RESEARCH ARTICLE

Enzymatic synthesis of γ-glutamyl dipeptides catalysed by mutant *E. coli* γ-glutamyltransferases

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Dedicated to Professor Cesare Gennari on the occasion of his 70th birthday

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Abstract: γ-Glutamyltransferases (GGTs) from different sources have been proposed in recent times as biocatalysts for the enzymatic synthesis of naturally occurring γ-glutamyl derivatives with flavor-enhancer properties and interesting biological activities. Although the enzymatic approach is considered as a viable alternative to both the troublesome and low-yielding extraction from natural sources and synthesis through peptide chemistry requiring protection/deprotection steps, yields are not completely satisfactory, due to the intervention of GGT-catalysed hydrolysis and autotranspeptidation side-reactions. Here, the design and the use as biocatalyst for preparative purposes of two mutants of *E. coli* GGT are described. The design of mutants was pursued by docking-guided identification of residues putatively involved in interaction with the acceptor substrate, thus probably representing a first identification of residues constituting the still elusive and poorly characterized acceptor substrate binding site of the enzyme.

Introduction

γ-Glutamyl dipeptides are small molecules in which the amino group of an amino acid is acylated by the γ-carboxyl group of a glutamic acid residue. They are naturally occurring compounds[1] with interesting biological activities[2] and raised an increasing interest as taste-active compounds with kokumi properties.[3] The word kokumi describes an increase in food palatability, in terms of enhanced roundness, thickness, balance and continuity of taste, elicited through the activation of a Calcium-sensing receptor (CaSR).[4] It has been reported that the activation of the CaSR is also responsible of other health-related effects of γ-glutamyl derivatives.[2d, 5]

An enzymatic approach for the obtainment of γ-glutamyl derivatives is usually considered as preferable with respect to both extraction from natural sources and synthesis through the classical peptide chemistry. Extraction from natural sources is laborious and time consuming; it requires a large amount of starting material and affords low and variable yields, depending on seasonal variation, development stage and storage conditions of the plant.[6] On the other hand, classical peptide synthesis relies heavily on the use of protecting groups, thus increasing the number of synthetic steps and limiting the yields,[3e, 4c] rendering the entire synthetic plan not economically viable for a large scale production.

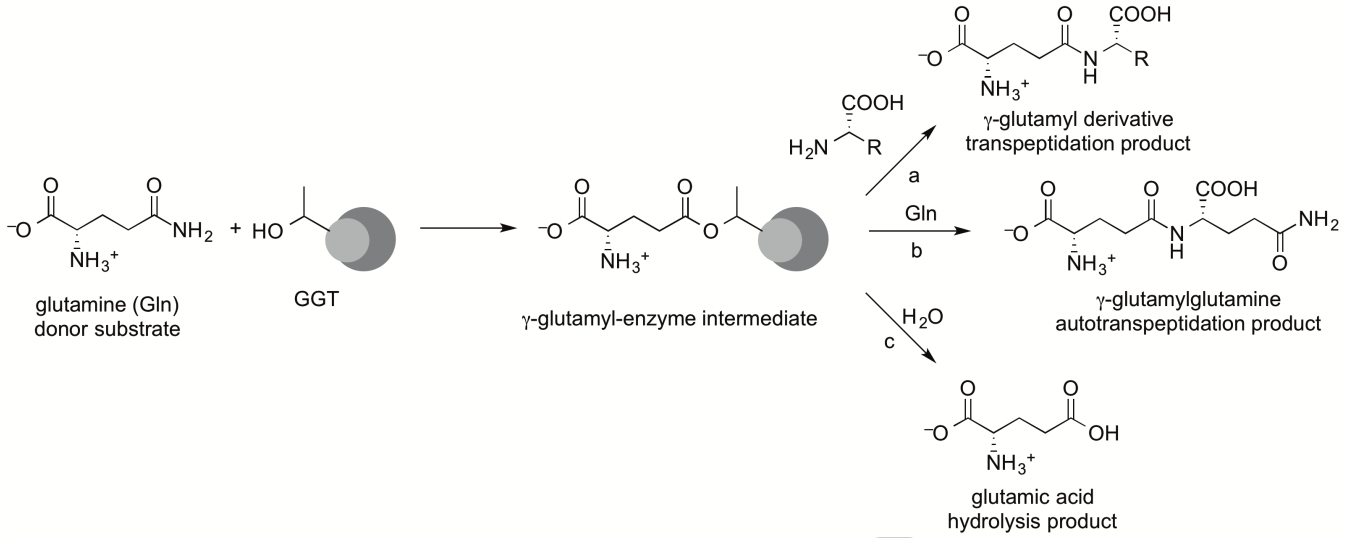
Among the enzymes currently exploited for the synthesis of γ-glutamyl derivatives, γ-glutamyltransferases (GGTs, E.C. 2.3.2.2 [7]) are considered as particularly promising.[7b, 8]

