Kinetics and thermodynamics of transpeptidation catalysed by *Bacillus subtilis* gamma glutamyl transferase

Sharath Balakrishna* & Asmita A Prabhune

Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road,
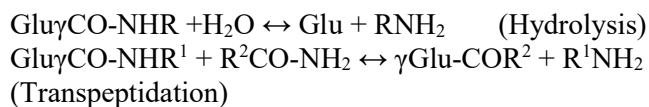Pune - 411 008, Maharashtra, India

Gamma glutamyl transferases (GGT) catalyse the removal (deglutamylation) of the terminal γ-glutamate residue from compounds such as glutathione and poly-γ-glutamic acid and its transfer either to a water molecule (hydrolysis) or to a peptide/amino acid (transpeptidation). We analysed the kinetics of *Bacillus subtilis* GGT (BsGGT) catalysed transpeptidation using γ-glutamyl-(3-carboxyl)-4-nitroaniline as the γ-glutamate-donor and glycylglycine (Gly-Gly) as the γ-glutamate-acceptor. Addition of Gly-Gly improved the affinity (K_m) of the enzyme for γ-glutamyl-(3-carboxyl)-4-nitroaniline by nearly 25 times with negligible impact on the rate of deglutamylation (V_{max}). The asymmetric changes in the kinetic parameters improved the specificity constant (k_{cat}/K_m) by about 43 times. BsGGT catalysed transpeptidation was pronounced in conditions that are unfavorable for hydrolysis. Maximum transpeptidation occurred near neutral pH and when the concentration of the γ-glutamate-donor substrate is lower. The effect of Gly-Gly on the kinetics of BsGGT is contrastingly different from that observed for eukaryotic GGTs. In the case of mammalian GGTs, the addition of Gly-Gly increases both K_m and k_{cat}; and, the specificity constant (k_{cat}/K_m) remains unaltered.

Keywords: Glycylglycine, Steady state kinetics

Gamma glutamyl transferases (GGT; E.C.2.2.3.2) are highly conserved enzymes that occur in archaebacteria, eubacteria, fungi, protozoa, nematodes, plants and mammals[1]. *Bacillus subtilis* produces a mucilaginous polymer (2×10^6 Da) composed of γ-polyglutamic acid during the early stationary phase and releases it into the extracellular medium. γ-Polyglutamic acid is consumed during the nutritionally limited late stationary phase as a source of nitrogen. The polymer is first degraded internally by an endo-type γ-polyglutamic acid hydrolase into 1×10^5 Da intermediates and then externally into glutamates by GGT. Inactivation of the *ggt* gene incapacitates the degradation of 1×10^5 Da intermediates and increases the frequency of sporulation[2]. In *Helicobater pylori* and *Niesseria meningitides*, GGT helps the bacteria to colonise the intestinal epithelium[3]and the brain[4] respectively. Mammalian

GGTs are involved in homeostasis of glutathione (a major antioxidant)[5], xenobiotic detoxification[6] and in pathologies like metastasis[7], drug resistance of malignant cells[8], cardiovascular diseases[9], inflammation[10], diabetes[11] and neurodegenerative diseases[12].

GGTs act on substrates of the general formula Gluγ(CO)-(NH)R. The enzyme removes the terminal γ-glutamyl moiety (deglutamylation) and then transfers it to a water molecule (hydrolysis) or an amine like peptides and amino acids (transpeptidation). The two reactions are represented below:

GluγCO-NHR $+H_2O \leftrightarrow$ Glu + RNH_2 (Hydrolysis)
GluγCO-NHR^1 + R^2CO-$NH_2 \leftrightarrow$ γGlu-COR^2 + R^1NH_2 (Transpeptidation)

GGTs undergo autocatalytic post-translational proteolysis to produce a heterodimeric active enzyme. GGTs use a nucleophilic substitution mechanism for catalysis. Upon formation of the enzyme-substrate complex, the γ-glutamyl moiety is esterified to an active site nucleophile (N-terminal Thr of the smaller subunit) to form an acyl-enzyme complex[13,14]. The γ-glutamyl moiety is then displaced from the active site (deacylation) when a non-enzymic nucleophile attacks the ester link and substitutes it with an amide bond.

