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Note

Molecular Cloning and Characterization of γ-Glutamyltranspeptidase from *Pseudomonas nitroreducens* IFO12694

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γ-Glutamyltranspeptidase from *Pseudomonas nitroreducens* IFO12694 (PnGGT) exhibited higher hydrolytic activity than transfer activity, as compared with other γ-glutamyltranspeptidases (GGTs). PnGGT showed little activity towards most of L-amino acids and towards glycyl-glycine, which is often used as a standard γ-glutamyl accepter in GGT transfer reactions. The preferred substrates for PnGGT as a γ-glutamyl accepter were amines such as methylamine, ethylamine, and isopropylamine.

Key words: *Pseudomonas nitroreducens*; γ-glutamyltranspeptidase; γ-glutamylmethylamide

γ-Glutamyltranspeptidase (GGT) catalyzes the transfer of the γ-glutamyl moiety from γ-glutamyl compounds, such as glutathione, to amino acids and peptides, as well as the hydrolysis of γ-glutamyl compounds. It is thought to play key roles in glutathione metabolism in both prokaryotic and eukaryotic organisms. Since its discovery in the sheep kidney,[1)] it has been isolated and characterized from various sources such as humans,[2)] rats,[3)] radishes,[4)] fungi,[5)] *Escherichia coli*,[6)] *Helicobacter pylori*,[7)] and *Bacillus subtilis*.[8)] Enzymatic synthesis of useful γ-glutamyl compounds such as γ-L-glutamyltaurine and γ-glutamylethylamide (theanine) using GGT has been reported.[9,10)] Efficient theanine synthesis from glutamine and ethylamine by immobilized cells of *P. nitroreducens* has also been reported.[11)] The enzyme catalyzing this transfer reaction is reported to be glutaminase based on a classification by Hartman.[12,13)] To ensure high production and for clarification of the reaction mechanism of this enzyme, molecular cloning of its gene is a prerequisite. Analysis of the *N*-terminal regions of the purified enzyme (heterodimeric polypeptides) clarified that the *N*-terminal amino acid sequences of the enzyme have high homology to those of microbial GGTs, whose heterodimeric polypeptides are generated by autocatalytic processing as found in the *N*-terminal nucleophile hydrolase superfamily. Consequently, the enzyme classified as a glutaminase based on Hartman's classification structurally belongs to GGTs. The DNA region encoding it was amplified by PCR using primers designed based on these *N*-terminal amino acid sequences and the nucleotide sequences of the highly conserved regions among microbial GGTs. In this study, PnGGT expression in *E. coli* and the properties of the enzyme, especially its novel substrate specificity, are reported.

E. coli JM109 was used as a host strain in preparing the plasmid. *E. coli* Rosetta-gami B (DE3) and pET22-b from Novagen (Madison, WI) were used for gene expression. Judging from the *N*-terminal amino acid sequences of large and small fragments of the purified enzyme and the nucleotide sequences of the other microbial GGTs, it is thought that its gene codes a signal peptide, a large subunit and a small subunit in a single peptide. At first, then, part of the PnGGT gene was amplified from *P. nitroreducens* genomic DNA using forward primer 5′-gtcaccctcgacggcggcgcggtt-3′ and reverse primer 5′-gtcgacgatggagaagtgcgtggt-3′, designed based on *N*-terminal amino acid sequences (large fragment, VTLDGGAVAAPDQYGAKVAA; small fragment, TTHFSIVDKDGNAVSNTYTL) and the nucleotide sequences of highly conserved regions among *Pseudomonas* GGTs. To obtain DNA fragments corresponding to the upstream and the downstream regions of the partial PnGGT gene, an LA-PCR *in vitro* cloning kit (Takara, Shiga, Japan) was used (*Bam*HI and *Eco*RI cassettes). The determined upstream and downstream sequences of the partial PnGGT gene allowed us to design PCR primers, forward primer 5′-taaggaagtcatatg-cgcgtgttccacttc-3′ (underlined region, *Nde*I restriction site) and reverse primer 5′-acggtgcgaattccggggcttaggg-tttga-3′ (underlined region, *Eco*RI restriction site), for amplification of the complete PnGGT gene. PCR was performed in a reaction mixture containing 10 ng of genome template DNA, 50 pmol of each primer, 10 nmol of each dNTP, and 2.5 U of ExTaq DNA polymerase (50 μl in ExTaq buffer). Thermal cycling was 1 cycle of 94 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 90 s. A PCR product was cloned into pET22-b and its nucleotide sequence was determined. *E. coli* Rosetta-gami B (DE3) harboring pPnGGT2 was cultured for PnGGT production.

