-zγGH or zγGH at less than 1 % of substrate concentrations were used for the reaction rate measurement. In brief, 0.5 to 250 pmole of purified NH-zγGH (in the volume of 0.5 µl) or 5 to 20

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µl of rat serum was added to 100 µl of reaction mixture containing 50 mM potassium phosphate, pH 6.0, 14 mM 2-mercaptoethanol, 2 % ascorbic acid and 2.5 µM 5-CHO-THF-Glu_3 or methotrexate-Glu_5 in a 1.5 ml centrifuge tube. Tubes were flushed with nitrogen before capping and incubated at 37 ^{o}C. After designated incubation times, tubes were boiled for 3 min and immediately chilled on ice to stop the reaction and precipitate protein simultaneously. The samples were centrifuged and the supernatants were filtered and analyzed on an Aquasil C_{18} column, 150 x 4.6 mm, 3 µm (ThermoFisher Scientific) using an HPLC system (Agilent 1100) with a fluorescence detector. The mobile phase used was acetonitrile-30 mM phosphate buffer (pH 2.3) under linear gradient elution conditions and the flow rate 0.4 ml/min. Retention times and peak areas of pure 5-CHO-THF-Glu_1 and 5-CHO-THF-Glu_3 of known concentrations were used for peak identification and standard curves construction. An estimation of the maximum rate was determined at the earliest time point when approximately 50% of 5-CHO-THF-Glu_3 had been converted to the monoglutamate. For kinetic studies, apparent K_m and k_{cat} were determined by performing reactions in the presence of 10 nM purified zγGH and 5-CHO-THF-Glu_3 ranging from 10 to 150 µM. The initial velocities were determined from the amount of 5-CHO-THF-Glu_1 generated within the first 30 sec at each substrate concentration after the reaction was initiated. The kinetic constants were determined from double-reciprocal plots of initial velocity versus substrate concentration.

Results

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Sequence and structural analysis of recombinant zγGH. The isolated full length zγGH cDNA (EU918170) is 939 bp and encodes a protein of 312 amino acids. Currently, γ-GH has been identified in several mammals including human. Substantial similarities of primary structure between zebrafish γ-GH and its mammalian counterparts reveal conservation during evolution of this enzyme. A high level of homology is observed in the primary sequences and more so for the amino acid residues essential for catalytic activity (Fig. 1). A 53.8 % identity between zebrafish and human γGHs primary sequences is observed. The residues crucial for catalytic activity: Tyr^{36}, His^{171}, His^{220} and Glu^{222}, are conserved in the zebrafish enzyme, as well as Cys^{110}, the residue mediating nucleophilic attack at the active site (Schneider and Ryan, 2006). Higher peptide sequence variation is observed in the postulated signal sequence (from Ile^{2} to Phe^{21}). This prediction is based on the comparison with γGH of other species with known signal peptide sequences (Yao, et al., 1996b;Yao, et al., 1996a). The predicted sequence patches present in the homodimer interface of mammalian enzymes also show similarity in zγGH, especially the first patch (Ala^{54} to Ser^{60}). Two of the consensus Asn-linked glycosylation sites are also observed in zγGH (Yao, et al., 1996b). The primary sequence of zγGH was subjected to on-line secondary structure prediction and compared with human γGH ["PredictProtein", http://www.predictprotein.org/newwebsite/submit.php; (Rost, et al., 1996)]. The predicted seven helices of zγGH strongly resemble those of human γGH, indicating structural similarity between zebrafish and human enzymes (data not shown).

Expression and purification of zγGH. Initially, full-length zγGH, including the prospective signal peptide, was cloned into pET43.1a with its start codon adjacent right after the ribosomal

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binding site on the vector. However, no significant expression was observed, despite the completely accurate and in-frame coding sequence. All of our attempts to solve this problem, including changing competent cells, culture medium, IPTG concentration, induction time and temperatures and cell density, were not successful. No enriched protein band at the expected range of molecular weight was observed in the whole cell extract (data not shown). Significant expression of recombinant zγGH was accomplished only when the coding sequence of zγGH without the signal peptide region was subcloned downstream of the Nus coding sequence and expressed as a Nus-fusion protein (Fig. 2).

The recombinant NH-zγGH was induced by adding 0.4 mM IPTG at 27 ^{o}C for 4 hrs. The majority of induced NH-zγGH remained in soluble fractions under this condition. The His-tag between Nus and zγGH allowed us to use Ni-Sepharose to greatly simplify the purification. The NH-zγGH eluted from Ni-Sepharose column was at least 95 % pure, judged from SDS-PAGE (Fig. 3). Starting from the harvested cell pellet, we were able to obtain 8 mg of active NH-zγGH from 1 liter of culture cells in 5 hours with good yield and purity.

To exclude the possible interference due to peptide fusion, we removed the N-terminal Nus peptide and His-tag by thrombin digestion of the NH-zγGH fusion protein combined with a second run of Ni-Sepharose column purification. The digestion efficiency and recovery rate were approximately 60 % under the conditions used, judging from SDS-PAGE and the quantity of obtained thrombin digested zγGH. Approximately 1.5 mg of thrombin digested zγGH was obtained from 8 mg of purified NH-zγGH protein.