GGT is expressed in the cell as a single polypeptide chain, and then it is converted into the catalytically active form by an autocatalytic, proteolytic cleavage affording a heterodimeric enzyme composed by a large and a small subunit.[9] The *N*-terminal threonine of the small subunit is the catalytically active residue, involved in both enzyme maturation and catalysis.[10]

GGT catalyzes the transfer of a γ-glutamyl moiety from a donor to an acceptor substrate, through the formation of a γ-glutamyl-enzyme intermediate in which the γ-glutamyl moiety is temporarily covalently bound to the catalytically active *N*-terminal threonine of the small subunit [11]. For application as biocatalysts, microbial γ-glutamyltransferases are usually preferred, as they accept glutamine as the donor substrate[7b, 12] in place of the more expensive glutathionre requested by GGTs from higher organisms.[8d]

Deacylation of the γ-glutamyl-enzyme intermediate by intervention of a nucleophile dictates the outcome of the reaction and restores the free enzyme for subsequent catalytic cycles.[13]

If an amino acid acts as the nucleophile, a new γ-glutamyl derivative is formed through transpeptidation. However, a water molecule from the solvent or a molecule of the donor compound itself can also be acceptors, leading to hydrolysis and autotranspeptidation, respectively. Thus, glutamic acid and the γ-glutamyl derivative of the donor (e.g. γ-glutamylglutamine) are commonly observed byproducts. Usually, a basic pH of the reaction medium is able to favor transpeptidation over hydrolysis to some extent.[13, 14] Therefore, although attractive, GGT-catalyzed syntheses of γ-glutamyl derivatives are usually low-yielding. This is mainly attributed to the depletion of the donor



Scheme 1. GGT-catalysed reactions. The donor substrate glutamine reacts with the *N*-terminal threonine of the small subunit of GGT affording the γ-glutamyl-enzyme intermediate. Nucleophilic attack by an acceptor amino acid leads to a transpeptidation product, with the formation of the γ-glutamyl derivative of the acceptor (path a); if a molecule of the donor glutamine acts as the acceptor, the autotranspeptidation product γ-glutamylglutamine is formed (path b); if the γ-glutamyl-enzyme intermediate is resolved by a water molecule, hydrolysis occurs with formation of glutamic acid (path c).

substrate caused by the concomitant hydrolysis and autotranspeptidation reactions.[8f, 15] As a consequence, suppression or at least limitation of hydrolysis and autotranspeptidation reactions have been considered as strategies aimed to favor the transpeptidase activity of the enzyme.[16]

Site-directed mutagenesis techniques have been applied to GGTs from various sources for the identification of residues relevant for catalysis and enzyme maturation,[9b, 17] but only seldom enzyme variants were obtained with the intended aim of affecting hydrolase and transpeptidase activities[18] or for other, special purposes.[19]

It is to noticed that improved transpeptidase activity has been pursued through mutations of residues involved in donor recognition and binding, in an attempt to affect its orientation and binding affinity in such a way to modify the reaction outcome.[20]

In another approach, a mutant enzyme with enhanced transpeptidase activity was obtained by inserting the lid-loop from *Escherichia coli* GGT (EcGGT) on the structure of *Bacillus subtilis* GGT (BsGGT), naturally lacking it.[20] However, the ability of the mutant enzyme to catalyze repeatedly the addition of several γ-glutamyl units onto a single acceptor molecule, inherited by the parent BsGGT, limits its application as biocatalyst for preparative purposes.[18c]

As the binding site for the acceptor substrate of GGTs has been not yet identified with certainty,[17e, 21] at the best of our knowledge no reports exist about mutants with altered recognition or binding capacity towards the acceptor substrates.