GGT activity was measured as follows: For hydrolytic activity, the reaction mixture contained 2.5 mM of γ-L-glutamyl-*p*-nitroanilide (γ-Glu*p*NA), 100 mM imidazole buffer (pH 9.0), and the enzyme in a final volume of 1 ml. The reaction was terminated by adding 0.5 ml of 10% acetic acid after incubation at 30 °C for 10–20 min. *p*-Nitroaniline formation was monitored at 410 nm. One unit of enzyme in the hydrolytic reaction was defined as the amount required to catalyze the formation of 1 μmol

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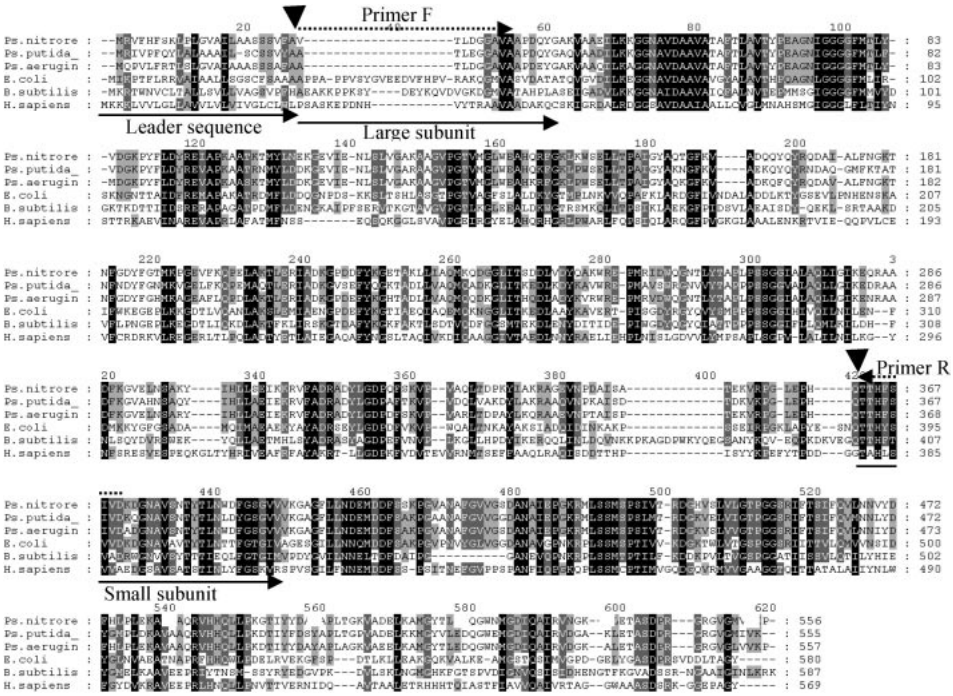

Fig. 1. Sequence Alignment of γ-Glutamyltranspeptidases from a Variety of Species.
Black boxes show amino acid residues identical and similar among all species, and shaded boxes show partially identical amino acid residues among them. Thick arrows show hydrolytic cleavage sites. Broken arrows for primer F and primer R indicate positions corresponding to those at which the forward primer and the reverse primer used in first PCR were designed, respectively.

of *p*-nitroaniline per min ($U_{hydrolysis}$). For transfer activity, the reaction mixture contained 40 mM L-glutamine, 20 mM hydroxylamine, 100 mM imidazole buffer (pH 7.5), and the enzyme in a final volume of 1 ml. After incubation for 10–20 min, the reaction was terminated by adding 2 ml of 0.2 M $FeCl_3 \cdot 6H_2O$, 0.12 M trichloroacetic acid, 0.25 N HCl, and water (8:2:1:13). The color of γ-L-glutamylhydroxamate that developed was determined spectrophotometrically at 540 nm. One unit of enzyme in the transfer reaction was defined as the amount required to catalyze the formation of 1 µmol of γ-L-glutamylhydroxamate per min ($U_{transfer}$). Gly-Gly and Gly-Gly-Gly were purchased from Watanabe Chemical (Hiroshima, Japan). γ-L-Glutamylmethylamide, γ-L-glutamylethylamide, γ-L-glutamylisopropylamide, and γ-glutamyl-N^{ε}-lysine were from Sigma (St. Louis, MO). The other reagents were chemically pure grades of commercial products.

