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ORIGINAL RESEARCH PAPER

Novel properties of γ-glutamyltransferase from *Pseudomonas syringae* with β-aspartyltransferase activity

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Abstract
Objectives Gene cloning, purification, and characterization of γ-glutamyltransferase from *Pseudomonas syringae* (PsGGT) were performed in *Escherichia coli*.
Results PsGGT was partially purified to 13-fold, with a specific activity of 0.92 U/mg. The molecule is presumed to be a heterodimeric consisting of large (37 kDa) and small (21 kDa) subunits. The optimal pH and temperature for hydrolytic activity were 8 and 37 °C, and those for transfer activity were 9 and 50 °C, respectively. PsGGT could transfer β-aspartyl moiety from asparagine to hydroxylamine and the γ-glutamyl moiety from glutamine to hydroxylamine.
Conclusion PsGGT demonstrated novel functionality on both γ-glutamyltransferase and β-aspartyltransferase.

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Keywords β-Aspartyl hydroxamate · β-Aspartyltransferase · γ-Glutamyltransferase · *Pseudomonas syringae*

Introduction

γ-Glutamyltransferase (GGT, EC 2.3.2.2) is a heterodimer enzyme that catalyzes both hydrolytic and transfer reactions. The enzyme cleaves the amides of γ-glutamyl compounds and transfers the γ-glutamyl moiety to mostly amino acids or peptides. It is widely distributed in humans, animals, fungi and bacteria. Although GGT has a central role in the γ-glutamyl cycle of GSH synthesis in animal and plant cells, different roles have been reported in bacteria (Ricci et al. 2014). GGT is produced by several types of bacteria.

GGT genes from several prokaryotes have been cloned, purified, and characterized. According to Hartman (1971), glutaminase and GGT can be grouped into four categories based on their abilities to hydrolyze and transfer γ-glutamyl moieties, i.e., (1) strict hydrolysis; (2) strict transfer; (3) hydrolysis prior to transfer; and (4) transfer prior to hydrolysis. Concealed proteinase and glutaminase activities have been found in GGT derived from kidney and *Bacillus substilis* (Gardell and Tate 1979; Minami et al. 2003). Using GGT, enzymatic synthesis of useful γ-glutamyl compounds such as γ-glutamyltaurine and γ-glutamylethylamide (theanine) has been reported (Suzuki and Kumagai 2002; Suzuki et al. 2003).

In our laboratory, we have been studying food-related enzymes such as asparaginase, glutaminase, and GGT. We have been investigating these enzymes from different species of *Pseudomonas* including *P. nitroreducens*, *P. syringae*, *P. putida*, and *P. aeruginosa*. We previously reported the properties of the GGT from *P. nitroreducens* (PnGGT) (Imaoka et al. 2010). During our exploration of *P. syringae* GGT (PsGGT), we found that it showed asparaginase activity. It could be a novel enzyme. Therefore, we were interested in investigating it further.

The gene encoding putative PsGGT was cloned and purified. To determine whether GGT was active toward not only glutamine but also other substrates, the activity of putative PsGGT toward asparagine was examined in addition to glutamine. We identified that recombinant putative PsGGT showed hydrolysis and transfer activities toward asparagine and glutamine. In this report, we describe the novel properties of PsGGT.

Materials and methods

Bacterial strains, plasmid and chemical compounds

Pseudomonas syringae pv. *phaseolicola* 1448A was obtained from American type culture collection (ATCC). *Escherichia coli* JM109 and *E. coli* Rosetta-gami B (DE3) were used for DNA recombination. The plasmid pET22b(+) was obtained from Novagen. γ-Glutamyl-*p*-nitroanilide (γ-G*p*NA), L-glutamine (L-Gln), D-glutamine (D-Gln), L-asparagine (L-Asn), D-asparagine (D-Asn), hydroxylammonium chloride, glycylglycine, methylammonium chloride, ethylammonium chloride were purchased from Wako Pure Chemical Industries Ltd. γ-Glutamyl ethylamide, γ-glutamyl glycylglycine, γ-glutamyl hydroxamate, γ-glutamyl ethylamide, β-aspartyl hydroxamate were purchased from Sigma-Aldrich Co. Other chemical compounds were analytical grade.

