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A mutant *Bacillus subtilis* γ-glutamyltranspeptidase specialized in hydrolysis activity

Hiromichi Minami, Hideyuki Suzuki *, Hidehiko Kumagai

Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan

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Abstract

γ-Glutamyltranspeptidase (GGT) catalyzes the hydrolysis of γ-glutamyl compounds and the transfer of their γ-glutamyl moieties to amino acids and peptides. The transpeptidation activity of *Bacillus subtilis* GGT is about 10-fold higher than its hydrolysis activity. In *B. subtilis* GGT, substitution of Asp-445 with Ala abolished its transpeptidation activity. The specific activity for hydrolysis of D445A GGT was 40.2% of that of the wild-type GGT. The K_m value for L-glutamine was 15.3 mM. D445A GGT was salt tolerant like the wild-type GGT. These results indicate that D445A GGT will be highly useful as a 'glutaminase' in food industry.

Keywords: γ-Glutamyltranspeptidase; Hydrolysis; Glutaminase; Site-directed mutagenesis; Salt tolerant; *Bacillus subtilis*

1. Introduction

γ-Glutamyltranspeptidase (GGT: EC 2.3.2.2), which is widely distributed in living organisms [1], catalyzes the hydrolysis of γ-glutamyl compounds such as glutathione, as well as the hydrolysis of the amide bonds of glutamine to yield glutamic acid and ammonia. GGT can also catalyze the transfer of γ-glutamyl moieties to amino acids and peptides. We have developed methods for the enzymatic synthesis of various γ-glutamyl compounds involving GGT from *Escherichia coli* K-12 [2–8]. Recently, we reported that *Bacillus subtilis* GGT is salt tolerant [9]. A *B. subtilis* strain producing large quantities of GGT was constructed, and its enzymatic properties were studied [9].

The delicious taste of soy sauce, a traditional Japanese seasoning, mainly depends on the amount of glutamic acid. Glutamic acid is an important flavor component and its taste is so-called 'umami'. During soy sauce fermentation, soy proteins are digested into peptides by proteases from *Aspergillus oryzae* or *Aspergillus sojae*, and the peptides are then cleaved into amino acids by their peptidases. The glutamine liberated is hydrolyzed to glutamic acid by glutaminase. When the level of glutaminase is insufficient, glutamine is converted spontaneously to tasteless or slightly sour pyroglutamic acid. Therefore, glutaminase is one of the most important enzymes for flavor enhancement in the manufacture of soy sauce. Soy sauce fermentation is performed in the presence of 18% NaCl to prevent contamination. 18% NaCl corresponds to more than 3 M. In the presence of this much NaCl, the activity of *Aspergillus* glutaminases is strongly inhibited. Therefore, salt-tolerant glutaminases were searched for in microorganisms for application to soy sauce fermentation. GGT catalyzes not only a hydrolysis reaction, but also a transpeptidation reaction. Therefore, although GGT has glutaminase activity, it also produces γ-glutamyl compounds as byproducts. Since *B. subtilis* GGT is salt tolerant, which is a distinguished character, a mutant GGT with only the hydrolysis reaction was searched for.

GGTs from various organisms have been studied to identify the amino acid residues involved in substrate binding and catalysis. Site-directed mutagenesis studies of human GGT suggested that Asp-423 interacted electrostatically with an α-amino group of a γ-glutamyl substrate [10], and that Arg-107 was significant as to binding to the

* Corresponding author. Tel.: +81 (75) 753-6278; Fax: +81 (75) 753-6275.
E-mail address: hideyuki@lif.kyoto-u.ac.jp (H. Suzuki).

Abbreviations: Gly-Gly, glycylglycine; γ-GCNA, γ-glutamyl-3-carboxy-4-nitroanilide; γ-G*p*NA, γ-glutamyl-*p*-nitroanilide; GGT, γ-glutamyltranspeptidase; KPB, potassium phosphate buffer; SDS, sodium dodecyl sulfate

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α-carboxyl group of a γ-glutamyl substrate [11]. In this study, the effects of amino acid substitutions at Arg-113 and Asp-445, corresponding to Arg-107 and Asp-423 of human GGT, on the activity of *B. subtilis* GGT were investigated to obtain a mutant GGT with only hydrolysis activity.

2. Materials and methods

2.1. Bacterial strains and plasmids, and growth of bacteria

The strains and plasmids used in this study are listed in Table 1. The *E. coli* strains were grown at 37°C in Luria–Bertani (LB) medium (Difco Laboratories). Antibiotics were added at the following concentrations: ampicillin, 100 μg ml^{-1}, and tetracycline, 30 μg ml^{-1}.

2.2. DNA manipulation

Plasmid DNA from *E. coli* was extracted using a Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA).