Recently we proposed that GGT from *E. coli* (EcGGT) is able to recognize and possibly to bind the acceptor amino acid for the transpeptidation reaction only through its alpha-amino acidic moiety, while the involvement of the side chain in substrate recognition seems to play a little role.[22]

In this paper we report the transpeptidase activity of two mutants of EcGGT, obtained through modifications of two residues (Y444F and T413L) probably involved in acceptor substrate binding and orienting, and identified by docking studies. EcGGT-T413L is the first mutant with improved transpeptidation-to-hydrolysis ratio arising from site-directed mutagenesis of a residue probably involved in the acceptor substrate binding.

Results and Discussion

In our previous work, we noticed that the outcome of *E. coli* GGT-catalysed reactions carried out in the presence of equimolar amount of donor and acceptor substrates gave quite similar conversion rates, independently from the used acceptor. This observation led to the hypothesis that the alpha amino-acidic moieties of the acceptor amino acids is recognized by the enzyme, and the contribution of the side chains for acceptor binding could be very little.[22] Some other studies support also this view.[21, 23]

With the aim to identify the residues possibly involved in acceptor substrate binding, docking experiments were carried out using the structure of the γ-glutamyl-enzyme intermediate as the receptor macromolecule and various amino acids as the ligands. The solved structure of the γ-glutamyl-enzyme intermediate for EcGGT is available at the Protein Data Bank with PDB-ID 2DBW.[24] In a preliminary docking experiment, the software Autodock was able to place glutamic acid inside the glutamyl-binding site of the enzyme, in a conformation very close to that experimentally observed for the EcoGGT-glutamic acid complex (PDB 2DBX). In the calculated best pose, all the interactions of the bound glutamic acid with binding residues of the enzyme's active site were correctly predicted. Docking results may thus be considered as reliable. However, being the binding site for the acceptor substrate of GGTs not yet clearly defined, docking of acceptor amino acids in the structure of the γ-glutamyl-enzyme intermediate was more challenging. Indeed, first experiments hardly converged towards productive conformations in which the nucleophilic amino group of the acceptor was within short distance (< 4 Å) from the scissile ester bond of the γ-glutamyl-enzyme intermediate. Recent computational studies through accelerated molecular dynamics techniques (aMD) suggest a role of the Tyr444 residue of the lid-loop in the recognition and

accommodation of the donor glutamine into the EcGGT active site.[25] It is then conceivable that the same mechanisms operate also for the recognition of the acceptor amino acid. Thus, if the recognition process involves the free enzyme and glutamine, then the formation of the γ-glutamyl-enzyme intermediate ensues; conversely, if it involves the γ-glutamyl-enzyme intermediate and an amino acid, the transpeptidation or autotranspetidation reaction would result, depending on the nature of the involved amino acid (the donor or the acceptor, respectively). If a water molecule is brought within bond distance from the ester carbon of the γ-glutamyl-enzyme intermediate, hydrolysis can occur.