Cloning of γ-glutamyltransferase

DNA manipulations were carried out according to standard methods. The complete ORF of γ-glutamyltransferase gene was amplified from *P. syringae* pv. *phaseolicola* 1448A using forward primer (5′-GAGAAACGCATATGTTCGATGATCTG-3′) and reverse primer (5′-GGTACTTTGGATCCATAAGCC GCG-3′). (*Nde*I and *Bam*HI cutting sites are indicated by underlined nucleotides in the forward and reverse primers, respectively). The PCR product and pET-22b(+) were digested with *Nde*I/*Bam*HI and ligated using High ligation ver. 2.1 (Toyobo Co.) The new constructed plasmid was amplified in *E. coli* JM109 on LB medium containing 50 μg ampicillin/ml.

Expression of γ-glutamyltransferase

The expression host, *E. coli* Rosetta-gami B (DE3) was transformed with the expression plasmid, pET-PsGGT. The cells were cultured in LB medium containing 50 μg ampicillin/ml, 30 μg kanamycin/ml, and 34 μg chloramphenicol/ml with continuous shaking at 37 °C. After induction using IPTG at 0.4 mM, incubation at 22 °C was continued for 16 h.

Partial purification of γ-glutamyltransferase

The harvested cell pellet was washed twice using 10 mM Tris/HCl buffer (pH 8) and re-centrifuged at 4 °C at 10,000×*g* for 15 min. Before disruption by sonication (1 h duty, 10 s on and 40 s off), the pellet was re-suspended with the same buffer. The cell debris was removed by centrifugation and the supernatant (cell-free extract) was dialyzed against the same buffer for 24 h. Cell-free extract was applied onto DEAE Cellufine column chromatography that was equilibrated with 10 mM Tris/HCl buffer (pH 8). Step-wise elution (0–250 mM NaCl in 10 mM Tris/HCl buffer, pH 8) was performed to obtain active fractions. Prior to butyl toyopearl chromatography, the enzyme was equilibrated with 10 mM Tris/HCl buffer (pH 8) containing 1 M $(NH_4)_2SO_4$. The active fractions were collected and dialyzed against 10 mM phosphate buffer (pH 8). Hitrap CM FF chromatography with gradient elution from 10 to 500 mM NaCl in 10 mM Tris/HCl buffer (pH 8) was performed. Pooled active fractions were used for further analysis.

Protein and molecular weight determination

Protein quantification was performed by the standard Lowry method with egg albumin as a standard. SDS-PAGE was used to determine the molecular weight of purified protein. SDS-PAGE analysis was performed with 12 % resolving and 4 % stacking gel.

Determination of enzymes activities

γ-Glutamyltransferase activity was evaluated by analyzing its hydrolytic and transfer reaction by colorimetric method. For hydrolysis assay, a total 0.4 ml reaction mixture comprised of 2.5 mM γ-G*p*NA, 100 mM Tris/HCl buffer (pH 8), and 50 µl enzymes. The reaction was stopped using 0.8 ml 10 % (v/v) acetic acid. The absorbance at 410 nm was then measured. One unit of hydrolysis activity was expressed as the amount of enzyme catalyzing the formation of 1 µmol *p*-nitroaniline/min at the standard condition described above.

Standard transfer reaction was performed in 2.5 mM γ-G*p*NA as a substrate donor, 20 mM hydroxylamine as a substrate accepter, 100 mM Tris/HCl buffer (pH 8), and 50 µl enzymes and distilled-water in 0.4 ml total volume. The reaction was terminated using 0.8 ml of 0.2 M $FeCl_3 \cdot 6H_2O$, 0.12 M trichloroacetic acid, 0.25 M HCl, and distilled water (8:2:1:13). γ-Glutamylhydroxamate was detected at 540 nm. One unit of transfer activity was defined as the amount of enzyme required to catalyze the formation of one µmol γ-glutamylhydroxamate/min. β-Aspartyltransferase (BAT) activity was measured in the reaction mixture contained 100 mM Tris/HCl (pH 9), 40 mM L-Asn, 160 mM hydroxylamine, and an appropriate amount of PsGGT in 0.4 ml. β-Aspartylhydroxamate was determined by HPLC analysis.

Characterization of PsGGT

The enzyme was characterized with respect to temperature (30–70 °C) and pH (5–11) dependence, effect of metal ions, and substrates specificity. The temperature and pH stabilities of enzyme were evaluated by its residual activity after the enzyme experienced preincubation for 10 min in the same range of temperature and pH conditions as described above. The enzyme activity toward four different acceptors (hydroxylamine, glycylglycine, ethylamine, and methylamine) was also examined.

