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Purification and Characterization of γ-Glutamyltranspeptidase from *Bacillus subtilis* SK11.004

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ABSTRACT: An extracellular γ-glutamyltranspeptidase (GGT) with a specific activity of 683.4 U/mg was purified to homogeneity from a culture filtrate of *Bacillus subtilis* SK11.004 in three steps and then characterized. The GGT is composed of one large subunit of 40 kDa and one small subunit of 21 kDa that was determined by SDS−PAGE and a molecular mass of 62 kDa that was determined by gel-filtration chromatography. The purified GGT had an optimal pH and temperature of 10 and 37 °C, respectively, and it was stable at pH 4.0−11.0 or <50 °C. The enzyme exhibited the highest affinity to imino acids (L-Pro) and then decreasing affinities for aromatic amino acids, ethylamine and basic amino acids. The K_m values of hydrolysis and of transpeptidation for L-Gln were 3.16 mM and 0.83 mM, respectively, suggesting that the GGT likely synthesizes valuable γ-glutamyl peptides using L-Gln as γ-glutamyl donor. The effects of inhibitors on the enzyme suggested that the tryptophan residues and hydroxy groups of Ser or Thr are essential to enzyme activity. Based on the biochemical characteristics of the enzyme and lack of homology to previously identified proteins, it can be concluded that the GGT from *B. subtilis* SK11.004 is a novel enzyme.

KEYWORDS: *Bacillus subtilis* SK11.004, gamma-glutamyltranspeptidase, purification, characterization

INTRODUCTION

Gamma-glutamyltranspeptidases (GGTs; EC 2.3.2.2) catalyze the cleavage of the γ-glutamyl bond of glutathione, and other related γ-glutamyl compounds, by transferring the γ-glutamyl group to either water (hydrolyzation) or other amino acids (transpeptidation).[1] These enzymes are involved in the metabolism of glutathione and found from bacteria to mammals. Whereas mammalian GGTs are embedded in the plasma membrane via a single N-terminal trans-membrane anchor and are heterologously glycosylated, bacterial homologous are generally soluble, not glycosylated and localized in the periplasmic space or extracellular space, especially in *Bacillus* species strains.[2−7] Many bacterial GGTs have been purified and characterized, but all have different molecular weights and subunit compositions that might lead to the differences in enzyme structure and properties. However, GGT-encoding genes from many bacterial species have been sequenced[8−13] and share extensive amino acid homology. The enzymes are translated as propolypeptides of approximately 580 amino acids and subsequently processed to form the two subunits of the mature enzymes. The N-terminal residue of the processed enzyme, a strictly conserved threonine, has been proposed to be the catalytic nucleophile in both the autoprocessing and the enzymatic reaction.[14−16]

By employing various γ-glutamyl acceptors, various γ-glutamyl compounds can be synthesized using GGT. Many functional γ-glutamyl compounds have been shown to be produced effectively by bacterial GGT, such as γ-L-glutamyl-L-DOPA, γ-glutamyl taurine, γ-D-glutamyl-L-tryptophan and γ-glutamyl-ethylamide (L-theanine).[17] With this method, modifications of reactive groups of the substrate and energy source such as ATP are not required.

Previously, we developed an efficient method for synthesizing theanine using GGT from *Bacillus subtilis* SK11.004 (BsGGT), however, we focused on the strain isolation and optimization of enzymatic synthesis.[18] This study aimed to characterize the molecular weight, subunit composition, optimum reaction conditions, stability, substrate specificity and inhibitors of BsGGT. The protein was also analyzed by MALDI-TOF-TOF MS, providing clues for the further study of its molecular characteristics.

MATERIALS AND METHODS

Materials. γ-Glutamyl-α-naphthylamide, γ-glutamyl-*p*-nitroanilide (γ-GpNA), fast garnet GBC salt and chemical modifiers (BD, DEPC, DTNB, EDC, EDTA, PMSF, NAI, NBS, and 2-ME) were purchased from Sigma (St. Louis, MO, USA). All chromatographic equipment, gels and standards were supplied by GE Healthcare (Uppsala, Sweden). Electrophoresis reagents were bought from Bio-Rad Laboratories Inc. (Richmond, CA, USA). Other chemicals were of analytical grade and were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Microorganism and Culture. *B. subtilis* SK11.004 (CCTCC No. M 208083), isolated from shrimp paste,[18] was used in the present investigation. *B. subtilis* SK11.004 was cultured in 30 mL of medium containing 2.5% sucrose, 0.5% tryptone, 1.5% corn step liquor, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.1% $K_2HPO_4 \cdot 3H_2O$ at pH 7.2 in a 250 mL flask and aerobically incubated at 37 °C in a rotary shaker at 200 rpm for 16 h. Then, the culture broth was centrifuged at 10000*g* and 4 °C for 30 min to remove the insoluble materials, and the supernatant was collected for enzyme purification.