Structure and activity of zγGH. Recombinant NH-zγGH and zγGH appeared on

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SDS-PAGE in the ranges of their estimated size of 92 kDa and 33 kDa, respectively (Fig. 3). Unexpectedly, the retention volumes during chromatography on a Superdex 200 column showed that NH-zγGH and zγGH had Stokes radii close to globular proteins of 400 kDa and 60 kDa, respectively (Fig. 4). These results suggest that zγGH exists as a homodimer, as observed for the human enzyme; whereas NH-zγGH is a tetramer (Eisele, et al., 2006). It appears that the presence of the N-terminal Nus and His-tag interferes with the quaternary structure of zγGH.

zγGH converts folate-polyglutamates to folate-monoglutamates. This activity was monitored by the conversion of 5-CHO-THF-Glu_3 to 5-CHO-THF-Glu_1 with HPLC. Shown in figure 5A are the chromatograms of 5-CHO-THF-Glu_3 and 5-CHO-THF-Glu_1 present in reaction mixtures after incubation for 5 min upon adding various amounts of NH-zγGH or rat serum to initiate the reactions. Judging from the peak areas corresponding to substrate and product, we found that NH-zγGH 0.1 µg (equivalent to 10 nM) was sufficient to convert more than 60 % of 5-CHO-THF-Glu_3 (2.5 µM) to 5-CHO-THF-Glu_1 at 37 ^{o}C in 5 mins. The better efficiency of de-conjugation mediated by NH-zγGH than by rat serum was also observed in the experiment performed in a time-dependent manner (Fig. 5B). The rate of product formation catalyzed by recombinant NH-zγGH in 2 mins was 65 nmole/nmole of protein/min. Conversion of all the substrate reached approximately 80 % at 20 mins and was completed in 2 hours using NH-zγGH. On the other hand, less than 2 % and 30 % of 5-CHO-THF-Glu_3 was de-conjugated when rat serum was added in the volume ratio of 1:10 (serum: sample) in 5 and 20 mins, respectively. More than 2 hrs was required to convert 80 % of substrate with rat serum. No significant loss of product was observed (2.4 µM) at

the end of 2-hr incubation with NH-zγGH; whereas only 80 % of the product was detected in the rat serum sample. Methotrexate-Glu_5, an antifolate drug commonly used in regimens for chemotherapy, was also a substrate of NH-zγGH and zγGH with comparable efficiency to 5-CHO-THF-Glu_3 (Fig. 5C). The presence of only methotrexate-Glu_5 and methotrexate-Glu_1 during hydrolysis suggests that zγGH is most likely an endopeptidase, as observed with rat γGH (Yao, et al., 1996b).

An approximately two-fold increase was observed for the catalytic activity of zγGH upon the removal of the N-terminal Nus peptide and His-tag, as compared with NH-zγGH. The 5-CHO-THF-Glu_1 generated was almost doubled when the same molecular concentration of NH-zγGH and zγGH were used (Fig. 6). The rates of product formation in the presence of 1.6 μM 5-CHO-THF-Glu_3 and 3 nM enzymes in 5 mins were 20 and 38 min^{-1} for NH-zγGH and zγGH with approximately 55 % and 95 % of product formed, respectively. These results suggest interference in enzyme activity due to the presence of the N-terminal Nus peptide and His-tag. No significant difference in the activities was observed when the catalytic reactions were performed at either pH 4 or pH 6 although slightly higher activity was observed in acidic condition. Catalytic efficiencies of both NH- zγGH and zγGH were lowered to 65 % when the reaction mixtures were performed at pH 12 (data not shown). The estimated apparent k_{cat} and K_m of zγGH for 5-CHO-THF-Glu_3, obtained from the average of three independent repeats, are 87.6 min^{-1} and 57.0 μM, respectively (Table 1).

Discussion

In this study, we report the cloning, characterization and significance of zebrafish γGH and

NH- zγGH fusion proteins. The purposes for obtaining pure and active zγGH are two-fold. First, it enables detailed studies for the properties of zγGH and its role in regulating the intracellular availability of folates and some antifolate drug. This will help not only to advance our knowledge in antifolate drug mechanisms and development but also to evaluate the feasibility of using zebrafish as a model for antifolate drug discovery. Second, it improves the efficiency of the critical step of folylpolyglutamate hydrolysis for folate measurements.

To our knowledge, this is the first report for the cloning and purification of zebrafish γGH. This recombinant zγGH expressed and purified from *E.coli* was active, suggesting that glycosylation is not essential for zγGH catalytic activity. This is in agreement to a previous observation with human enzyme (Yao, et al., 1996b). However, we could not exclude the possibility that glycosylation is important for the regulation of enzyme stability or function. The K_m of recombinant zebrafish γGH is comparable to that of human enzyme. However, the V_{max} is appoximately 35-fold higher than that of human GH (Chave, et al., 1999). Several factors might have contributed to the difference. First, to avoid potential product inhibition, the initial rate of reaction catalyzed by zγGH was determined for the first 30 secs after adding enzyme to initialize the reaction. That was when less than 2 % of substrate was converted to product. Human enzyme was measured when less than 15% of substrate was converted to product. Second, different substrates used in the assays for zebrafish and human enzymes might also affect the catalytic efficiency of the enzymes. What we used in the assay was 5-CHO-THF-Glu_3; whereas it was 4-NH_2-10-CH_3 PteGlu_2 for human enzyme. Nevertheless, we could not exclude the possible fundamental differences reside in these enzymes due to different species

although we think it is less likely.