Results

Amino acid sequence analysis

The full-length open reading frame of PsGGT consists of 1596-bp nucleotides, encodes 531 amino acids, and has a predicted molecular mass of 56.4 kDa. To examine proteins similarity to PsGGT, multiple sequence alignment of the deduced amino acid sequence of PsGGT was performed against several well-known GGTs, glutaminase–asparaginase, asparaginase, and plant-type asparaginase. Only a few amino acids were conserved (Fig. 1). Among GGTs, less than 25 % similarity was observed for all compared amino acids (Fig. 1a). The highest similarity of PsGGT to *E. coli* GGT (EcGGT) was only 24 %. In addition, compared with glutaminase–asparaginase, asparaginase, plant-type asparaginase and β-aspartyltransferase, PsGGT had a similarity of <10 % (Fig. 1b).

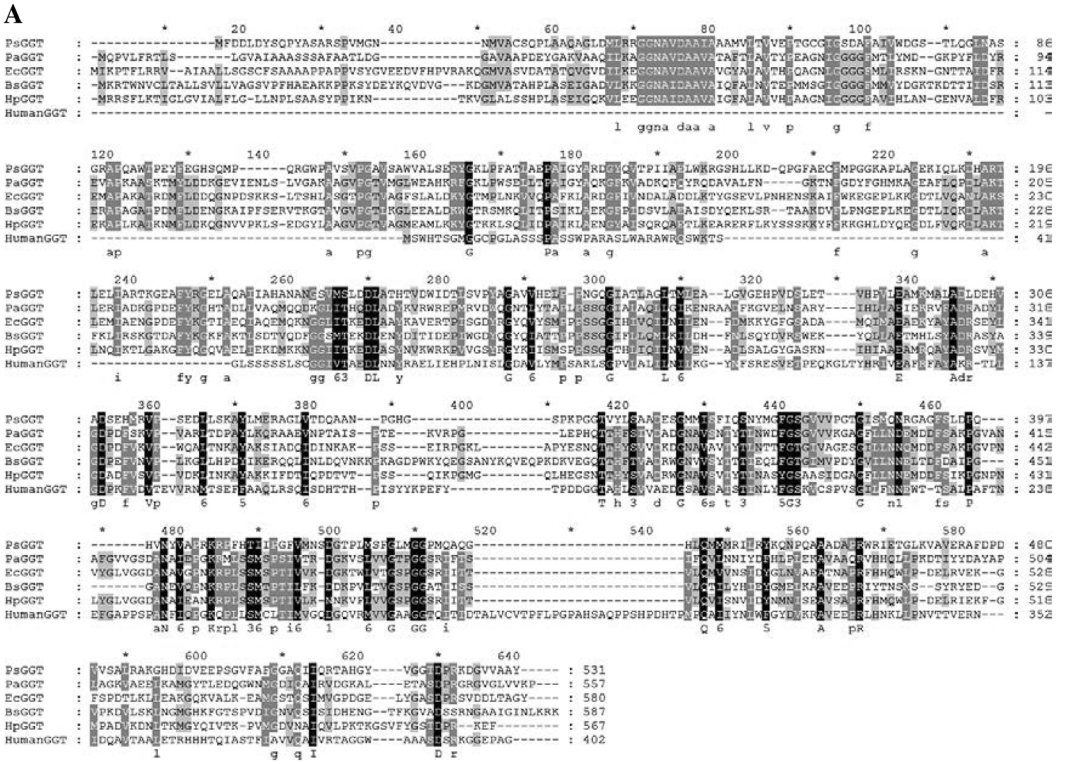
Cloning, expression and purification of PsGGT

The recombinant *E. coli* Rosetta-gami B (DE3) for PsGGT production was firstly cultured at 37 °C and continued at 22 °C after 0.5 mM IPTG induction. The activity of the target enzyme was detected in soluble fraction. Purification was summarized in Table 1. The specific activity of the transfer reaction after the final purification step was 0.92 U/mg. The result of an SDS-PAGE analysis is depicted in Supplementary Fig. 1. PsGGT was easily inactivated. Various stabilizing agents could prolong its activity (Supplementary Fig. 2). However, because of its instability, its activity was very low during several purification steps. Additional purification steps markedly decreased the activity. Therefore we have concluded that the unique reaction properties of PsGGT should be reported although the enzyme preparation is not purified to homogeneously. Comparison of *E. coli* harboring the PsGGT gene with *E. coli* harboring a control plasmid without an inserted gene indicated that no BAT and negligible GGT activities were found in the control *E. coli*. This result clearly indicates that BAT-GGT activity depends on only the PsGGT gene and does not arise from any contamination.