Purification of GGT from *B. subtilis* SK11.004. After a 30 min centrifugation at 10000*g* and filtration through a 0.45 μm membrane, crude GGT from the culture broth was precipitated by 60−80%

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ammonium sulfate saturation. After 20 min of centrifugation at 10000*g*, the precipitate was dissolved in 50 mM Tris-HCl buffer (pH 8.0, buffer A) and dialyzed against the same buffer at 4 °C overnight. The resulting crude enzyme was purified on a Hiprep DEAE-sepharose FF 16/10 column with a stepwise elution 2 column volumes each of 0.1 M, 0.2 M, 0.3 M, 0.4 and 0.5 M NaCl in buffer A at a flow rate of 1 mL/min. Fractions with GGT activity were collected and concentrated by ultrafiltration with an Amicon PM-10 membrane (Millipore Co., Bedford, MA, USA). The concentrated enzyme solution was then eluted from a Superdex 75 10/300 GL column with buffer A at a flow rate of 0.5 mL/min. The molecular mass of the enzyme was determined using the same column, which was previously calibrated with a range of reference proteins: BSA (67 kDa), ovalbumin (43 kDa), ribonuclease (13.7 kDa), aprotinin (6.5 kDa) and vitamin B_{12} (1.4 kDa). Blue dextran was used to determine the void volume of the column. Fractions with GGT activity were collected and subjected to enzyme activity assay and electrophoresis (SDS−PAGE). The above-mentioned procedures were performed at <5 °C.

GGT Activity Assay and Protein Determination. The enzyme assay was performed according to the method previously described.[18] The assay mixture (1 mL) was composed of 50 mM borate−NaOH (pH 10), 5 mM γ-GpNA, 20 mM Gly-Gly and the enzyme solution. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 0.1 M HCl. The transferase activity was calculated from the difference in absorbance between reaction mixtures with and without Gly-Gly at 410 nm. One unit of GGT was defined as the amount of enzyme that released 1 μmol of *p*-nitroaniline per minute from γ-GpNA by the transpeptidation reaction.

Protein concentration was determined by the Lowry method with BSA as a standard.[19]

Electrophoresis and Activity Staining. SDS−PAGE was carried out according to the procedure of Laemmli[20] to determine the molecular mass and subunit composition of the enzyme using a 12% acrylamide separating gel and 4% acrylamide stacking gel. Gels were run at 20 mA for approximately 50 min using a Bio-Rad PowerPac Basic (Bio-Rad, Hercules, CA). The gel sheets were stained for proteins with 0.25% Coomassie brilliant blue (CBB)-R250.

Native PAGE was carried out on a 12% polyacrylamide gel under constant electric current at 20 mA for approximately 50 min at 4 °C. Gels were then cut into two parts. One was stained for proteins with 0.25% Coomassie brilliant blue (CBB) R250. The other was stained for GGT activity using a modification of the method from Albert et al.[21] The gel was incubated at 37 °C for 15 min in 50 mL of the staining mixture (25 mL of a 1.1 mg/mL concentration of γ-L-glutamyl-α-naphthylamide, 2 mL of 1 M K-phosphate buffer, pH 7.0, 10 mL of 0.3 M Gly-Gly, 25 mg of fast garnet GBC, and 13 mL of distilled water) and then washed once in 20 mM Tris-HCl buffer (pH 8.0).