―――――――――――
*Correspondence:
Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar - 563 101, Karnataka, India
Phone: 9611228095
E-mail: sbk.sharath@gmail.com
Abbreviations: *Bs*GGT, *Bacillus subtilis* Gamma Glutamyl Transferase; γ-GCN, γ-Glutamyl-(3-carboxyl)-4-nitroaniline; Gly-Gly; Glycylglycine

Glutamic acid is the end product when water serves as the deacylating agent and the overall reaction is hydrolysis. Transpeptidation arises when the deacylating function is provided by a compound with a free amino group. The end product in the case of transpeptidation is a γ-glutamylated peptide. Among amino acids and peptides, Gly-Gly depeptide serves as the best deacylating agent (hereon denoted as acyl-acceptor) for *Bacillus subtilis* GGT (hereon referred to as *Bs*GGT) mediated transpeptidation[15].

Transpeptidation activity of mammalian GGTs has been the subject of much interest as it can increase the rate of deglutamylation by up to 1000 fold relative to hydrolysis[16]. In contrast, acyl-acceptors have a marginal effect on the rate of deglutamylation catalysed by bacterial GGTs; therefore, the transferase activity of the bacterial homologues was assumed to be unimportant. Here we show that *Bs*GGT is capable of catalyzing a robust transpeptidation reaction which is markedly different from that of the eukaryotic homologues.

Materials and Methods

Chemicals

Glutamyl-(3-carboxyl)-4-nitroaniline ammonium salt was obtained from Fluka (Switzerland). Glycylglycine and all other reagents were from Sigma-Aldrich (USA).

Cloning, expression, and purification of *Bs*GGT

These details are published elsewhere[17]. In brief, the GGT ORF was amplified (by PCR method) from the genomic DNA of *B. subtilis* and cloned into pET-26b plasmid DNA between *NcoI* and *XhoI* sites. The resultant construct was expressed in *E. coli* strain BL21 at 16°C overnight. The IPTG induced cell pellet was homogenised using French press and the enzyme was purified from the homogenate by a combination of nickel affinity chromatography and size-exclusion chromatography using Hi-Load Superdex S200.

Enzyme assay

The hydrolase activity was assayed by incubating ~0.4 μg of the enzyme sample at 30°C for 5 min with the ammonium salt of γ-glutamyl-(3-carboxyl)-4-nitroaniline in 100 mM buffer in a final volume of 100 μL. Buffers used were tricine for pH 7.5 and 9.0 and CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) for pH 11. The reaction was stopped by the addition of 900 μL of 2 M acetic acid and the absorbance was measured at 410 nm. Suitable blanks were used to correct for absorbance due to the substrate. The One unit of activity was defined as the amount of enzyme required to produce 1 μmole of 3-carboxyl-4-nitroaniline in a minute. The assay was linear with respect to the time of incubation. For measuring the transferase activity, 100 mM Gly-Gly was included in the reaction after adjusting its pH to that of the assay conditions.

Steady state kinetics

Substrate saturation plots were prepared by measuring the reaction rates in a range of substrate concentrations. Concentrations were chosen to give a relatively even distribution of the data points. K_m and V_{max} were determined by fitting the velocities to Michaelis-Menten equation (Eq. 1) by non-linear regression method using Origin 6.1 software:

$$v = V_{max} \frac{[S]}{K_m + [S]} \qquad \text{Eq. 1}$$

The velocities determined in pH 11 buffer were fitted to Hill equation of the form:

$$v = V_{max} \frac{[S]^h}{K_{0.5} + [S]^h} \qquad \text{Eq. 2}$$

where v is the initial velocity, V_{max} is the maximal velocity, $K_{0.5}$ is the substrate concentration for half maximal activity and h is the measure of cooperatively between n interacting sites.

Thermodynamic analysis

The value of k_{cat} for the respective reaction was determined at 20, 30, 40 and 50°C in 100 mM tricine HCl, at pH 7.5. Arrhenius plot was used to calculate the activation energy. The data were fitted by linear regression to the equation:

$$\ln k_{cat} = -\frac{Ea}{RT} + \ln A \qquad \text{Eq. 3}$$

where Ea is the activation energy, R is the gas constant, T is the absolute temperature and A is the frequency of collision. The slop of this plot is equal to –Ea/R.

Eyring's plot was used to determine the enthalpy and the entropy of activation. The data were fitted by linear regression to the equation:

$$\ln \frac{k_{cat}}{T} = \ln \frac{k_B}{h} + \frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{RT} \qquad \text{Eq. 4}$$

where $\Delta H^\ddagger$ is the enthalpy of activation, $\Delta S^\ddagger$ is the entropy of activation, h is Planck's constant, k_B is Boltzmann's constant, R is the gas constant and T is the absolute temperature.