It was unexpected that our initial attempt to over-express zγGH without the Nus-expression partner was not successful. We had carefully examined the coding sequence for accuracy and codon usage and found several rarely used codons in *E.coli*. However, the use of the Rosetta strain for expression should have overcome this potential problem (Chang, et al., 2007). We also tested for inclusion bodies and culture broth with SDS-PAGE for the possibility of aggregation or export of expressed zγGH, since secretion of this protein had been reported in mammalian tumor cell lines (Gourdon, et al., 2008). However, no enriched protein band was found in these fractions. Although we still have no confirmed answer for this result, several possibilities might help explain this observation. It is possible that the change of enzyme structure due to the presence of Nus-His-Tag expression partner had altered the quaternary structure and the overall conformation, rendering this recombinant NH-zγGH less susceptible to protease degradation. This hypothesized change in protein conformation might also contribute to the observed decreased activity. An additional possibility is the lack of an ER system in *E.coli*. The presence of a leader sequence on the N-terminal of zγGH implies an ER-associated expression and/or organelle-location for this enzyme. It has been suggested that the signal sequence must be removed for efficient expression in an *E. coli* system (Gourdon, et al., 2008).

Adding purified NH-zγGH to a de-conjugation reaction has increased the hydrolysis efficiency significantly. A 1:5 to 1:10 ratio between mammalian serum and biological sample is commonly used for folate de-conjugation in most of the folate measurement assays used currently. Incubation at 37 ^{o}C for at least 4 hours is usually required for complete hydrolysis. However, this long

incubation time has been reported to be detrimental to folates and therefore decreases accuracy significantly (Quinlivan, et al., 2006). The incubation time required to reach 80 % conversion with purified NH-zγGH was less than 15 % of that when rat serum was used. Besides, the dilution effect due to the volume of added enzyme (0.5 µl) was negligible. We even needed to dilute the purified enzyme before adding to the reaction mixture. This high efficiency allows a wide-range for the amount of enzyme added without affecting folate concentrations in the samples. For example, a second boost with more purified enzyme after the first 5-min incubation might significantly reduces the time needed for complete conversion. In addition, we used NH- zγGH in most of our assays since small amounts of this fusion enzyme had provided sufficient activity for our experiments. Nevertheless, using thrombin digested zγGH is expected to improve the hydrolysis efficiency and also decrease the incubation time.

There are still unanswered questions remaining about zγGH. It is important to investigate whether γGH is the enzyme responsible for hydrolyzing folylpolyglutamates in zebrafish intestines, since that will affect the absorbance of potential antifolate drugs to be tested in zebrafish. In addition, comparable catalytic efficiencies of zebrafish γGH at both neutral and acidic environments raises the question whether this enzyme resides only in lysosomes. The enzyme purified in this study can be used to make an antibody that will help answer the above questions. Using the zebrafish model for drug discovery is foreseen to significantly speed up the translation of drug or treatments from bench research to bedside reality by revolutionizing the cell-based assays to embryo-based screening (Kari, et al., 2007). Previously, we have shown that zebrafish folate enzymes are comparable to their human

and mammalian counterparts for their catalytic and structural properties, as well as susceptibility to antifolate compounds (Chang, et al., 2006;Chang, et al., 2007;Kao, et al., 2008). This purified recombinant zγGH will allow for further investigation to unravel the role of this key enzyme in regulating the intracellular availability of folates and antifolate drugs and to use zebrafish as an in vivo model for folate-related studies and antifolate drug discovery.

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Footnotes

1. The amino acid numbering used for zγGH in this study is numbered starting from the first methionine in the full-length peptide with the signal peptide.

2. We acknowledge the support from the grant NSC 96-2320-B-006-023-MY3 funded by National Science council, Taiwan to Tzu-Fun Fu and the grants funded by the Program for Promoting Academic Excellence of Universities, National Cheng Kung University (NCKU): D96-3500 to Tzu-Fun Fu and D96-2200 to Hua-Lin Wu, Director of Cardiovascular Research Center, NCKU.

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Figure legends

Fig. 1 Alignment of γGH peptide sequences. The shaded characters indicate amino acids that differ among compared species. Gaps, indicated by hyphens, are introduced for optimal alignment. The arrowheads indicate the conserved amino acid residues essential for enzyme activity. The brackets indicate the conserved sequences residing at the dimer interface. The boxes indicate the potential consensus residues for glycosylation. The sequences were aligned using the Clustal W method (Combet, et al., 2000) with MegAlign/DNAstar sequence analysis software (DNASTAR, Madison, WI). The GenBank accession numbers of the aligned sequences are NP_003869 for human γGH, NP_034411 for mouse γGH, NP_037092 for rat γGH and EU 918170 for recombinant zebrafish γGH in the current report.

Fig. 2 Map of plasmid pET43.1a::NH-zγGH. The construction of this plasmid is described in detail under *Materials and Methods*. The crossed and shaded areas represent the Nus peptide and zebrafish γGH without the signal peptide, respectively.