Indentifying BsGGT by MALDI-TOF-TOF MS. After native PAGE analysis, the GGT band was excised and cut into small pieces. The gel was destained with 50% acetonitrile in 100 μL of 50 mM ammonium bicarbonate and digested with 10 μL of 12.5 ng/μL porcine trypsin (Promega, Madison, WI) overnight at 37 °C. Peptides were extracted from the gels with 60 μL of elution buffer containing 50% ACN and 0.1% TFA. The digested peptides were spotted onto the MALDI sample plate and dried in air, followed by the addition of 0.7 μL of the matrix solution containing α-cyano-4-hydroxycinnamic acid in 50% ACN, containing 0.1% TFA. The target plate was loaded into a Proteomics 4700 MALDI-TOF-TOF Analyzer (Applied Biosystems, Foster City, CA), and the spectra were acquired in reflector mode on positively charged ions. Raw spectrum files were opened and searched in GPS Explorer TM software (Applied Biosystems, USA) and MASCOT (Matrix Science, London, U.K.).

Effect of pH and Temperature on Activity. The effect of pH on the transpeptidase activity and hydrolytic activity of BsGGT was determined at 37 °C by performing 30 min incubations according to the assay procedure at various pH values. The relative activities are expressed as a percentage of the maximum enzyme activity. For pH stability measurements, the BsGGT was maintained at pH 3−11 for 12 h at 4 °C before assaying. For the determination of the optimum temperature, the incubations were carried out for 30 min at various temperatures ranging from 25 to 75 °C under the standard assay conditions. The thermostability of the enzyme was measured after preincubation in the same buffer and at the same pH but at various temperatures and for different incubation times. Relative and residual activities were measured in the same conditions used to determine the GGT activity; the activity of the preincubated control sample at 4 °C was regarded as 100%. All experiments were carried out a minimum of three times.

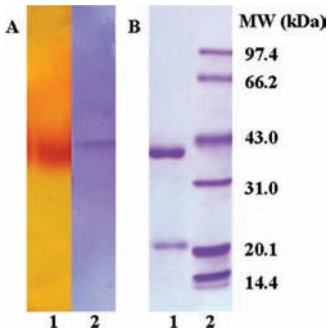

Figure 1. Native PAGE (A) and SDS−PAGE (B) of BsGGT. A: Lane 1, activity staining; lane 2, protein staining. B: Lane 1, BsGGT; lane 2, molecular weight markers with indicated molecular masses in kDa on the right. Molecular mass markers from the top to the bottom are rabbit phosphorylase b (97.4 kDa), BSA (66.2 kDa) and rabbit actin (43.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (20.1 kDa) and hen egg-white lysozyme (14.4 kDa), respectively.

Table 1. Purification Summary of the BsGGT

procedure	total protein (mg)	total act. (U)	sp act. (U/mg)	purification (fold)	recovery (%)
crude enzyme	28.5	672	23.57	0	100
ammonium sulfate fractionation (60−80%)	5.97	520.5	87.18	3.7	77.45
DEAE-Sepharose FF	0.662	389.33	587.8	24.9	57.9
Superdex 75	0.346	232.36	683.4	28.99	34.57

Substrate Specificity for γ-Glutamyl Acceptors. The substrate specificity for γ-glutamyl acceptors was measured by the GGT activity assay method, using γ-glutamyl acceptors instead of glycylglycine. A steady-state kinetics study of the purified GGT was performed at 37 °C in 50 mM borate−NaOH (pH 10.0) with different substrates. The K_m values for γ-glutamyl donors (γ-GpNA, L-Gln) and acceptors (Gly-Gly, ethylamine) were calculated by the Michaelis−Menten equation.

Effects of Metals and Inhibitors on Enzyme Activity. To investigate the effects of metal ions on enzyme activity, the assay was performed in a reaction mixture containing different metal ions. The metal ions tested were Al^{3+}, K^{+}, Na^{+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, Fe^{3+}, Mg^{2+}, Co^{2+}, Zn^{2+}, and Fe^{2+}. To determine the influence of protease inhibitors (BD, DEPC, DTNB, EDC, EDTA, PMSF, NAI, NBS, and 2-ME) on enzyme activity, the enzyme was preincubated with the respective compound, and 50 mmol L^{-1} borate−NaOH (pH 10) for 1 h at 37 °C and the reaction was initiated by addition of the substrates. The studies were performed under the standard test conditions. The level of inhibition is expressed as a percentage of the remaining activity