Docking experiments nicely converged when the side chain of Tyr444 was considered as flexible during computation (Fig. 1A and Fig. S1). As anticipated, the carboxyl group of each amino acid was invariably involved in the binding within the enzyme active site, assisting the putative acceptor substrate to reach a productive conformation (Fig. S1). Beside the phenolic hydroxyl group of Tyr444, also the backbone NH group and the hydroxyl group of the side chain of Thr413 seem to be responsible for acceptor substrate binding and orienting (Fig. 1B and Fig. S1). It is to note that the hydroxyl groups of Tyr444 and Thr413 can be involved also in binding water molecules in proximity to the scissile bond of the γ-glutamyl-enzyme intermediate, thus favoring hydrolysis, as can be seen in the solved structure of the γ-glutamyl-enzyme intermediate.[24] On the other hand, removal of the hydroxyl groups of Tyr444 or Thr413 could change the polarity of the microenvironment around the reaction site, preventing water molecules to enter the active site and thus hampering hydrolysis. Thus, although slightly affecting the binding of the acceptor, mutations of these residues through removal of the respective hydroxyl groups can be beneficial for the transpeptidation-to-hydrolysis ratio. Indeed, the aptitude of an amino acid to act as an acceptor substrate appears to be primarily related to the pK_a of its amino group. Stated another way, the rate of the transpeptidation reaction appears dependent mostly on the concentration of the neutral, non protonated amino group able to act as a good nucleophile with respect to a water molecule.[14b] Mutants EcGGT-Y444F and EcGGT-T413L were thus obtained.

Mutant enzymes EcGGT-T413L and EcGGT-Y444F were preliminarily tested through the standard spectrophotometric assay based on the use of γ-glutamyl-p-nitroanilide (GPNA) as the donor substrate. In this assay, the enzymatic activity is related to the liberation of *p*-nitroaniline following the formation of the γ-glutamyl-enzyme intermediate. A new catalytic cyclic can ensue only after the resolution of the γ-glutamyl-enzyme intermediate either through hydrolysis or transpeptidation. Measurements carried out in the absence of an added acceptor are usually taken as a measure of the hydrolytic activity, while the rate of the transpeptidation reaction is evaluated in the presence of an added acceptor substrate, using glycylglycine as the reference standard acceptor.[26] In the same experimental conditions, for most GGTs the measured activity is higher in the presence than in the absence of the added GlyGly, leading to the common notion that GGTs-catalysed transpeptidation reaction is faster than hydrolysis. However, enzymes from different sources show variable transpeptidation-to-hydrolysis ratio. The ratio is higher for GGTs from animal origin, while bacterial GGTs show only a modest prevalence of the transpeptidation reaction over hydrolysis, ranging from 2- to 4-6 fold, depending on the producing microorganism.[17e]

Specific activities of wt EcoGGT were calculated to be 1.38 μmol $min^{-1}gr^{-1}$ and 3.30 μmol $min^{-1}gr^{-1}$ for measurements in the absence and in the presence of GlyGly as the acceptor substrate, respectively. The mutant Y444F showed the higher improvement in specific activities in both experimental conditions (5.60 μmol $min^{-1}gr^{-1}$ in the absence of GlyGly and 25.4 μmol $min^{-1}gr^{-1}$ in its presence). Mutant T413L showed instead specific activity as low as 0.68 μmol $min^{-1}gr^{-1}$ in the absence of the acceptor substrate, and an improvement in reactions carried out in the presence of GlyGly (14.1 μmol $min^{-1}gr^{-1}$). From these results it can be concluded that the ratio of reaction velocities with or without GlyGly as the acceptor revealed to be similar for the wt enzyme and for mutant Y444F, showing a 4-fold higher activity in the