Fig. 3 SDS-PAGE of zγGH at each step of purification. Lane 1, un-induced cell lysate; lane 2, IPTG-induced cell lysate; lane 3, fraction flow through from Ni-Sepharose; lane 4, NH-zγGH eluted from Ni-Sepharose; lane 5, ammonium sulfate precipitated NH-zγGH after elution from Ni-Sepharose; lane 6, thrombin digested NH-zγGH before fractionation on the second round of

Ni-Sepharose; lane 7, Ni-Sepharose flow-through of thrombin digested NH-zγGH; lane 8, fraction eluted from the second round Ni-Sepharose; M, molecular weight marker. The upper and lower arrows indicate NH-zγGH and zγGH, respectively.

Fig. 4 Size-exclusion chromatographic profiles showing quaternary structures of recombinant zebrafish γGH. Approximately 50 μg of recombinant zebrafish NH-zγGH (A), NH-zγGH thrombin digestion mixture (B) and zγGH cleaved from NH-zγGH and purified by a second Ni-Sepharose chromatography (C) were analyzed on Superdex 200 (0.46 x 30.0 cm) and compared with molecular weight standard markers (D). Peak 1, NH-zγGH; peak 2, Nus-His-tag; peak 3, zγGH; peak 4, apo-ferritin (443 kD); peak 5, β-amylase (200 kD); peak 6, alcohol dehydrogenase (150 kD); peak 7, albumin (66 kD); peak 8, carbonic anhydrase (29 kD); peak 9, ribonuclease A (14 kD). X, molecule with unknown identity. The inset shows the plot of log M (molecular weight of standard markers) versus elution volume (close circle) and from which the molecular weight of NH- zγGH (open circle) and zγGH (open square) were estimated.

Fig. 5 HPLC chromatograms showing the concentration and time dependence of 5-CHO-THF-Glu_3 and methotrexate-Glu_5 hydrolysis. (A) 5-CHO-THF-Glu_1 generated in hydrolysis reactions catalyzed by recombinant NH-zγGH (left panel) and rat serum (right panel) was analyzed by HPLC on a C_{18} reversed phase column. The purified NH-zγGH or rat serum of indicated amounts were incubated with 2.5 μM of 5-CHO-THF-Glu_3 at 37 ^{o}C for 5 mins before

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stopping the reaction and loading on column. The number on the top of the peak represents the peak area. Peak A, 5-CHO-THF-Glu_3; peak B, 5-CHO-THF-Glu_1; peak C, 2-mercaptoethanol; peak D, 5-CHO-THF-Glu_1 standard; peak E, 10-CHO-THF-Glu_1; peak F, THF-Glu_1 (B) The concentrations of 5-CHO-THF-Glu_1 generated in the hydrolysis reactions were plotted against the reaction time. 5-CHO-THF-Glu_3 (2.5 μM) was incubated with 0.1 μg of NH-zγGH (dotted line) or rat serum of 1: 10 ratio (solid line) at 37 ^{o}C for indicated time. The concentrations of 5-CHO-THF-Glu_1 in the reaction mixtures were calculated by a linear interpolation of the peak areas of 5-CHO-THF-Glu_1 in the HPLC chromatograms from a standard curve constructed with 5-CHO-THF-Glu_1 of known concentrations (inset). (C) Methotrexate-Glu_5, instead of 5-CHO-THF-Glu_3, was used to examine the catalytic activity of NH-zγGH with HPLC using the condition as described for 5-CHO-THF-Glu_3. Peak A, MTX-Glu_5; peak B, MTX-Glu_1. The concentrations of MTX-Glu_5 (dotted line) and MTX-Glu_1(solid line) generated in the hydrolysis reactions were plotted against the reaction time (inset).

Fig. 6 HPLC chromatograms showing the higher catalytic activity observed with thrombin digested zγGH than with NH-zγGH fusion enzyme for 5-CHO-THF-Glu_3 hydrolysis.

5-CHO-THF-Glu_3 (1.6 μM) was incubated with buffer only (top), 3 nM NH-zγGH (middle) and 3 nM thrombin digested zγGH (bottom) at 37 ^{o}C for 5 mins before being analyzed with HPLC. The number on the top of each peak represents the area under the corresponding peak.

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Table 1 Comparison of kinetic parameters for recombinant zebrafish and human γ–GH.

Species	[1]K_m (μM)	k_{cat} (min^{-1})	V_{max} (nmole/min•μg)	Reference
Zebrafish	57.0±9.8	2890±215	87.6 ±6.5	Present report
Human	49.0±14.8	NA	2.6±1.0	Chave et al. (Chave, et al., 1999)

[1] The substrate used in the assays for zebrafish enzyme was 5-CHO-THF-Glu_3; while the substrate used for human enzyme was 4-NH_2-10-CH_3 PteGlu_2. NA, not available