Table 2. Comparison of Biochemical Properties of the Purified Enzyme with Other Existing GGTs

strain for enzyme source	molecular mass (kDa)	subunit composition	optimal temp (°C)	optimal pH	sp act. (U/mg)	ref
Escherichia coli K-12	58,000	39,200 + 22,000 (A); 38,600 + 22,000 (B)	50	8.73	1.40 (A)	23
	57,000				1.41 (B)	
Bacillus sp. KUN-17	70,000	42,000 + 22,000	50	7.0	425	2
Bacillus pumilus	45,000	38,000 + 23,000	62	8.9	nr[a]	3
Bacillus subtilis 168	50,000	45,000 + 21,000	37	9.0−9.5	100	4
Bacillus subtilis NX-2	nr	43,000 + 32,000	40	8.0	73.36	7
Bacillus subtilis (natto)	58,000	45,000 + 22,000	60	8.5	62.9	6
Helicobacter pylori	60,000	2 × 20000 + 2 × 40000	nr	nr	nr	10
Bacillus licheniformis	6,0974	40,475 + 20,517	40	6−8	185.6	12
Bacillus subtilis TAM-4	105,000	40,000 + 39,000 + 23,000 (A); 39,000 + 22,000 (B)	55	8.8	49.8 (A)	25
	51,000				81.1 (B)	
Saccharomyces cerevisiae	90,000	64,000 + 23,000	nr	nr	121	24
Bacillus subtilis SK11.004	62,000	40,000 + 21,000	37	10	683.4	this study

[a] Not reported.

1 MKKKKFMNLC FIVLLSTLLA AGSIPYHAQA **KKHPFSYDDY KQ**VDVGKDGM VATAHPLASQ
61 IGADVLKKGG NAIDAAVAIQ FALNVTEPMM SGIGGGGFMM VYDAKTKDTT IIDSRERAPA
121 GATPDMFLDE NG**KAIPFSER** V**TKGTAVGVP GTLKGLEKAL DKWGTRSMKQ LITPSIKLAS**
181 KGFPIDSVLA DAISDYKDKL SHTAAKDVFL PDGEPLKEGD TLIQKDLAKT FTAIKYKGT**K**
241 **AFYDGAFSKK** LAETVQEFGG SMTEKDIKNF NVTIDEPIWG DYQGYHIATA PPPSSGGVFL
301 LQMLNLLDDF **KLSQYDIRSW QKYQLLAETM HLAYADRAAF** AGDPEFVNIP L**KGLLNPDYI**
361 **NARRQLIDID** KVNKKPKAGD PWAYQEGSAN YKQVEQPTDK QEGQTTHFTV TDRFGNVVSY
421 TTTIEQLFGS GIMVPGYGVV LNNELTDFDA VPGGANEVQP N**KRPLSSMTP TILFKN**NEPV
481 LTVGSPGGAT IISSVLQTIL NKVEYGMDLK AAVEEPRIYT NSMTSYRYEK GVPEEARTKL
541 NEMGHKFGSS PVDIGNVQSI LIDRENGTFT GVADSSRNGA AIGVNLKKCE K

Figure 2. MALDI-TOF-TOF MS protein sequence coverage. The experimental peptides that matched to GGT [*Bacillus amyloliquefaciens* FZB42] as reported by Mascot are shown in bold.

(with either metal ion or inhibitor) to the control activity (without metal ion or inhibitor).

RESULTS AND DISCUSSION

Purification and Molecular Weight Determination of GGT. GGT was purified from the culture supernatant of *B. subtilis* SK11.004 by ammonium sulfate precipitation, ion exchange chromatography and gel filtration. Among the five different fractions which were eluted from the Hiprep DEAE-Sepharose FF 16/10 column, the second peak eluted by a 0.2 M NaCl solution showed a high transpeptidation activity. Further purification was performed by gel filtration. The elution profile of the Superdex 75 gel filtration chromatography showed one peak having transpeptidation activity at 10.5 mL. The fractions were collected and concentrated by ultrafiltration. The homogeneity of the purified GGT was confirmed by the presence of a single band on the native PAGE gel (Figure 1A). Activity staining confirmed the activity of the protein band. The purification results are summarized in Table 1. The enzyme was purified at 28.99-fold with a 34.57% recovery and 683.4 U/mg of specific activity.