From the plot, $\Delta H^{\ddagger}$ was calculated from the slope ($= -\Delta H^{\ddagger}/R$) and $\Delta S^{\ddagger}$ from the y-axis intercept. Free energy of activation ($\Delta G^{\ddagger}$) was calculated from the equation:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \qquad \text{Eq. 5}$$

Results and Discussion

Kinetics of transpeptidation

Addition of 100 mM Gly-Gly to the reaction mixture was found to stimulate the breakdown of 2 mM γ-glutamyl-(3-carboxyl)-4-nitroaniline (γ-GCN) by about 4 times at pH 9.0 (Fig. 1). We, therefore, examined the kinetics of transpeptidation catalysed by BsGGT using γ-GCN as the acyl-donor and Gly-Gly dipeptide as the acyl-acceptor substrates. BsGGT was assayed with a range of Gly-Gly concentrations (10-200 mM) and a fixed amount of γ-GCN (10 mM) in pH 7.5 buffer; from this, K_m for Gly-Gly was determined to be 50 mM. All the subsequent transpeptidation reactions were performed by including 100 mM Gly-Gly in the assay mixture. The effect of Gly-Gly on the kinetics of γ-GCN deglutamylation is shown in Table 1. In the absence of Gly-Gly, K_m of BsGGT for γ-GCN was 25.9 mM at pH 7.5. The K_m decreased to 0.97 mM when 100 mM Gly-Gly was added to the reaction mixture. In contrast, the addition of Gly-Gly increases the

V_{max} marginally by 1.6 times. Inspection of the substrate saturation curves (Fig. 1) shows that the stimulatory effect of Gly-Gly is inversely related to the concentration of the acyl-donor. The largest stimulation is produced when the concentration of γ-GCN, the acyl-donor, is at its lowest. The asymmetric changes in the kinetic parameters increase the specificity constant (k_{cat}/K_m) by about 43 times (Table 1). Thus, Gly-Gly stimulates deglutamylation of γ-GCN by improving the enzyme's affinity for the acyl-donor.

Effect of pH on transpeptidation

Our previous study showed that the nature of hydrolytic kinetics of BsGGT is significantly affected by the assay pH[17]. The kinetics followed standard Michaelian form producing hyperbolic substrate saturation curves between pH 7.0 and 9.0. The substrate saturation curves changed to sigmoid form when the assay pH was at or above 10.0. A Similar trend was observed with the kinetics of transpeptidation also (Fig. 1).

The stimulatory effect of Gly-Gly on deglutamylation of γ-GCN was significantly affected by the assay pH (Table 1). In pH 7.5, the addition of 100 mM Gly-Gly lowered the K_m for γ-GCN by 26 times. The decrease in K_m was 4.5 times in pH 9.0 and 1.1 times at pH 11.0. The effect of pH on transpeptidation activity is opposite to that on its hydrolytic activity. The
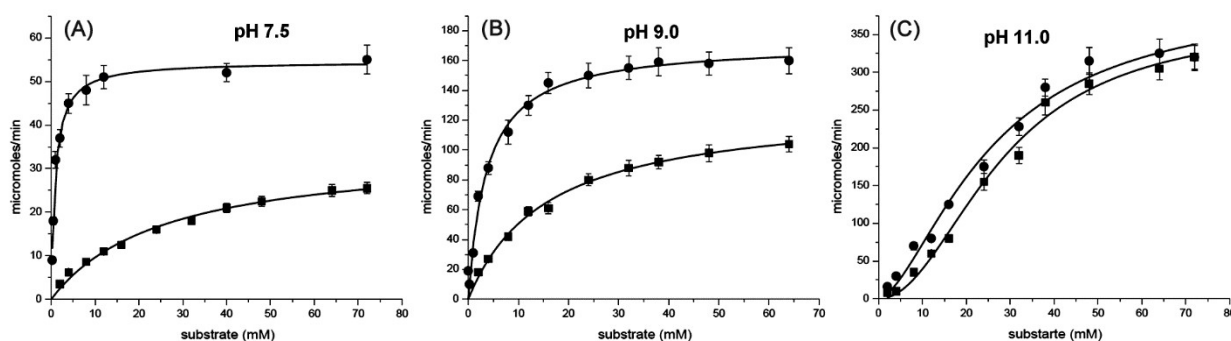

Fig. 1 — Substrate saturation plots showing (●) BsGGT catalyzed hydrolysis and (■) transpeptidation of γ-GCN at pH 7.5, 9.0 and 11.0.