Fig. 1

Fig. 2

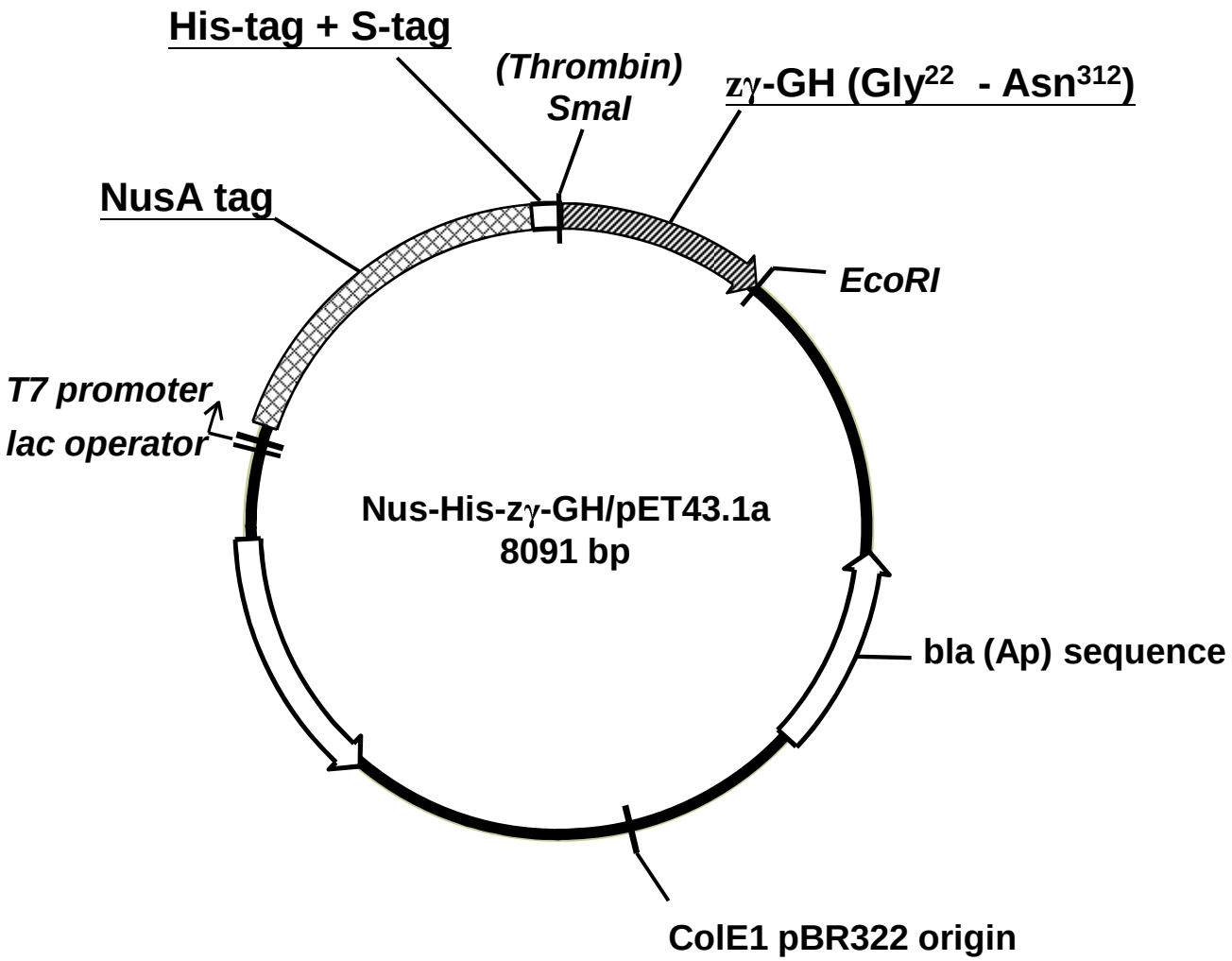


Fig. 3