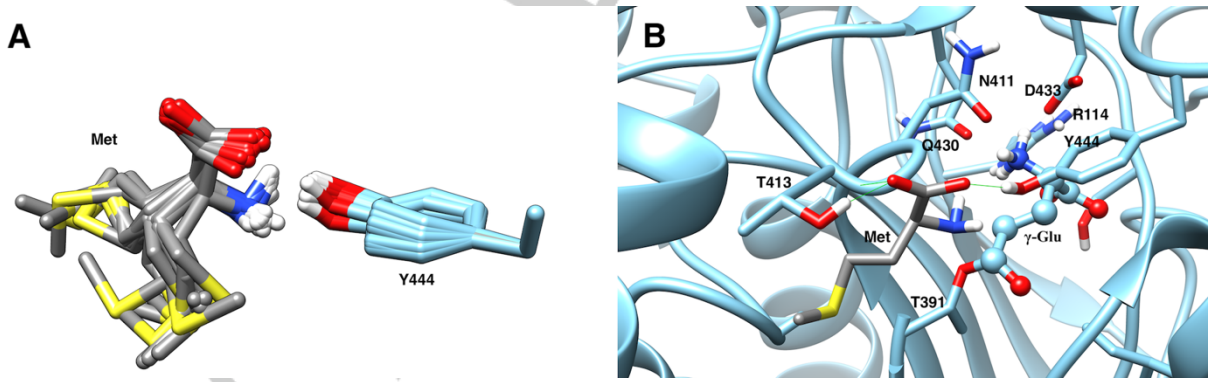


Fig. 1 Results for the docking experiments of the representative acceptor substrate methionine on the γ-glutamyl-enzyme intermediate of EcGGT (PDB 2DBW). A) Superimposition of representative productive poses of methionine. Three docking experiments were carried out and 100 docked poses were calculated in each experiment. For each experiment, the 100 calculated poses were clustered at 1.5 Å rmsd and the minimum-energy conformation of each productive cluster is reported in the figure, together with the corresponding conformations of the side chain of Tyr444, considered as flexible during docking calculations. It is evident the conformational variability of the side chain of methionine with respect to its alpha-amino acidic moiety, in agreement with the reported experimental observations. B) Best docking pose of methionine in the active site of EcGGT. The bound conformations calculated by the three independent docking experiments were clustered altogether at 1.5 Å rmsd. The bound conformation of methionine in the figure is the lowest-energy conformation of the lowest-energy and most populated cluster. Met is held in a productive conformation, with the nucleophilic amino group pointing towards the scissile ester bond of the γ-glutamyl-enzyme intermediate (in balls and sticks representation), through hydrogen bonds involving its carboxylic group with both Y444 and T413 (green lines).

Table 1. Relative activity of EcGGT-Y44F and EcGGT-T413L towards selected acceptor substrate, referenced to the activity measured using GlyGly as the acceptor substrate taken as 100%.

Acceptor	EcGGT-Y444F	EcGGT-T413L
GlyGly	100	100
None	25.3±1.80	5.1±0.77
L-Met	116.8±11.18	71.2±8.68
L-Phe	22.6±9.77	29.0±5.48
L-His	60.2}13.25	13.7±5.90
L-Ala	9.8±2.49	3.1±1.02
L-Val	25.0±1.02	12.1±1.75
L-Leu	24.5}6.31	10.3±1.62
L-SAC[a]	66.2±4.14	46.7±2.88

[a] SAC = *S*-allyl-L-cysteine

presence of GlyGly than in its absence. Conversely, the activity of the T413L mutant was very poor in the absence of the acceptor substrate, but raised consistently in the presence of GlyGly, reaching a 20 : 1 ratio, despite the overall low specific activity. Various, selected amino acids were tested as acceptors substrates to gain a first, rough indication about substrate specificity (Table 1). As stated before, being transpeptidation a faster reaction than hydrolysis, compounds are considered to be good acceptor substrates if the enzyme activity measured in their presence is higher than the activity measured in the absence of any added acceptor. Transpeptidase activity is given as a percentage with respect to the activity in the presence of the standard acceptor GlyGly. Mutant Y444F showed transeptidase activity in the presence of methionine, histidine and *S*-allyl-cysteine. Activity in the presence of methionine was higher than that in the presence of the reference acceptor GlyGly. With alanine, a lower activity was observed than that measured in the absence of any added acceptor. This aspect was not investigated further, but this behavior strongly suggests the occurrence of inhibition phenomena.[14b] In the presence of phenylalanine and the branched-chain amino acids valine and leucine, the measured activities were very close to that recorded in the absence of acceptors, thus suggesting that these amino acids could be poor acceptor substrates.

For mutant T413L, only in the presence of alanine an activity was observed very similar to that recorded in the absence of any acceptor. With all the other tested substrates a promising, albeit sometime low activity, was observed. Best results were obtained using methionine and *S*-allyl-cysteine; phenylalanine gave intermediate results, while histidine, valine and leucine were anticipated to be poor acceptors.