Table 1 — Effect of 100mM Gly-Gly on the kinetics of degulamylation of γ-GCN

pH	γ-GCN hydrolysis			γ-GCN transpeptidation			T/H[*]
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1}mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k'_{cat}/K_m (s^{-1}mM^{-1})	
7.5	34.5 ±1.03	25.9±1.98	1.3	55.5± 2.18	0.97± 0.12	57.2	43
9.0	132 ± 5.4	16.7 ±1.8	7.9	171 ± 2.1	3.7 ±0.2	46.2	5.8
11.0	365 ± 26.3	26.9 ± 2.7	13.6	386.4 ± 28	24.1 ± 2.8	16.1	1.2

* Fold increase in k_{cat}/K_m of transpeptidation over that of hydrolysis

hydrolytic function, which is almost negligible at pH 7.5, progressively increases as a function of pH and produces maximum activity at pH 11[17]. The specificity constant (k_{cat}/K_m) for γ-GCN increased as a function of pH during hydrolysis but decreased in case of transpeptidation (Table 1). Improvement of the specificity constant for γ-GCN, due to 100 mM Gly-Gly, was about 43 times at pH 7.5 but negligible at pH 11.0. Thus, assay pH and the rate of transpeptidation are inversely related.

Thermodynamics of transpeptidation

The dependence of k_{cat} on the assay temperature was measured in the range 20-50°C and used to construct Eyring and Arrhenius plots (Fig. 2). The plots were linear in the experimental range. The thermodynamic parameters for hydrolysis and transpeptidation reactions are given in Table 2. The activation enthalpy is positive in both the cases as is expected for reactions involving bond breakage which are endothermic. The free energy of activation ($\Delta G^{\ddagger}$) was calculated from $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ at 25°C. The positive values of $\Delta G^{\ddagger}$ are expected as the formation of the transition state is non-spontaneous. Thus, hydrolysis and transpeptidation differ primarily in the enthalpy of
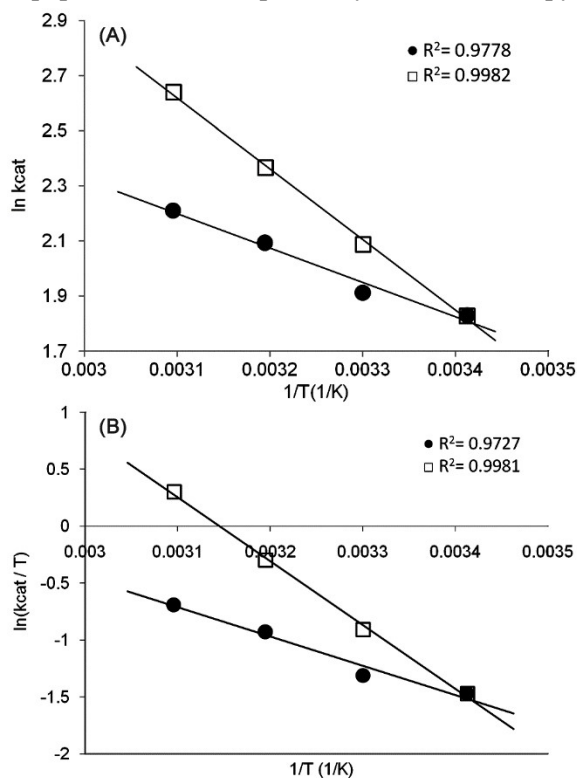
activation. Changes in the enthalpy of activation represent changes in the formation and breakage of chemical bonds and thus the mechanism of the reaction. In contrast, the entropy of activation measures the extent of the order of the activated complex in the transition state. From Table 2, we see that the activational enthalpy for transpeptidation is almost two-times the value for hydrolysis. This indicates that Gly-Gly alters the rate limiting step of γ-GCN deglutamylation. This is in agreement with the reported pre-steady state studies with rat kidney GGT, wherein deglutamylation of the acyl-donor was limited by the rate of deacylation of the enzyme. Deacylation was improved upon addition of Gly-Gly[18]. Both hydrolysis and transpeptidation share the first catalytic step (acylation). So the formation of 3-carboxyl-4-nitroaniline represents the rate of depletion of the acyl-donor in both the reactions.