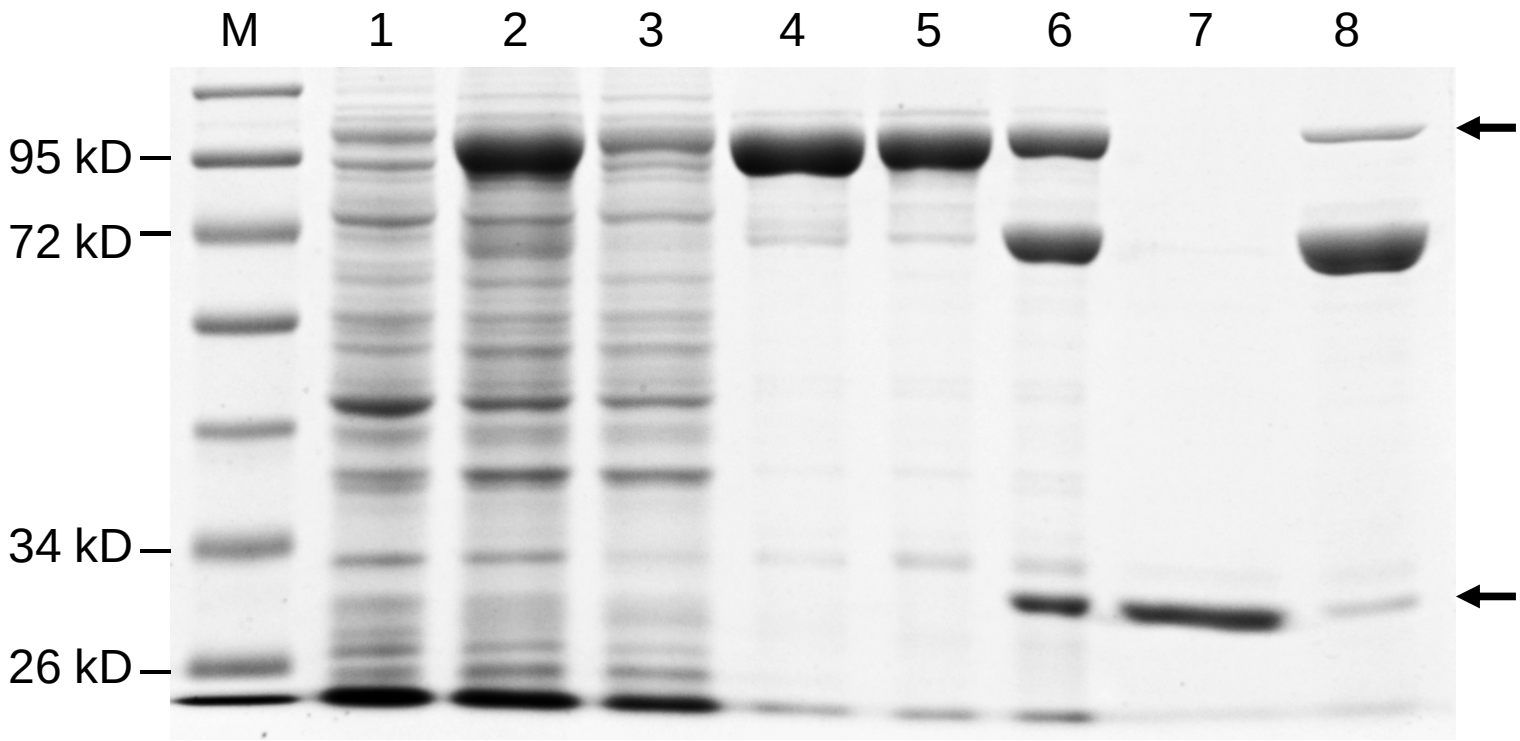


Fig. 4

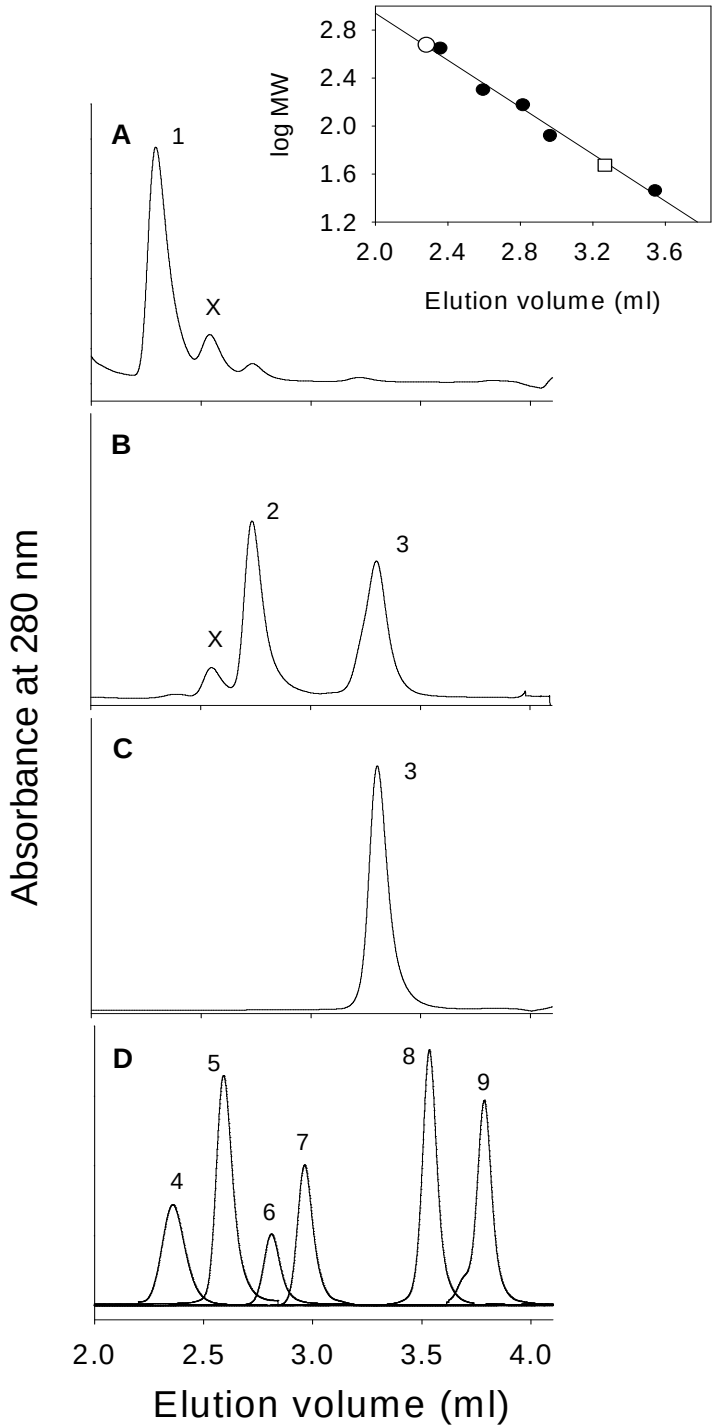


Fig. 5A