The complete PnGGT gene consisted of 1,671 bps encoding a protein of 557 amino acids. The nucleotide sequence and deduced amino acid sequence were deposited in the DDBJ databases under accession no. AB548627. As shown in Fig. 1, PnGGT had 88% sequence identity with the GGTs of *P. putida* and *P. aeruginosa*, while PnGGT showed 58, 50, and 46% sequence identity with the GGTs of *E. coli*, *B. subtilis*, and *Homo sapiens* respectively.

Recombinant PnGGT showed 4.9 U/mg of specific activity of hydrolytic reaction in the cell-free extract. This value was about 70-fold higher than that in the parental strain, *P. nitroreducens*. The enzyme was purified to homogeneity by 6.1-fold purification with a yield of 10.4% using DEAE-Cellulofine and Butyl-FF column chromatography (Table 1). The purified enzyme showed the specific activities of hydrolyase and transferase, 30.2 $U_{hydrolysis}$/mg and 2.98 $U_{transfer}$/mg respectively. SDS–PAGE and *N*-terminal amino acid sequence analysis of purified enzyme revealed that it consisted of large and small subunits of the molecular sizes and *N*-terminal amino acid sequences of which were the same as those of the enzyme purified from the parent strain. The mechanism of formation of the heterodimeric structure of the gene product, however, remains to be resolved in further investigation.

The enzyme was characterized enzymologically. While the optimum pH of the hydrolytic reaction was about 9, the optimum pH of the transfer reaction was about 10.5. The ratios of hydrolytic activity to transfer

Table 1. Purification of PnGGT

Steps[a]	Total protein (mg)	Total activity[b] (U)	Sp. Activity (U/mg)	Yield (%)
Cell-free extract	1424	6998	4.9	100
DEAE-Cellulofine (pH 8.0)	91	2247	24.6	32.1
DEAE-Cellulofine (pH 7.0)	82	2280	28.5	32.6
Butyl FF	24	725	30.2	10.4

[a]Cell-free extract dialyzed against the 10 mM Tris–HCl buffer (pH 8.0) was loaded onto a DEAE-Cellulofine A-500m column (ϕ3 cm × 30 cm) equilibrated with the same buffer. After the column was thoroughly washed with the buffer, the enzyme was eluted with a linear gradient of NaCl (0 to 180 mM) in the buffer. The active fractions were collected and dialyzed against potassium phosphate buffer (pH 7.0). The enzyme was loaded onto a DEAE-Cellulofine A-500m column (ϕ2 cm × 10 cm) equilibrated with the same buffer. It was recovered in the unabsorbed fractions. Ammonium sulfate was added to the active fractions to 30% saturation. The enzyme solution was applied to a Butyl-FF column (ϕ0.5 × 2 cm) equilibrated with potassium phosphate buffer (pH 7.0) containing ammonium sulfate to 30% saturation. The enzyme was eluted by reducing the ammonium sulfate concentration from 30% to 0% saturation. The active fractions were dialyzed and concentrated.
[b]Activity is expressed for the hydrolytic reaction with γ-Glu*p*NA as substrate ($U_{hydrolysis}$).

Table 2. Substrate Specificity of PnGGT

Hydrolyase			Transferase		
Substrate	conc. (mM)	Rel. activity (%)	Accepter	conc. (mM)	Rel. activity (%)
γ-Glu-*p*NA	2.5	51.8	*p*-Nitroaniline	25	0
γ-Glu-hydroxamate	10	140	Hydroxylamine	100	160
Gln	40	100	Methylamine	100	100
γ-Glu-methylamide	40	100	Ethylamine	100	92.8
γ-Glu-ethylamide	40	110	Isopropylamine	100	60.8
γ-Glu-isopropylamide	40	29.1	Gly	100	0
γ-Glu-Gly	40	22.7	Gly-Gly	100	5.3
γ-Glu-Gly-Gly	40	31.7	Gly-Gly-Gly	100	0
γ-Glu-Gly-Gly-Gly	40	29.3	Gln	100	33.9
γ-Glu-α-Gln	40	134	Phe	100	0
γ-Glu-Phe	40	137	Glu	100	0
γ-Glu-Glu	40	44.2	Leu	100	0
γ-Glu-Leu	40	61.9	Tyr	5	0
γ-Glu-Tyr	20	57.3	Lys	100	173
γ-Glu-ε-Lys	5	94.2	Cys-Gly	25	0
γ-Glu-Cys-Gly	40	93.4	Methanol	100	0
(γ-Glu-Cys-Gly)$_2$	40	165	Ethanol	100	0
γ-Glu-methyester	40	144	Phenol	100	0
γ-Glu-ethylester	40	153			
γ-Glu-benzylester	8	71.3			