Properties of PsGGT

Effects of temperature and pH

The effect of temperature is shown in Fig. 2. The hydrolytic activity was optimal at 37 °C (Fig. 2a). In contrast, the transfer activity exhibited different behavior. The optimum temperature for transfer activity was 50 °C (Fig. 2b). Moreover, it was


A
PsGGT
PaGGT
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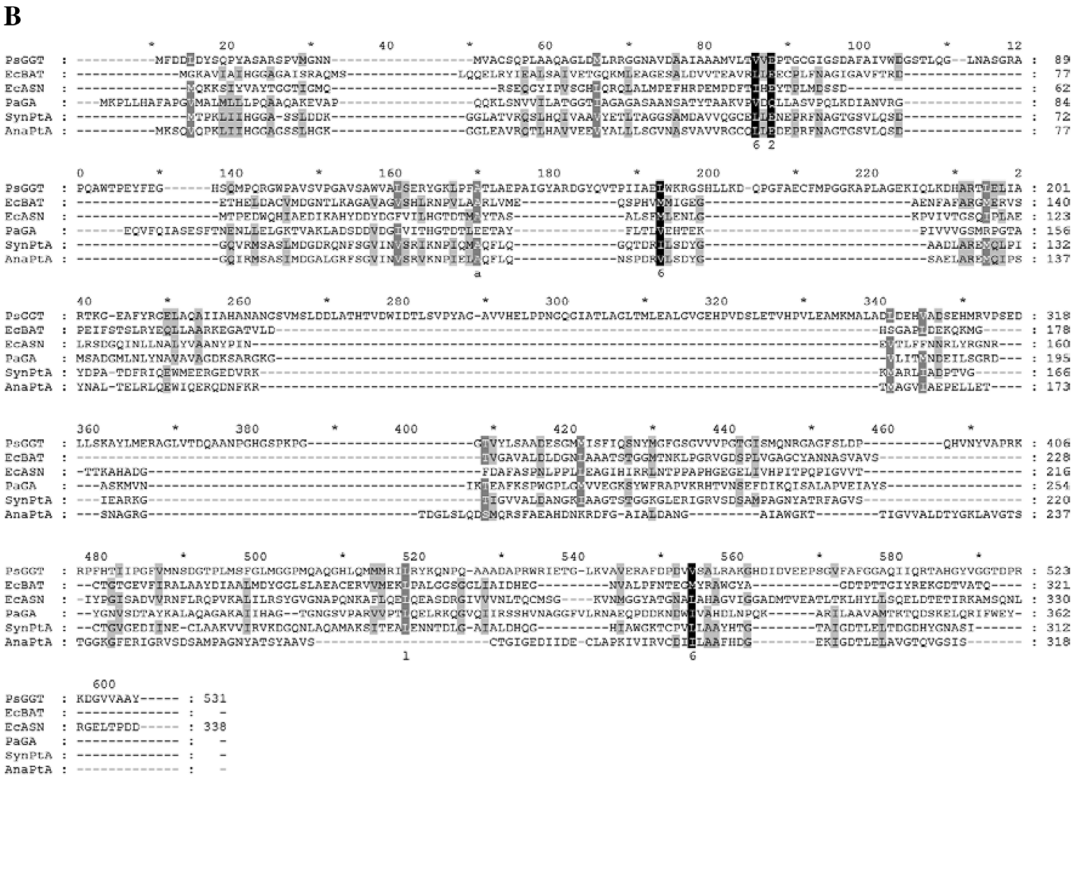
BsGGT
HpGGT
HumanGGT


B
PsGGT
EcBAT
EcASN
PaGA
SynPtA
AnaPtA
HumanGGT

◀**Fig. 1** Multiple sequence alignments of the regions conserved among GGTs (**a**), and among the enzymes with β-aspartyl amidohydrolase activity (**b**). Homologous or identical amino acids are shaded. Conserved amino acids are in *black shaded*, and similar amino acids are in *gray shaded* (lower similarity are indicated by *lighter gray shaded*)

interesting to note that the enzyme stability was higher in the transfer reaction than in the hydrolytic reaction. The optimum pH values for the hydrolytic and transfer reactions over a range of 5–11 were examined. The optimum pH for hydrolytic activity was pH 8 (Fig. 3a), and that for the transfer reaction was pH 9 (Fig. 3b). With respect to the effect of temperature and pH on enzyme stability, the enzyme might be reactivated to some extent under the condition of transfer reaction.

Effect of metal ions, reducing and chelating agents

Both hydrolytic and transfer activities of PsGGT were slightly inhibited by tested metal ions, except for Zn^{2+} at a concentration of 1 mM. DTT and EDTA slightly enhanced both hydrolytic and transfer activities at a concentration of 5 mM.