Comparison of transpeptidation activities of *B. subtilis* and mammalian GGTs

Addition of 100 mM Gly-Gly to *Bs*GGT catalysed deglutamylation of γ-GCN significantly improves the K_m with minor effect on the V_{max}. The asymmetric changes in K_m and V_{max} result in an increase of the specificity constant (k_{cat}/K_m). The changes in the kinetic parameters are contrary to those observed for mammalian GGTs (Table 3). Addition of Gly-Gly results in an increase in both K_m and V_{max} for GGTs from human[16] and rat[19] tissues. Also, the specificity constant mostly remains unaffected. Transpeptidation catalysed by bovine GGT is an unusual case; K_m decreases at lower concentrations of Gly-Gly, reaches a minimum and thereafter increases[20]. In general, bacterial GGTs have insignificant transpeptidation activity. Addition of acyl-acceptors like Gly-Gly increases the velocity of deglutamylation mostly by about 2 fold in GGTs from *Helicobacter pylori*[21], *Escherichia coli*[22], *and Proteus mirabilis*[23]. Our results show that *Bs*GGT is capable of catalyzing robust transpeptidation, which is kinetically different from that catalyzed by the mammalian GGTs.

Fig. 2 — (A)Arrhenius and (B) Eyring plots showing thermodynamics difference between (●) *Bs*GGT catalyzed hydrolysis and (□) transpeptidation of γ-GCN.

Table 2 — Comparison of the thermodynamic parameters for the reactions catalyzed by *Bs*GGT

Reaction	Free Energy of Activation ($\Delta G^{\ddagger}$) KJ/mol	Activation Enthalpy ($\Delta H^{\ddagger}$) KJ/mol	Activation Entropy ($\Delta S^{\ddagger}$) J/molK	Activation Energy (Ea) KJ/mol
Hydrolysis	(109.2)	49.2	−201.3	23.9
Transpeptidation	(163.4)	107.3	−190.9	49.1

Table 3 — Comparison of the effect of acyl-acceptor on the kinetics of acyl-donor deglutamylation catalyzed by various GGT homologs.

GGT	Absence of Acyl-Acceptor			Presence of Acyl-Acceptor		
	V_{max}	K_m (μM)	V_{max}/K_m	V_{max}	K_m (μM)	V_{max}/K_m
B. subtilis	25.9[a]	25900	1×10^{-3}	41.7[a]	950	40×10^{-3}
Human[16]	4.3[a]	7.2	0.6	800[a]	1000	0.8
Rat[19]	0.59[b]	13.9	23.6	5.46[b]	113	20.1
Helicobacter pylori[21]	5.81[a]	12.5	2.2	6.81[a]	10.9	0.6
Proteus mirabilis[22]	0.67[a‡]	310	0.002	0.8[a‡]	400	0.002
Escherichia coli[23]	0.42[c‡]	35	0.012	0.48[c‡]	68	0.007

[a]μmoles/min/mg; [b]μmoles/L; [c]nmoles/min; ‡Specific activity under standard assay conditions is shown as values for V_{max} were not available

Conclusions

Our study shows that Gly-Gly significantly improves the affinity of *Bs*GGT for the γ-glutamyl donor substrate. Comparative analysis shows that the effect of Gly-Gly on the kinetics of transpeptidation catalysed by *Bs*GGT is different from that observed for the mammalian GGTs.

References:

1 Balakrishna S & Prabhune AA, Gamma-glutamyl transferases: A structural, mechanistic and physiological perspective. *Fron Biol*, 9 (2014) 51.

2 Luo Z, Guo Y, Liu J, Qiu H, Zhao M, Zou W & Li S, Microbial synthesis of poly-γ-glutamic acid: current progress, challenges, and future perspectives. *Biotechnol Biofuels*, 29 (2016) 9.

3 Ricci V, Giannouli M, Romano M & Zarrilli R, *Helicobacter pylori* gamma-glutamyl transpeptidase and its pathogenic role. *World J Gastroenterol*, 20 (2014) 630.

4 Schoen C, Kischkies L, Elias J & Ampattu BJ, Metabolism and virulence in Neisseria meningitidis. *Front Cell Infect Microbiol*, 4 (2014) 114.

5 Lu SC, Regulation of glutathione synthesis. *Mol Aspects Med*, 30 (2009) 42.

6 Lee DH & Jacobs DR, Is serum gamma-glutamyltransferase a marker of exposure to various environmental pollutants? *Free Radic Res*, 43 (2009) 533.