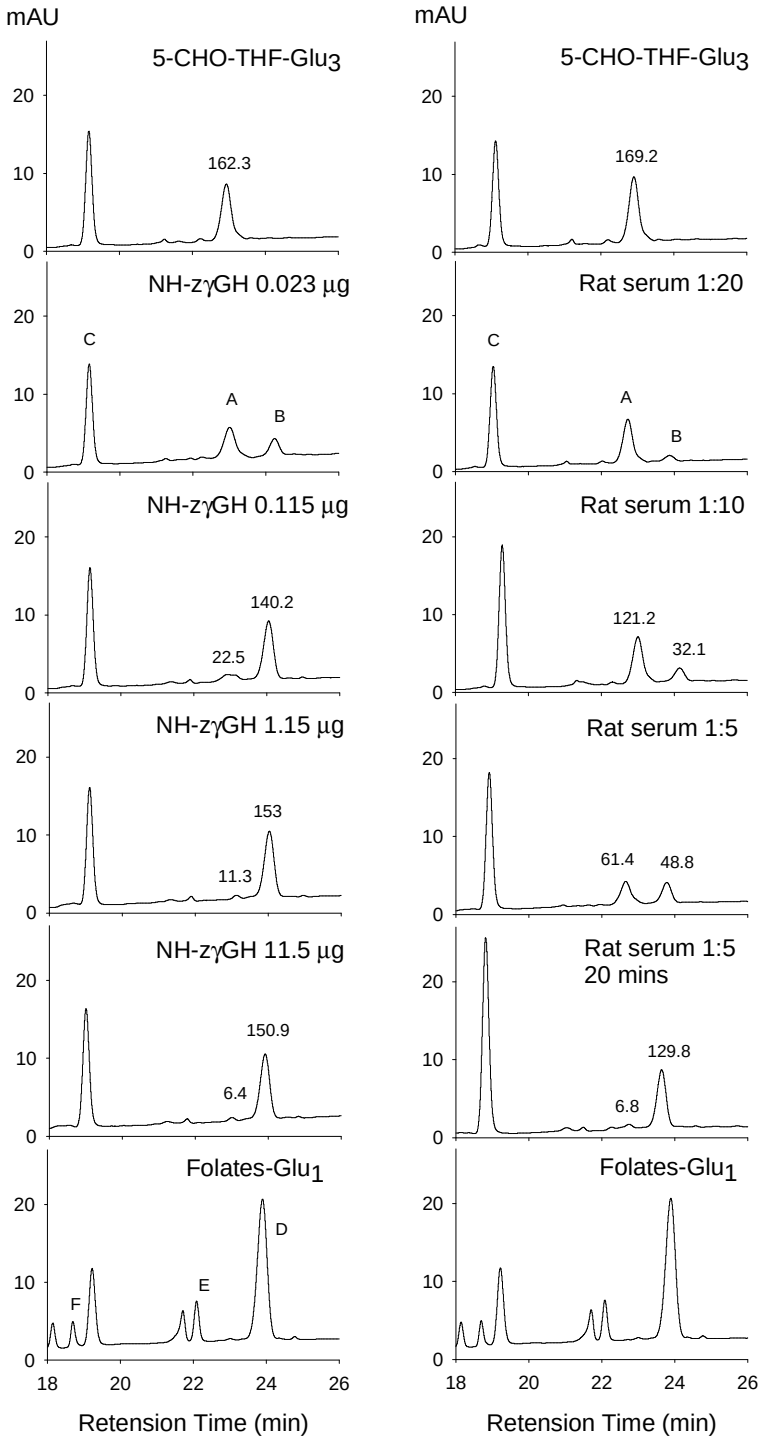
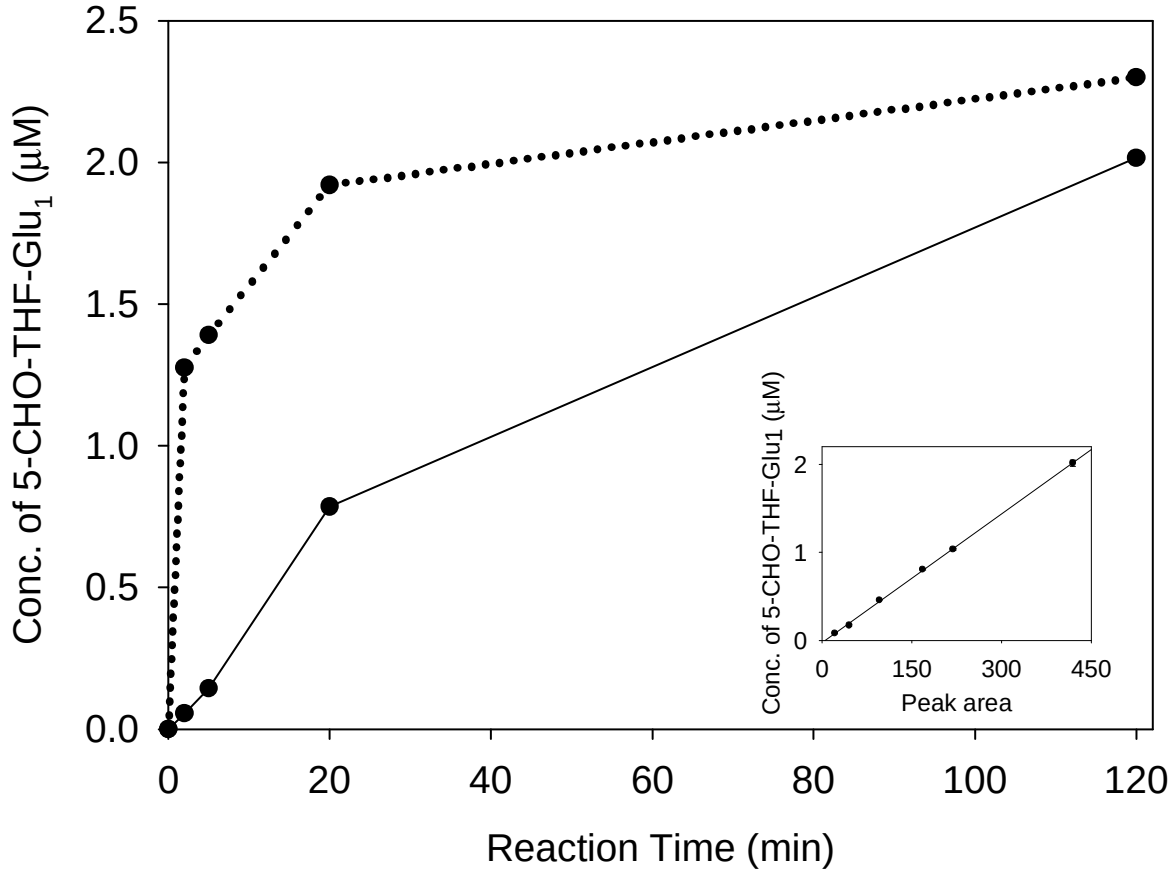