The molecular mass of the enzyme was estimated at 62 kDa by size exclusion chromatography on the Superdex 75 column. The subunit composition of the purified protein was analyzed by SDS−PAGE under reducing conditions. The purified GGT exhibited two bands of apparent molecular masses of 40 and 21 kDa, which is in good agreement with the molecular mass (62 kDa) determined by size exclusion chromatography (Figure 1B). The subunit compositions of GGTs have various forms, such as a monomer,[22] dimer,[2−4,6,7,12,23,24] trimer,[25] and tetramer[10] (Table 2). However, independent of the origin of the GGT, its small subunit always presents a molecular weight of about 22,000 and its amino acid sequence appears highly conserved and contains the active site.[4,6,23,25] In contrast, the molecular weight of the large subunit can vary widely from one organism to another.

Identification of BsGGT. To identify BsGGT, the band corresponding to GGT was excised from the native PAGE gel and subjected to trypsin digestion. The resulting peptides were analyzed by mass spectrometry. The peptide-mass data were used to query the Mascot proteomics database (http://www.matrixscience.com), resulting in one significant match, GGT [*Bacillus amyloliquefaciens* FZB42] (BaGGT). Mascot protein scores greater than 81 are considered significant ($p < 0.05$). After in-gel digestion, we obtained a Mascot score of 84 with a sequence coverage of 18% (matched peptides shown in bold, Figure 2). The result strongly suggests the purified GGT was a novel enzyme and different from GGT previously isolated from *Bacillus subtilis*.[11]

Effects of pH and Temperature on Enzyme Activity. The pH dependence of the GGT activity indicated the hydrolase reaction reaches maximum activity at pH 8, but transpeptidase activity is maximized at pH 10 (Figure 3A,B). The optimum pH for the transpeptidation activity was different from those of other bacterial GGTs (Table 2), most of which have optimal activity at approximately pH 8−8.9.[3,6,7,23,25] The ratio of transferase activity/hydrolase activity of BsGGT is maximal (13.2) at pH 10. Results revealed that, at pH 10, the transpeptidation reaction is preferred. The enzyme remained active at pH values ranging from 5.0 to 11.0; however, when the pH value was lower than 5.0,