A reaction mixture (hydrolysis) containing γ-glutamyl compounds (2.5–40 mM), 100 mM borate buffer (pH 10.5), and the enzyme (0.05 $U_{transfer}$) was incubated at 30 °C for 2 min. A reaction mixture (transfer) containing accepter (5–100 mM), 40 mM L-Gln, borate buffer (pH 10.5), and the enzyme (0.1 $U_{transfer}$) was incubated at 30 °C for 20 min. For hydroxylamine, the reaction was done with 100 mM imidazole buffer (pH 7.5). Glutamate (hydrolyase activity) and γ-glutamyl compounds (transferase activity) were measured by HPLC equipped with Cosmosil 5C18-MS-II column (4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan) and a fluorescence detector (model L-2485, Hitachi, Tokyo). Hydrolyase activity and transferase activity were expressed relative to those measured towards L-Gln (1.51 μmol of L-Glu formed) and methylamine (2.61 μmol of γ-glutamylmethylamide formed) respectively (as 100%).

activity at pH 9 and pH 10.5 were 4.5 and 0.75 respectively. The other enzymatic properties of the recombinant enzyme, including optimal temperature and pH stability, were identical to those of the enzyme purified from the parental strain. The enzyme showed quite different reactivity towards the same acceptor molecule between the hydrolysis and transfer reactions. For example, it hydrolyzed γ-Glu-Phe sufficiently to L-Glu and L-Phe even at pH 10.5, which is favorable to the transfer reaction, but it produced no γ-Glu-Phe from L-Gln and L-Phe at pH 10.5. To estimate the ratio of transfer activity to hydrolyase activity towards the same acceptor at a pH appropriate to both reactions, a pH of 10.5, that of the reaction mixture for hydrolyase activity, was selected because both reactions can occur sufficiently at pH 10.5 (Table 2). Glycyl-Glycine, which is used as a standard γ-glutamyl accepter in the GGT transfer reaction, was found to be a rather poor substrate for PnGGT. For the GGT of *E. coli* (EcGGT) and the GGT of *B. subtilis* (BsGGT), Glycyl-Glycine is a preferable accepter, as is L-Lys. When L-Lys and Glycyl-Glycine were used as accepters, the ratios of transpeptidase activities towards L-Lys to Glycyl-Glycine of PnGGT, EcGGT, and BsGGT were 32.4, 1.21, and 0.21 respectively.[6,8]

Furthermore, standard L-amino acids including glycine are scarcely used as γ-glutamyl accepters, except for L-glutamine and L-lysine. Such a preference for the γ-glutamyl-accepter of PnGGT is not to be found in previously reported GGTs.[2,6,8] On the other hand, methylamine, ethylamine, and isopropylamine were good γ-glutamyl accepters (Table 2). The recombinant enzyme can use ethylamine as an accepter, and then can be just as useful as the original enzyme in theanine production. When L-lysine was used as γ-glutamyl accepter, the HPLC retention time of the product of a transfer reaction by PnGGT coincided completely with that of an authentic γ-glutamyl-N^{ε}-lysine (data not shown). These results indicate that compounds with a bulky group or carboxyl groups adjacent to the amino group in a γ-glutamyl accepter might be unsuitable as substrates for transfer reactions. However, PnGGT catalyzed the hydrolysis of γ-glutamyl-glycyl-glycine to glutamic acid and glycyl-glycine, and glutathione to glutamic acid cysteinyl-glycine, respectively. The difference between the hydrolytic and transfer reactions in terms of recognition of substrate structure is interesting and remains to be explained. Such specificity for γ-glutamyl accepters might allow PnGGT to synthesize theanine from L-glutamine and ethylamine effectively without producing by-products such as γ-glutamyl-glutamic acid. Further investigations, including X-ray crystallographic analysis, are being performed to clarify the structural characteristics of PnGGT in substrate recognition.

We think that the classification of the enzyme acting on γ-glutamyl compounds should be revamped based on both structural features and reaction characteristics. Moreover, if the enzyme has transfer activity of the γ-glutamyl moiety to amino acids, peptides, and amines even though its hydrolyase activity towards γ-glutamyl compounds is higher than its transfer activity, it should be classified with γ-glutamyltranspeptidase or γ-glutamyltransferase. The enzyme in this study was structurally discriminated from the enzyme catalyzing only the hydrolysis of L-glutamine, so called L-glutamine amidohydrolases such as glutaminase from *Micrococcus luteus* K-3.[14] In this study, we classified the enzyme from *P. nitroreducens* IFO12694 in γ-glutamyltranspeptidase.

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