Substrate specificity

Five different donor substrates (γ-G*p*NA, L-Gln, D-Gln, L-Asn and D-Asn) were used to examine the substrate specificity of PsGGT, and four accepters (glycylglycine, ethylamine, methylamine, and hydroxylamine) were chosen for this enzyme reaction. For the hydrolysis reaction, γ-G*p*NA was considered to be a control substrate. The hydrolytic activity of the PsGGT reaction toward L-Asn was twice as high as that of the control reaction.

During 30-min reaction, the three acceptors, glycylglycine, ethylamine, and methylamine, failed to produce noticeable γ-glutamyl and β-aspartyl compounds. In contrast, when hydroxylamine was used as a substrate acceptor, a 30-min reaction was enough to markedly form reaction products (γ-glutamyl-hydroxamate and β-aspartyl-hydroxamate). These results are summarized in Table 2.

β-Aspartyltransferase activity

HPLC analysis of the reaction showed that the products were formed in a time-dependent manner (Fig. 4). β-Aspartylhydroxamate (peak no. 2) was

Table 1 Summary of PsGGT purification

Step	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	5432	409.4	0.07	100	1
DEAE-Cellufine pH 8	129.2	71.06	0.55	17.36	7.85
Butyl Toyopearl pH 8	10.42	6.5	0.63	1.58	9
Hitrap CM FF (concentrated)	6.42	5.89	0.92	1.43	13.14

[a] Calculated on the basis of GGT transfer activity

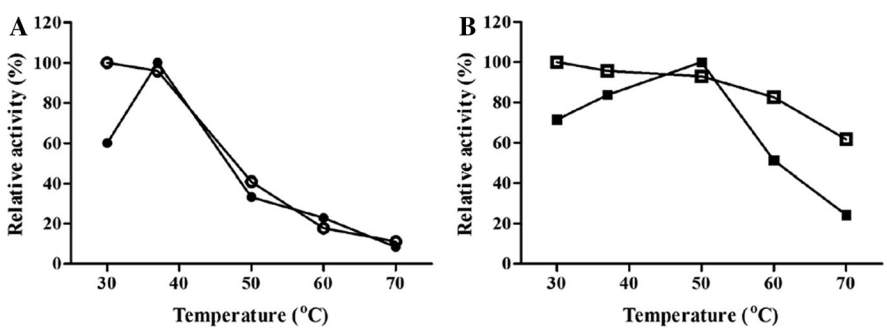


Fig. 2 The effect of temperature on PsGGT. Hydrolytic activity (**a**), *closed-circle* is optimum activity and *open-circle* is enzyme stability. Transfer activity (**b**), *closed-square* is optimum activity and *open-square* is enzyme stability. The values were mean from two independent experiments

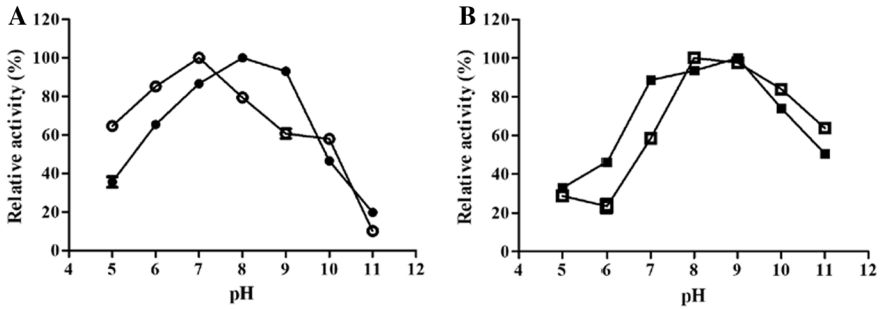


Fig. 3 The effect of pH on PsGGT. Hydrolytic activity (**a**), *closed-circle* is optimum activity and *open-circle* is enzyme stability. Transfer activity (**b**), *closed-square* is optimum activity and *open-square* is enzyme stability. The values were mean from two independent experiments

detected in a 30-min reaction and the area of peak no. 2 increased in a 3-h reaction. On comparing the standard and the zero time reaction HPLC peaks with another time-dependence reaction peak profile, a new peak at retention time of ~18 min was observed and was thought to be β-aspartylhydroxamate. After a 3 h reaction, the concentration of the product was about 20 mM. This suggested that β-aspartylhydroxamate was definitely synthetized by PsGGT.