Fig. 5B



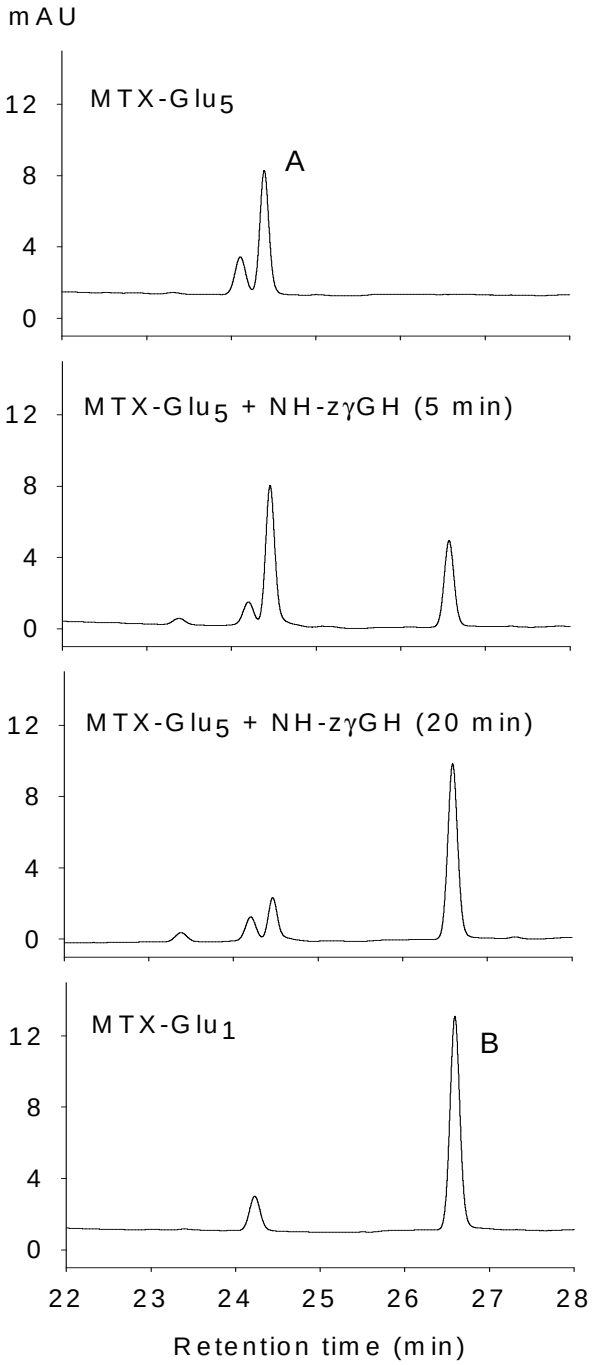


Fig. 5C

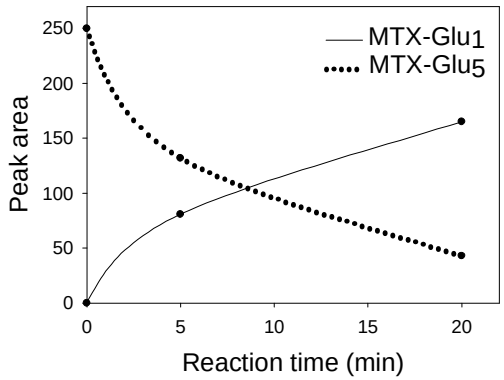


Fig. 6