7 Staudigl C, Concin N, Grimm C, Pfeiler G, Nehoda R, Singer CF & Polterauer S, Prognostic Relevance of Pretherapeutic Gamma-Glutamyltransferase in Patients with Primary Metastatic Breast Cancer. *PLoS One*, 10 (2015) e0125317.

8 Corti A, Franzini M, Paolicchi A & Pompella A, Gamma-glutamyltransferase of cancer cells at the crossroads of tumor progression, drug resistance and drug targeting. *Anticancer Res*, 30 (2010) 1169.

9 Jiang S, Jiang D & Tao Y, Role of gamma-glutamyltransferase in cardiovascular diseases. *Exp Clin Cardiol*, 18 (2013) 53.

10 Ali SS, Oni ET, Blaha MJ, Veledar E, Feiz HR, Feldman T, Agatston AS, Blumenthal RS, Conceicao RD, Carvalho JA, Santos RD & Nasir K. Elevated gamma-glutamyl transferase is associated with subclinical inflammation independent of cardiometabolic risk factors in an asymptomatic population: a cross-sectional study. *Nutr Metab*, 18 (2016) 37.

11 Rajarajeswari D, Krishna TS, Naidu MP & Naidu JN, Serum gamma glutamyl transferase levels in association with lipids

and lipoproteins in type2 diabetes mellitus. *Int J Res Med Sci*, 2 (2014) 838.

12 Yavuz BB, Yavuz B, Halil M, Cankurtaran M, Ulger Z, Cankurtaran ES, Aytemir K & Ariogul S, Serum elevated gamma glutamyltransferase levels may be a marker for oxidative stress in Alzheimer's disease. *Int Psychogeriatr*, 20 (2008) 815.

13 West MB, Chen Y, Wickham S, Heroux A, Cahill K, Hanigan MH & Mooers BH, Novel insights into eukaryotic γ-glutamyltranspeptidase 1 from the crystal structure of the glutamate-bound human enzyme. *J Biol Chem*, 288 (2013) 31902.

14 Wickham S, West MB, Cook PF & Hanigan MH, Gamma-Glutamyl Compounds: Substrate Specificity of Gamma-Glutamyl Transpeptidase Enzymes. *Anal Biochem*, 414 (2011) 208.

15 Morelli CF, Calvio C, Biagiotti M & Speranza G, pH-dependent hydrolase, glutaminase, transpeptidase and autotranspeptidase activities of Bacillus subtilis γ-glutamyltransferase. *FEBS J*. 281 (2014) 232.

16 Ikeda Y, Anderson M E, Taniguchi N & Meister A, Involvement of Ser-451 and Ser-452 in the catalysis of human gamma-glutamyl transpeptidase. *J Biol Chem*, 270 (1995), 22223.

17 Balakrishna S & Prabhune A, Effect of pH on the Hydrolytic Kinetics of Gamma-Glutamyl Transferase from *Bacillus subtilis*. *The Sci World J*, 2014 (2014) 216270.

18 Castonguay R, Halim D, Morin M, Furtos A, Lherbet C, Bonneil E, Thibault P, Keillor JW, Kinetic characterization and identification of the acylation and glycosylation sites of recombinant human gamma-glutamyl transpeptidase. *Biochemistry*, 46 (2007) 12253.

19 Wickham S, West MB, Cook PF & Hanigan MH, Gamma-glutamyl compounds: substrate specificity of gamma-glutamyl transpeptidase enzymes. *Anal Biochem*, 414 (2011) 208.

20 Gololobov MY & Bateman RC, Gamma-Glutamyltranspeptidase-catalysed acyl-transfer to the added acceptor does not proceed *via* the ping-pong mechanism. *Biochem J*, 304 (1994) 869.

21 Boanca G, Sand A & Barycki JJ, Uncoupling the enzymatic and autoprocessing activities of Helicobacter pylori gamma-glutamyltranspeptidase. *J Biol Chem*, 281 (2006) 19029.

22 Suzuki H, Kumagai H & Tochikura TT, gamma-Glutamyltranspeptidase from Escherichia coli K-12: purification and properties. *J Bacteriol*, 168 (1986) 1325.

23 Nakayama R, Kumagai H & Tochikura T, Purification and properties of gamma-glutamyltranspeptidase from Proteus mirabilis. *J Bacteriol*, 160 (1984) 341.