Discussion

The hydrolytic and transfer activities of PsGGT toward glutamine and asparagine have been examined. PsGGT was capable of transferring the γ-glutamyl- and β-aspartyl moieties of these substrates. This characteristic of PsGGT has not been described for other GGTs and β-aspartyl transfer activity has not been reported in any GGTs. Human GGT only transfers the γ-glutamyl moiety from γ-glutamyl compounds to amines or dipeptides, such as glycyl-glycine and glycyl-L-alanine, which are the two best acceptors (Tate and Ross 1977) and *E. coli* GGT prefers L-arginine as an acceptor (Suzuki et al. 1986). Other reports on GGT also have indicated that this particular enzyme only catalyzes the transfer reaction of γ-glutamyl moieties (Shuai et al. 2011; Murty et al. 2012).

GGT is an *N*-terminal nucleophile (Ntn) hydrolase and transfers γ-glutamyl moieties to other compounds, mainly amino acids, peptides or water. Several bacterial GGTs have been biochemically characterized (Lin et al. 2006; Song et al. 2011) but there have been no reports that GGTs catalyze hydrolysis and transfer of L-Asn reactions.

Glutaminase–asparaginase is only able to hydrolyze Gln and Asn. Our investigation of *Pseudomonas aeruginosa* glutaminase–asparaginase indicated that this enzyme failed to transfer γ-glutamyl- and β-aspartyl moiety to hydroxylamine (unpublished work). β-Aspartyltransferase (BAT; EC 2.3.2.7) has only been reported in *Mycobacterium tuberculosis* (Jayaram et al. 1969). However, BAT from *M. tuberculosis* cannot act on L-Gln. Therefore, the enzyme from *P. syringae* that has both GGT and BAT is a novel enzyme that has not yet been reported.

Multiple sequence alignment of the amino acid sequence of PsGGT with two other GGTs, glutaminase–asparaginase, glutaminase, asparaginase, and two plant-type asparaginases showed that there was low similarity with all molecules. EcGGT shared the maximum similarity of 24 %. A region near the *N*-terminus (around amino acid residues 22–74) and the region considered to be the active site (around amino acid residues 349–433) shows relatively high similarity with the other GGTs. However there are few amino acid residues identical to the other GGTs in the other regions of PsGGT. Furthermore, the overall length of the PsGGT amino acid sequence is approx. 50 residues shorter than those of EcGGT and *Bacillus subtilis* GGT (BsGGT). The lid-loop structure found in the active sites of both EcGGT and PaGGT is absent in PsGGT similar to BsGGT. These differences in amino acid sequence between PsGGT and the other GGTs may contribute to the instability and unique reaction properties of PsGGT, which have not been observed in other GGTs.

Table 2 Substrate specificity of PsGGT

Hydrolytic activity[a]			Transfer activity[b]		
Substrates	Conc. (mM)	Rel. activity (%)*	Donor	Acceptor	Rel. activity (%)*
γ-G*p*NA	2.5	100	γ-G*p*NA	HA	100
L-Gln	40	120 ± 1.8		GG	nd
D-Gln	40	78 ± 3.1		EA	nd
L-Asn	40	207 ± 6.8		MA	nd
D-Asn	40	25 ± 2.5	L-Gln	HA	106.3 ± 2.7
				GG	nd
				EA	nd
				MA	nd
			D-Gln	HA	92.3 ± 5.3
				GG	nd
				EA	nd
				MA	nd
			L-Asn	HA	301 ± 2.2
				GG	nd
				EA	nd
				MA	nd
			D-Asn	HA	48 ± 8.2
				GG	nd
				EA	nd
				MA	nd

[a] Except for γ-G*p*NA, hydrolytic activity was calculated using Nessler method. A mixture of 150 µl crude enzyme, 50 µl 1 M Tris/HCl buffer (pH 8.0), 200 µl deionized water, and 100 µl substrate at the defined final concentration was incubated for 30 min at 30 °C. To terminate the reaction, 125 µl 20 % (w/v) trichloroacetic acid was added. The mixture was then centrifuged at 1000×*g* for 15 min. To the 450 µl supernatant, 125 µl Nessler solution was added an incubated for 15 min at room temperature. The results were read at 480 nm. One unit of enzymatic activity was defined as 1 µmol ammonia produced/min under the described conditions

[b] Concentration of substrate donor was in the same amount as in hydrolysis reaction. *HA* hydroxylamine, *GG* glycylglycine, *EA* ethylamine and *ME* methylamine at 20 mM were used for substrates acceptor. Colorimetric and HPLC analyses were used to determine the products in the reaction

* The values represent the mean ± SD (n = 2)

Determining subunit structure of PsGGT in a semipurified state is difficult. However, on the basis of multiple sequence alignment analysis (Fig. 1) and comparison of the present study data on PsGGT with our previous data on PnGGT (Imaoka et al. 2010), threonine at the position of 349 (T349) is likely to be a cleavage site in PsGGT. Hence, by considering T349 as a cleavage site, the deduced large and small subunits of PsGGT were 37 and 21 kDa, respectively. Fragments corresponding in size to these subunits were found among the fragments on SDS-PAGE (Supplementary Fig. 1). Further investigation is necessary to confirm the subunit structure of PsGGT.