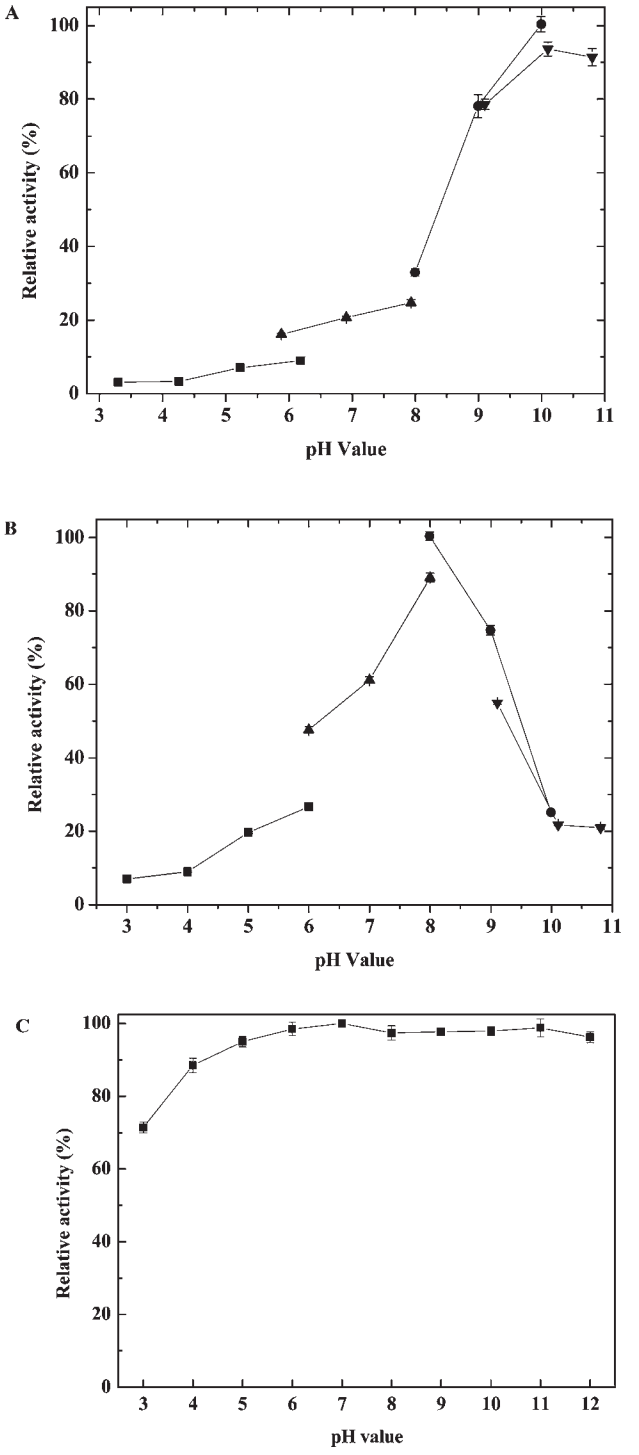

Figure 3. Effect of pH on the transpeptidase activity (A), hydrolytic activity (B), and pH stability (C) of BsGGT: (■) citrate−phosphate, (▲) phosphate buffer, (●) glycine−NaOH buffer, (▼) carbonate−sodium bicarbonate. Hydrolytic activity was measured without γ-glutamyl acceptor in the buffer. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitroaniline per minute from γ-GpNA. Relative activity was expressed as a percentage of the maximum enzyme activity under the two assay conditions respectively. Activity means transpeptidase activity if not mentioned. Each point represents the mean (n = 3) ± standard deviation.

enzyme activity decreased remarkably. These results suggest that the enzyme is active in a very wide range of pH, especially in alkaline

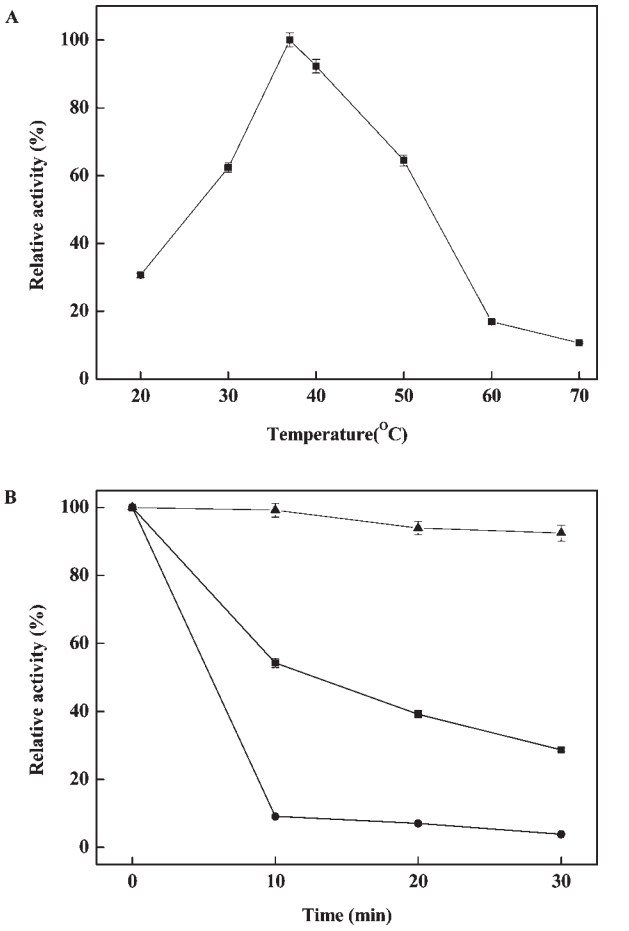

Figure 4. Effects of temperature on the activity (A) and stability (B) of BsGGT: (■) 50 °C, (▲) 60 °C, (●) 70 °C. Each point represents the mean (n = 3) ± standard deviation.