2.3. Method of transformation

E. coli strains were transformed by the method of Inoue et al. [12].

2.4. Site-directed mutagenesis

Site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The *Aat*I–*Acc*I fragment of pMH2279, containing amino acid residues 1–351 of the GGT, was ligated with pUC18, which had been digested with *Acc*I and *Sma*I. And the *Aat*I–*Eco*RI fragment of pMH2279, containing amino acid residues 352–587 of the GGT, was ligated with pUC18, which had been digested with *Aat*I and *Eco*RI. These plasmids were named pMH2282 and pMH2283, respectively. pMH2282 and pMH2283 were used as the DNA templates for Arg-113 and Asp-445 mutagenesis, respectively. The oligonucleotides used for mutagenesis are listed in Table 2. The nucleotides altered are underlined. After Asp-445 mutagenesis, *Bst*EII–*Psh*AI fragments of the Asp-445 mutants were ligated with pMH2285, which had been digested with *Bst*EII and *Eco*RV. The resulting plasmids of the D445A, D445E, D445N, and D445Y mutants were named pMH2288, pMH2300, pMH2328, and pMH2329, respectively. For Arg-113 mutagenesis, the *Eco*RI–*Xba*I fragment of pMH2279 was ligated with pHY300PLK, which had been digested with *Eco*RI and *Xba*I. This plasmid was named pMH2280. The *Nde*I–*Xba*I fragment of an Arg-113 mutant was ligated with pMH2280, which had been digested with *Nde*I and *Xba*I. And then the *Psh*AI–*Eco*RV

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source (reference)
E. coli		
DH5α	*supE44 ΔlacU169* (ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	
SH641	F$^-$ Δ*ggt-2 rpsL recA56 srl300*::Tn*10*	[19]
MH2288	SH641 containing pMH2288	this study
MH2300	SH641 containing pMH2300	this study
MH2328	SH641 containing pMH2328	this study
MH2329	SH641 containing pMH2329	this study
MH2334	SH641 containing pMH2334	this study
Plasmid		
pHY300PLK	p15A and pAMα1 replicons *bla*$^+$ *tet*$^+$	Takara Shuzo Co. (Ishiwa and Tsuchida [20])
pMH2279	pSC101 replicon *bla*$^+$ *ggt*$^+$; the 2.5-kb *Hin*cII–*Xba*I fragment of 168 containing the *ggt* gene was ligated with pMW119 cleaved with *Sma*I and *Xba*I	[9]
pMH2280	p15A and pAMα1 replicons *bla*$^+$ *tet*$^+$ *ggt*$^+$; the *Eco*RI–*Xba*I fragment of pMH2279 was ligated with pHY300PLK cleaved with *Eco*RI and *Xba*I	this study
pMH2282	ColE1 replicon *bla*$^+$ *ggt*; the *Aat*I–*Acc*I fragment of pMH2279 was ligated with pUC18 cleaved with *Acc*I and *Sma*I	this study
pMH2283	ColE1 replicon *bla*$^+$ *ggt*; the *Aat*I–*Eco*RI fragment of pMH2279 was ligated with pUC18 cleaved with *Eco*RI and *Sma*I	this study
pMH2285	p15A and pAMα1 replicons *bla*$^+$ *tet*$^+$ *ggt*$^+$; the *Eco*RI–*Ssp*I fragment of pMH2279 was ligated with pHY300PLK cleaved with *Eco*RI and *Sma*I	[9]
pMH2288	pMH2289 but with D445A substitution	this study
pMH2289	p15A and pAMα1 replicons *bla*$^+$ *ggt*$^+$; pMH2285 was digested with *Eco*RV and *Psh*AI	this study
pMH2300	pMH2289 but with D445E substitution	this study
pMH2328	pMH2289 but with D445N substitution	this study
pMH2329	pMH2289 but with D445Y substitution	this study
pMH2334	pMH2289 but with R113K substitution	this study

Table 2
Oligonucleotides used for mutagenesis

Mutation	Sequence of oligonucleotide primer
R113K	5′-CGATAATCGACAGCAAAGAGCGTGCTCCAGCAGGCGC-3′
D445A	5′-CAATGAATTAACGGCTTTTGATGCGATACCAGGCGG-3′
D445E	5′-CAATGAATTAACGGAATTTGATGCGATACCAGGCGG-3′
D445N	5′-CAATGAATTAACGAATTTTGATGCGATACCAGGCGG-3′
D445Y	5′-CAATGAATTAACGTATTTTGATGCGATACCAGGCGG-3′

fragment was removed from the resulting plasmid. This plasmid was named pMH2334. GGT-deficient strain SH641 was transformed with these plasmids, yielding strains expressing mutant GGTs.