Because this enzyme is unstable, obtaining PsGGT in a homogeneous state is not straightforward. We have tested various stabilizers to overcome this drawback (Supplementary Fig. 2). Although these could prolong PsGGT activity for a certain time, its stability is shorter under the purification conditions. After four purification steps lasting approx. 5 days, its activity is low. It is completely inactive after 6 days. Therefore, further purification is impossible. We have attempted various chromatography methods to shorten purification times. However, these attempts failed to manage PsGGT instability. Therefore, we reported the best state of the enzyme, in which it is possible to assay

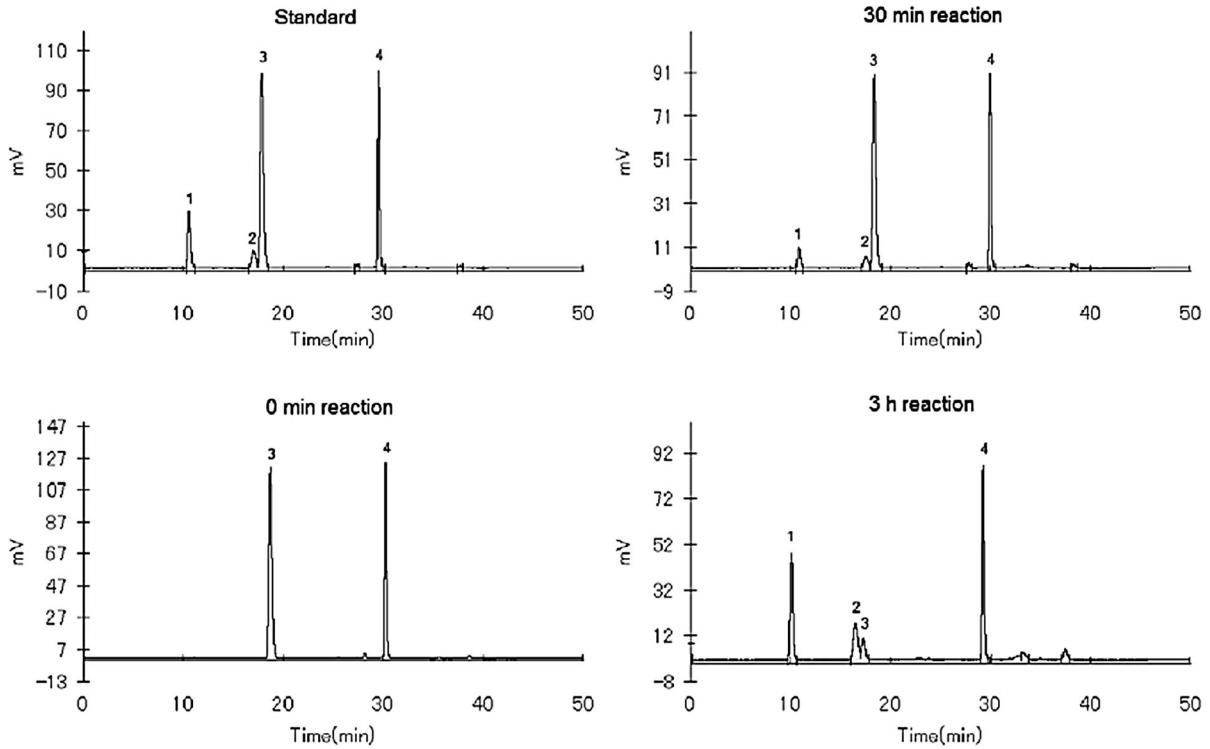


Fig. 4 HPLC profile of time-dependent BAT reaction. Peak No. 1, 2, 3, 4 were L-aspartic acid, β-aspartyl hydroxamate, L-Asn, and Hydroxylammonium chloride, respectively. Standard concentration used for L-aspartic acid, β-aspartyl hydroxamate, L-Asn, and hydroxylammonium chloride were 10, 10, 40, 160 mM, respectively. HPLC analysis using Cosmosil 5C18-MS-II ID 10 × 250 mm pre-packed column. The solvent buffer for the analysis of hydrolytic and transfer reaction was buffer A [0.1 M acetate buffer (pH 8) with 7 % acetonitrile and 30 % tetrahydrofuran) and buffer B (0.1 M acetate buffer (pH 8) with 47 % acetonitrile and 3 % tetrahydrofuran]. The system was run with 50-min linear gradient with increasing in acetonitrile from 15 to 47 % from 0 to 40 min and back to 15 % acetonitrile from 40.1 to 50 min. Flow rate was set to 1 ml/min. The peak profiles were detected by RF-10A$_{XL}$ Fluorescence detector (Shimadzu) with 344 nm excitation and 433 nm emission wavelength. The assay was done as follows, the reaction with 500 µl total volume consist of 40 mM substrate donor, 200 mM substrate acceptor, 100 mM Tris/HCl buffer (pH 8.0) was incubated at 30 °C for several different times to observe optimum time for GGT reaction. The reaction mixture (5 µl) was converted to derivatized-fluorescence compound using *o*-phthalaldehyde (OPA) and *N*-(tert-butyloxycarbonyl)-L-cysteine (BOC) in 0.4 M borate buffer (pH 9), and 5 µl of the mixture was applied to HPLC

PsGGT for further investigation of its characteristics. PsGGT showed a unique activity profile toward temperature. The hydrolytic activity of this enzyme was more sensitive to temperature than the transfer activity was. The hydrolytic activity sharply decreased after reaching the maximum activity of approx. 37 °C, which was not the case for the transfer activity. For transfer activity, a bell-shaped graph was obtained and the relative activity of transferase at all temperature examined was >60 %. It appeared that in the transfer reaction, the enzyme might be stabilized or reactivated, which was most probably due to the differences in the reaction mixture substances between the hydrolytic and transfer reactions. Hydroxylamine had a buffering effect in the reaction (Supplementary Fig. 3). A study by Zera (1987) demonstrated that the substances in the reaction medium greatly affected the enzyme thermostability.