Table 3. Substrate Specificity of BsGGT for γ-Glutamyl Acceptors[a]

substrate	reactive act. (%)	substrate	reactive act. (%)
L-Pro	140.07	L-Met	62.72
L-Try	130.26	L-Cys	46.49
L-Phe	128.5	D-Met	46.10
L-His	120.17	D-Thr	45.32
Gly-Gly	100	L-Thr	26.31
L-Leu	99.12	L-Ala	21.05
L-Val	97.37	L-Gly	18.86
L-Tyr	89.47	L-Glu	16.88
L-Arg	83.98	L-Ser	6.578
ethylamine	72.2		

[a] Transferase activity was measured by a spectrophotometric method as described in Materials and Methods. Activity is expressed relative to that found with 20 mM Gly-Gly (100%).

environment (Figure 3C). Concerning temperature dependence, maximal enzymatic activity was measured at 37 °C (Figure 4A). The thermal stability of the GGT was also examined by measuring residual enzyme activity at various time points during incubations at 50, 60 and 70 °C. These results revealed that the enzyme maintained 92% of its activity following treatment at 50 °C for

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Table 4. K_m Values[a]

parameter	K_m (mM)
Transferase Activity	
donor	
γ-GpNA[b]	1.73
L-Gln[c]	0.83
acceptor	
Gly-Gly[d]	68.37
ethylamine[e]	557
Hydrolyase Activity	
donor	
γ-GpNA	7.5
L-Gln	3.16

[a] The apparent K_m values, calculated by Michaelis−Menten equation. [b] GGT activity was assayed at various concentrations of γ-GpNA ranging from 10 to 3000 μM, 20 mM Gly-Gly was used as an acceptor. [c] The theanine synthesis reaction condition, described in the last paper, with 0.06 U/mL GGT and 50 mM ethylamine as an acceptor, was incubated at 37 °C for 1 h, and theanine formed in the mixture was measured by HPLC. [d] GGT activity was assayed at various concentrations of Gly-Gly ranging from 1 to 20 mM; 5 mM γ-GpNA was used as a donor. [e] Synthesis of theanine was performed at various concentrations of ethylamine ranging from 10 to 50 mM.

Table 5. Effect of Ions and Protease Inhibitors on the Activity of BsGGT[a]

protease inhibitors (1 mM)	rel act. (100%)	metal ions (2 mM) and salts (10 mM)	rel act. (100%)
2-ME	97.19 ± 1.23	Al^{3+}	120.1 ± 2.56
DEPC	97.13 ± 2.02	Mg^{2+}	116.4 ± 2.07
DTNB	93.77 ± 1.54	K^+	111.8 ± 1.34
TNBS	89.77 ± 1.76	Na^+	105.4 ± 1.16
EDC	88.58 ± 1.16	Ca^{2+}	106.2 ± 1.60
EDTA	88.06 ± 2.26	Mn^{2+}	82.4 ± 1.56
BD	87.35 ± 1.45	Co^{2+}	79.7 ± 1.63
PMSF	75.55 ± 1.18	Fe^{3+}	82.1 ± 1.92
NBS	2.943 ± 0.19	Cu^{2+}	56.3 ± 2.35
control	100.0	Fe^{2+}	61.2 ± 1.74
		Zn^{2+}	47.9 ± 1.62
		NaCl	106.3 ± 1.82
		Na NO_3	96.6 ± 1.56
		$(Na)_2SO_4$	92.2 ± 1.87
		control	100.0

[a] Data are expressed as mean values of three replications with their corresponding standard deviations.

30 min, indicating the enzyme was highly stable below 50 °C. The enzyme was inactivated rapidly at temperature higher than 70 °C and was completely inactivated within 10 min (Figure 4B). However, the enzyme produced by *Bacillus subtilis* natto and *Bacillus subtilis* TAM-4 showed higher optimal temperatures (60 and 55 °C, respectively) while also being more sensitive to thermal inactivation.[6,25]

Substrate Specificity of GGT for γ-Glutamyl Acceptors. The transfer of γ-glutamyl from γ-GpNA to various acceptors through catalysis by BsGGT was studied, and the results are shown in Table 3. The transfer activity with Gly-Gly as substrate was taken as 100%. It was revealed that the enzyme has very broad substrate specificity; various amino acids and peptides could serve as γ-glutamyl acceptors from γ-GpNA. Besides Gly-Gly control, imino acid (L-Pro), aromatic amino acids (L-Try, L-Phe, and L-Met), and ethylamine and basic amino acids (L-Arg, L-Lys, and L-His) were good acceptors; L-Ala, L-Gly, L-Glu (acidic amino acid) and L-Ser were poor acceptors. This phenomenon indicated that alkalinity and steric hindrance were major factors affecting the enzyme activity. BsGGT showed low affinity to L-Ala, L-Gly, L-Glu and L-Ser, which was similar to GGTs from *Bacillus subtilis* 168[4] and *Bacillus subtilis* (natto).[6] However, the enzyme exhibited high affinity to imino acid (L-Pro) and aromatic amino acids (L-Try, L-Phe, and L-Met), which indicated that BsGGT has different catalytic characteristics, when compared with other GGTs reported.[2,4,6,7,23] The enzyme exhibited different activities in catalyzing L-amino acids and their racemic forms, which also indicated an influence of stereochemistry on the enzymatic activity of the γ-glutamyl transfer.