2.5. Purification of a mutant GGT from E. coli

The enzyme isolation procedures were performed at 4°C. Strain MH2288 expressing D445A GGT was grown in 4 l LB medium for 24 h at 37°C. The cells were harvested by centrifugation at 11 600 × *g* for 20 min. Spheroplasts were prepared as described by Suzuki et al. [13]. The spheroplasts were collected by centrifugation at 11 600 × *g* for 10 min. The supernatant, the periplasmic fraction, was dialyzed against 10 mM potassium phosphate buffer (KPB) (pH 7.0). The dialyzed supernatant was applied to a Gigapite column (Seikagaku Kogyo, Tokyo, Japan) (3 × 14 cm) equilibrated with 10 mM KPB (pH 7.0), and eluted with a linear gradient of 0–1 M KPB (pH 7.0). The active fractions were collected and dialyzed against 50 mM KPB (pH 8.0). The dialyzed solution was applied to a SOURCE 15Q PE 4.6/100 column (Amersham Pharmacia Biotech) equilibrated with 50 mM KPB (pH 8.0), and eluted with a linear gradient of 0–0.5 M NaCl. The active fractions were collected and dialyzed against 50 mM KPB (pH 7.0) containing 1.5 M ammonium sulfate. The dialyzed solution was applied to a Phenyl Superose HR 5/5 column (Amersham Pharmacia Biotech) equilibrated with 50 mM KPB (pH 7.0) containing 1.5 M ammonium sulfate, and eluted with a linear gradient of 1.5–0 M ammonium sulfate. The active fractions were collected and dialyzed against 50 mM KPB (pH 7.0).

2.6. Gel electrophoresis

The purified enzyme was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 10% polyacrylamide gels and staining with Coomassie blue R-250.

2.7. Enzyme assay

GGT activity was measured by the standard assay method described previously [14]. One unit of enzyme was defined as the amount of enzyme that released 1 μmole of *p*-nitroaniline per min from γ-glutamyl-*p*-nitroanilide (γ-G*p*NA). The transpeptidation activity was defined as the activity enhanced in the presence of an acceptor substrate, glycylglycine (Gly-Gly). For the transpeptidation reaction, a γ-glutamyl compound, γ-glutamyl-Gly-Gly, was measured with an automated amino acid analyzing system (model LC-9; Shimadzu, Kyoto, Japan) equipped with a Shim-pack amino-Na column (Shimadzu) as described previously [9]. For kinetic analysis, γ-glutamyl-3-carboxy-4-nitroanilide (γ-GCNA) was used as the γ-glutamyl donor, because it is more soluble than γ-G*p*NA.

Glutaminase activity was measured as the level of conversion of Gln to Glu. One unit of enzyme was defined as the amount of enzyme that produced 1 μmole of Glu per min from Gln through the hydrolysis reaction. The concentration of Glu was measured with an automated amino acid analyzing system (model LC-9; Shimadzu).

2.8. Measurement of protein concentrations

Protein concentrations were measured by the method of Lowry et al. [15] using bovine serum albumin as a standard.

3. Results and discussion

3.1. Comparison of the activities of the wild-type and mutant GGTs

The wild-type and mutant GGTs were expressed in *E. coli*. The strains were grown in 50 ml of LB medium at 37°C. The cells were harvested by centrifugation at 11 600 × *g* for 20 min. After resuspension in 5 ml of 50 mM KPB (pH 8.0), the cells were sonicated. The supernatants were used as enzyme preparations. The respective activities are shown in Table 3. In the wild type, the transpeptidation activity was 2.38 times higher than the hydrolysis activity. The proportion of transpeptidation activity of R113 or D445 mutants was lower than that of the wild

Table 3
Comparison of the activities of the wild-type and mutant GGTs

	Relative activity (mU mg^{-1})		
	T[a] ± S.D.[c]	H[b] ± S.D.[c]	T/H ± S.D.[c]
Wild type	0.499 ± 0.0376	0.211 ± 0.0129	2.38 ± 0.159
R113K	0.111 ± 0.0019	0.156 ± 0.0105	0.72 ± 0.047
D445A	< 0.001	0.665 ± 0.0078	< 0.001
D445E	0.071 ± 0.0041	0.176 ± 0.0051	0.40 ± 0.035
D445N	0.054 ± 0.0029	0.131 ± 0.0069	0.42 ± 0.040
D445Y	0.044 ± 0.0044	0.110 ± 0.0082	0.40 ± 0.026

Transpeptidation and hydrolysis activities were measured using cell-free extracts as described in Section 2. γ-G*p*NA (final concentration, 0.5 mM) was used as the donor substrate.
[a]Transpeptidation.
[b]Hydrolysis.
[c]Standard deviation.

type. In particular, the D445A mutant exhibited no detectable transpeptidation activity.