The optimum pH for transfer was 9, which was slightly higher than that for hydrolysis activity (pH 8). GGTs from *P. nitroreducens* IFO12694 and *B. subtilis* also demonstrated the same property (Imaoka et al. 2010; Morelli et al. 2014). The substrate specificity for hydrolysis and transfer exhibited the same trend, and the L-isomer of donor substrates was preferable to the D-isomer. A similar result was also

reported for GGT from *B. substilis* TAM 4 (Abe et al. 1997). For the transfer reactions of GGTs from *E. coli* and *Helicobacter pylori*, L-Gln was used as the preferred substrate donor (Suzuki et al. 2003; Song et al. 2011).

Hydroxylamine was the only substrate acceptor for BAT reported in this study, and this finding has not been previously reported. The only enzyme that has been reported to exhibit BAT activity is aspartotransferase from *M. tuberculosis*, but its characteristics remain unclear (Jayaram et al. 1969). Plant-type asparaginase, isoaspartyldipeptidase hydrolyzes β-aspartyl peptides but has no transfer activity (Hejazi et al. 2002). Glutaminase–asparaginase (EC 3.3.2.2), glutaminase (EC 3.5.1.2), and asparaginase (EC 3.5.1.1) only hydrolyze Gln and Asn, Gln, and Asn, respectively, but all do not catalyze transfer reactions. These data suggest that PsGGT is a new type of enzyme.

In conclusion, although our investigation was performed using semipurified enzyme, we have characterized the GGT from *P. syringae* pv. *phaseolicola* 1448A and show that it had unique BAT activity. Considering this novel activity and the higher transfer activity toward β-aspartyl- than toward γ-glutamyl moieties, we named this enzyme as β-aspartyl- γ-glutamyltransferase (BAT-GGT).

Supporting information Supplementary Fig. 1 SDS-PAGE of PsGGT. Lane: M, molecular size markers; A, control (pET-22b); B, crude extract; C, DEAE cellufine; D, Butyl Toyopearl; E, Hitrap CM FF.

Supplementary Fig. 2 The effect of osmolytes and polyols on PsGGT stability. The residual activities were calculated by incubating the enzyme containing 5 % osmolyte and polyol agents at the final concentration at 4 °C.

Supplementary Fig. 3 The effect of hydroxylamine on the hydrolytic reaction of PsGGT. The residual activities were calculated by performing the hydrolysis reactions with and without hydroxylamine 20 mM. Closed circle; standard reaction without hydroxylamine. Closed square; reaction with hydroxylamine. The values represent the mean from three independent experiments.

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