However, the experimental conditions adopted for activity assay are very different with respect to those suited for the use of GGTs as biocatalysts for preparative purposes, thus representing merely a preliminary, rough indication. Indeed, the wild-type EcGGT is able to catalyse the transpeptidation reaction of different acceptor substrates very similarly, regardless their apparent activities measured spectrophotometrically.[22] Mutants Y444F and T413L were then employed in reactions intended for preparative purposes. Glutamine was used as the donor substrate and selected amino acids were tested as acceptors.

First experiments catalysed by the mutant EcoGGT-Y444F, carried out at a preparative level using glutamine as the donor substrate and *S*-allylcysteine as a representative acceptor in equimolar amount (100 mM) at pH 10 and 40 °C, showed the formation of the usual products noticed in all the GGT-catalysed reactions (Fig. 2). The expected transpeptidation product reached a maximum concentration within 3-4 hours reaction time. After this time, its concentrations started to decrease slowly due to the irreversible hydrolysis. The autotranspeptidation product γ-glutamylglutamine formed transiently at the beginning of the reaction and, when the concentration of the donor glutamine fell below a certain threshold, it was in turn recognized by the enzyme as a donor substrate. Further glutamylation of the first transpeptidation product was also evident, with the formation of γ-glutamyl-γ-glutamyl-*S*-allyl-L-cysteine. Products formation appears to be slower in comparison with the reactions catalysed by the wild-type enzyme in the same experimental conditions (data not shown) and, more interesting, the subsequent hydrolysis of the newly formed γ-glutamyl derivatives appears also to be slower. As a consequence, the concentration of the desired product in the reaction mixture remains quite stable for a comparatively longer time and the concentration of glutamic acid raises more slowly, albeit steadily. However, the concentration of the transpeptidation product did not exceed a moderate yield (ca 35-40%) and this was ascribed to its further conversion into the corresponding γ-glutamyl-γ-glutamyl derivative. Reaction carried out using methionine as the acceptor substrate showed an initial faster production of γ-glutamyl-methionine with respect to the autotranspeptidation product γ-glutamyl-glutamine, but it gave anyway comparable results in terms of final yield of the desired compound. Attempts were made in order to limit the further γ-glutamylation of the transpeptidation product during the reaction, still maintaining the equimolar ratio of the substrates. Lowering the pH of the reaction mixture up to pH 8.5 only favored the hydrolysis reaction, with the production of substantial amounts of glutamic acid (Table 2, entry 5). Lowering the temperature to 21-25 °C did not affect significantly product distribution, but lengthened the reaction times. By using the enzyme at 0.4 U/mL concentration at 21-25 °, transpeptidation products reached maximum concentration in the reaction mixtures within 4 hours reaction time. Leucine, phenylalanine, methionine and *S*-allyl-

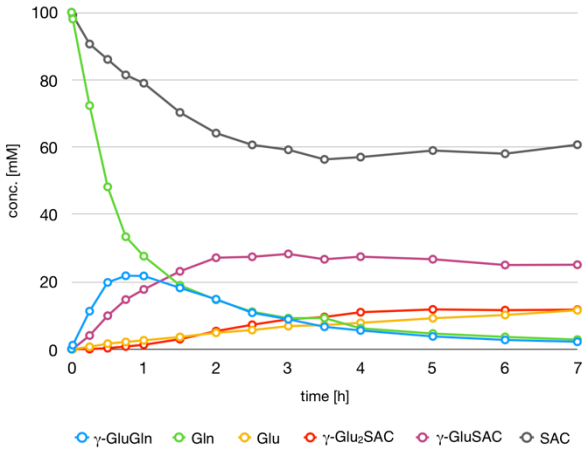


Fig. 2 Time course of EcGGT-Y444F mutant-catalysed reaction. Donor glutamine and acceptor *S*-allyl-L-cysteine were 100 mM each, pH 10, 40 °C, 0.25 U/mL enzyme.