3.2. Purification of D445A GGT from E. coli

D445A GGT was purified to electrophoretic homogeneity from MH2288 by periplasmic fractionation, and three subsequent chromatographic steps on Gigapite, SOURCE 15Q, and Phenyl Superose. SDS–PAGE of D445A GGT gave two bands corresponding to molecular masses of 45 and 21 kDa (data not shown). D445A GGT had the same subunit composition as the wild type [9].

3.3. Glutaminase activity of D445A GGT

The glutaminase activity of D445A GGT was determined. The specific activity of the wild type was 4.82 U mg^{-1} [9], and that of D445A GGT 1.30 U mg^{-1}, which was 27.0% of that of the wild type. The optimum pH for the glutaminase activity of D445A GGT was 7.5–8.0, which was similar to that of the wild type [9].

3.4. Effect of the NaCl concentration on GGT activity

The salt tolerance of D445A GGT was determined as described previously [9]. In the presence of 18% NaCl, which is the concentration used for the fermentation of soy sauce, 82% of the hydrolysis activity remained. This was the same as the level of the wild-type GGT [9]. Therefore, D445A GGT retained the salt-tolerant GGT activity. This indicated that D445A GGT is suitable for food fermentation under high salt conditions, such as in the fermentation of soy sauce and miso (fermented soybeans).

3.5. Kinetic parameters for the purified D445A GGT

The kinetic parameters for the purified D445A GGT were compared with those for the wild type. The specific activity, K_m for γ-GCNA, and k_{cat} values of the wild-type and D445A GGTs are shown in Table 4. It was confirmed with an automated amino acid analyzing system that the purified D445A GGT exhibited no transpeptidation activity, that is, no transpeptidation product, γ-glutamyl-Gly-Gly, was detected.

The specific activity for the hydrolysis reaction of D445A GGT was 40.2% of that of the wild type. The optimum pH for the hydrolysis activity of D445A GGT with γ-G*p*NA as the substrate was 8.5 (Fig. 1), which was the same as that of the wild type [9]. However, the activity decreased at an acidic pH. The K_m of D445A GGT was 15.3 mM, i.e. 1.47-fold higher than that of the wild type. And the k_{cat} of D445A GGT was 3380 min^{-1}, i.e. 1.30-fold higher than that of the wild type. As compared with the human mutant GGT, D445A GGT of *B. subtilis* catalyzed hydrolysis well without transpeptidation [10,11].

Although K_m for D445A GGT of *B. subtilis* increased, it was the same as the levels of glutaminases, 4–36 mM [16–18], and k_{cat} did not decrease. It was indicated that the hydrolysis activity of D445A GGT was superior to that of the wild type with a high concentration of substrate. D445A GGT is suitable for industrial applications, i.e. for obtaining large amounts of products from high levels of substrates.

Table 4
Kinetic parameters for the wild-type and D445A GGTs

Enzyme	Specific activity (U mg^{-1})[a]	K_m (mM)	k_{cat} (min^{-1})
Wild type	38.3	10.4	2600
D445A	15.4	15.3	3380

Hydrolysis activity was measured as described in Section 2. γ-GCNA was used as the substrate.
[a]Final concentration, 5 mM.

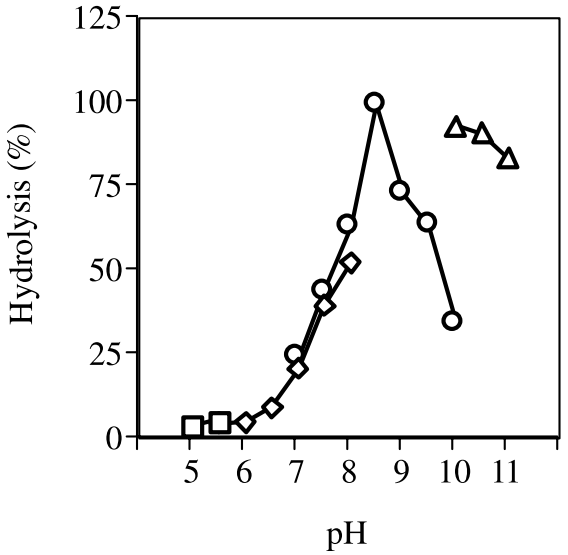

Fig. 1. The optimum pH of the hydrolysis activity of D445A GGT. The optimum pH was determined for the purified enzyme (31.9 mU ml^{-1} of hydrolysis activity under the standard conditions) in the following buffers: 50 mM Na-acetate buffer (pH 5.0–5.5); 50 mM KPB (pH 5.5–8.0); 50 mM Tris–HCl buffer (pH 7.0–10.0); and 50 mM imidazole-HCl buffer (pH 10.0–11.0). The reaction mixtures without the enzyme were incubated similarly as controls. Practically, γ-G*p*NA was not cleaved without the enzyme under our reaction conditions.

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