The K_m values for γ-glutamyl donors (γ-GpNA, L-Gln) and acceptors (Gly-Gly, ethylamine) were determined and are shown in Table 4. The results show that GGT has low K_m values for γ-glutamyl donors, but high ones for acceptors, and low affinity for γ-GpNA, but high for L-Gln. This finding suggests that transpeptidation is not the major function of this enzyme and that L-Gln is more effective as a donor than γ-GpNA. K_m values for hydrolysis and transpeptidation for L-Gln were 3.16 mM, and 0.83 mM, respectively. This results demonstrates that the synthesis of theanine by GGT in the presence of glutamine as a donor and ethylamine·HCl as an acceptor is effective. Furthermore, the GGT is promising for the synthesis of other valuable γ-glutamyl peptides which use L-Gln as donor.

Effects of Metals and Inhibitors on Enzyme Activity. Results from activity assays with metal ions and protease inhibitors showed that some characteristics of the purified enzyme were different from those of GGTs previously mentioned. As shown in Table 5, Al^{3+}, Mg^{2+}, K^+, and Na^+ stimulated the enzyme activity, whereas Cu^{2+}, Fe^{2+} and Zn^{2+} caused its inhibition. Mn^{2+} and Ca^{2+} had a negligible influence on the enzyme activity. Zn^{2+} and Fe^{2+} inhibited the enzyme activity up to approximately 30%. The acid radicals $SO4^{2-}$, Cl^-, and NO_3^- did not influence enzyme activity either. In addition, its activity was slightly inhibited by EDTA. Chemicals selective for glutamic acid or aspartic acid (EDC), histidine residues (DEPC), disulfide bonds (2-ME) or mercapto groups (DTNB) had little effect on the enzyme activity. However, a strong inhibition of enzyme activity by NBS supports the hypothesis tryptophan residue is necessary in the active site of the enzyme. This is the first example of a tryptophan residue being essential to the enzyme activity of GGT. PMSF, which specifically inhibits enzymes containing a serine or threonine residue at the active site, produced a moderate inhibition of the activity (Table 5); the enzyme activity decreased with increasing PMSF concentrations, and was completely inhibited at 5 mM (data not shown). This effect can be compared to those described by Moallic et al. for *Bacillus pumilus* GGT[3] and Suzuki et al. for *E. coli* GGT,[23] which suggest the presence of an essential threonine residue in the active site. Moreover, Suzuki et al. and Boanca et al. have proved the N-terminal residue, threonine, to be the catalytic nucleophile in both the autoprocessing and enzymatic reactions.[14,15] Further investigation is needed to determine which amino acids are important in maintaining native conformation, substrate binding and the catalysis reaction of the

enzyme. In addition, the cloning, sequencing, and expression of its gene from chromosomal DNA of *B. subtilis* SK11.004 is envisaged in the future.

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ABBREVIATIONS USED

GGT, γ-glutamyltranspeptidase; BsGGT, *Bacillus subtilis* SK11.004 GGT; γ-GpNA, L-γ-glutamyl-*p*-nitroanilide; Gly-Gly, glycylglycine; BD, 2,3-butanedione; DEPC, diethylpyrocarbonate diethylpyrocarbonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NAI, *N*-acetylimidazole; NBS, *N*-bromosucinimide; PMSF, phenylmethanesulfonyl fluoride; 2-ME, 2-mercaptoethanol; EDTA, ethylene diamine tetraacetic acid tetrasodium; K_m, Michaelis–Menten constant; PAGE, polyacrylamide gel electrophoresis; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MALDI-TOF-TOF MS, matrix assisted laser desorption ionization time-of-flight time-of-flight mass spectrometry; TFA, trifluoroacetic acid; ACN, acetonitrile

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