Table 2. Results of EcGGT-Y444F- and EcGGT-T413L-catalysed reactions. Unless otherwise specified, all reactions were carried out at 21-24 °C, pH 10, in the presence of 0.4 U/mL enzyme.

entry	Mutant enzyme	Donor (conc. mM)[j]	Acceptor (conc. mM)[j]	Isolated yield %	Estimated yield % (HPLC)	Glu% (HPLC)	γ-Glu_2-acceptor % (HPLC)
1	EcGGT-Y444F	Gln (100)	Leu (100)	32	44	8	7
2	EcGGT-Y444F	Gln (100)	Met (100)	34	38	10	18
3	EcGGT-Y444F	Gln (100)	Phe (100)	35	39	8	15
4	EcGGT-Y444F	Gln (100)	SAC[d] (100)	21	27	8	12
5	EcGGT-Y444F[b]	Gln (100)	SAC[d] (100)	nd[c]	30	35	4
6	EcGGT-Y444F	Gln (100)	SAC[d] (300)	51	57	18	16
7	EcGGT-T413L	Gln (100)	Met (100)	23	35	3	18
8	EcGGT-T413L	Gln (200)	Met (600)	47	49	0.5	19
9	EcGGT-T413L[a]	Gln (100)	SAC[d] (100)	nd[c]	36	2	12
10	EcGGT-T413L[b]	Gln (100)	SAC[d] (100)	nd[c]	33	7	7
11	EcGGT-T413L[a]	Gln (100)	SAC[d] (200)	nd[c]	48	4	10

[a] reaction carried out 40 °C in the presence of 025U/mL enzyme.
[b] reaction carried out at pH 8.5.
[c] reaction carried out only at an analytical level.
[d] SAC = *S*-allyl-L-cysteine.

cysteine were used as acceptor substrates at a preparative level and the corresponding γ-glutamyl derivatives were isolated through ion-exchange column chromatography (Table 2, entries 1-4). They were usually obtained in moderate isolated yields, in good agreement with those estimated by HPLC analysis of the reaction mixture at 4 hours reaction time. In order to improve the low yield of γ-glutamyl-*S*-allyl-cysteine, the reaction was repeated using the acceptor substrate *S*-allyl-cysteine in 3-fold molar amount with respect to the donor glutamine, obtaining the product in 51% isolated yield (Table 2, entry 6).

Transpeptidation reaction catalysed by mutant EcoGGT-T413L was mainly studied using methionine and SAC as the acceptor substrates (Table 2, entries 7-11). Although the hydrolysis product is present in the reaction mixture in lower amount, usually less than 5 mM, enzyme-catalysed γ-glutamylation of the newly formed transpeptidation product limits its obtainment in satisfactory yield. Maximum concentration of the transpeptidation product was observed between 1.5 and 4 hours reaction time. In this time interval, the concentration of the transpeptidation product remains fairly constant, but the double-glutamilation product accumulates in the reaction mixture (data not shown). After this time, compounds able to act as donor substrates are depleted and also the concentrations of the produced γ-glutamyl derivatives start to decrease due to hydrolysis. Preparative reactions were thus stopped after two hours reaction time. In addition to the expected product, HPLC analyses of the reaction mixtures showed the presence of residual, unreacted donor glutamine (10-15%), the double γ-glutamylation product (10-18%) and a small amount of glutamic acid (< 5%). γ-Glutamylimethionine was isolated in 23% yield after ion exchange column chromatography (Table 2, entry 7). The yield improved to 47% using a three-fold molar excess acceptor methionine (Table 2, entry 8).

Conclusion

Two mutant enzymes were obtained by removing the hydroxyl groups from residues Y444 and T413 of *E. coli* GGT, obtaining mutants Y444F and T413L. On the basis of docking studies, it was anticipated that these mutations could affect slightly the binding of the acceptor substrate, decreasing at the same time the hydrophilicity of residues surrounding the entrance of the active site, thus possibly improving the transpeptidation-to-hydrolysis ratio of the enzymes.

Mutant Y444F showed an improved specific activity with respect to the wild-type enzyme, still conserving a similar activity. On the other hand, mutant T413L showed a quite low specific activity, but an interesting 20-fold increased transpeptidase activity over hydrolysis. These results could be related to an active involvement of T413 in acceptor substrate binding and orienting, for which no experimental precedents are found in the literature. The main drawback hampering the obtainment of the desired products in high yield remains the enzyme-catalysed transfer of a γ-glutamyl moiety onto the first transpeptidation product, affording the γ-glutamyl-γ-glutamyl derivative of the acceptor substrate. Using different acceptor amino acids, the corresponding γ-glutamyl dipeptides were indeed obtained in moderate yield, being the di-glutamylated derivatives the main byproducts observed in the reaction mixtures.

Our results reinforce the hypothesis that the binding site for the acceptor substrate in *E. coli* GGT, and perhaps in other bacterial GGTs, is only loosely defined. *E. coli* GGT has therefore a very broad substrate specificity, according to our previous observations. Therefore, improving the transpeptidase activity of bacterial GGTs through enzyme modification would mainly imply the suppression of the autotranspeptidase activity.

Experimental Section

General

L-Glutamine, L-glutamic acid, L-serine, L-methionine, L-phenylalanine, L-leucine, L-glutamic acid 5-(*p*-nitroanilide), glycilglycine and 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) were from Sigma Aldrich (Darmstadt, Germany). All reagents were used as received without further purification. HPLC-grade solvents were from Aldrich. *S*-allylcysteine was prepared as described.[27]

GGT from *E. coli* was obtained as previously described.[20]

Obtainment of mutant enzymes will be reported elsewhere.

Analytical TLC was performed on silica gel F_{254} pre-coated aluminum sheets (0.2 mm layer) (Merck, Darmstadt, Germany). Eluent was a mixture of n-BuOH/water/AcOH 3 : 1 : 1. Detection: 5% w/v ninhydrin solution in ethanol, followed by heating at 150 °C ca.

HPLC analyses were carried out using a 250 x 4.6 mm Gemini RP C18 column (Phenomenex, Torrance, CA, USA) on a Jasco instrument equipped with UV/Vis detector. Eluent A was 0.1% trifluoroacetic acid; eluent B was a 80:20 mixture of acetonitrile and eluent A. The following gradient was used: 0 - 10 min, isocratic elution with eluent A : eluent B 80 : 20; 10 - 15 min, linear gradient to eluent A : eluent B 70 : 30; 15 - 25 min, linear gradient to eluent A : eluent B 40 : 40; 25 - 35 min, linear gradient to eluent A : eluent B 40 : 60; 35 - 40 min, isocratic elution with eluent A : eluent B 40 : 60; 40 - 60 min, column re-equilibration through linear gradient to eluent A : eluent B 80 : 20. Flow rate was 1 ml/min and detection was at 356 nm.

Ion exchange column chromatography was performed with Dowex 1x8 resin 200-400 mesh (Aldrich, Darmstadt, Germany) in the acetate form.

^{1}H-NMR spectra were acquired at 400.13 MHz on a Bruker Advance 400 spectrometer (Bruker, Karlsruhe, Germany) interfaced with a workstation running Windows operating system and equipped with a TOPSPIN software package. Chemical shifts are given in ppm (δ) and are referenced to solvent signal (δ_H D_2O 4.79 ppm). Spectra analyses were carried out with inmr Reader software (ww.inmr.net).

ESI-MS spectra were recorded on a Thermo Finnigan LCQ Advantage spectrometer (Hemel Hempstead, UK).

UV measurements were carried out with a Jasco V-360 Spectrophotometer (Jasco International, Tokyo, Japan).

Docking simulation was carried out using Autodock 4.2.5 and is detailed in the supplementary material.

Enzyme activity assay

The release of *p*-nitroaniline from a 2 mL solution of 1 mM GPNA, 100 mM GlyGly and 20 μL of the enzyme solution in TRIS buffer at pH 8.5 was continuously monitored at 410 nm recording data every 10 s for 3 minutes. One enzyme unit was defined as the amount of enzyme that liberates 1 μmol mL^{-1} min^{-1} of *p*-nitroaniline in the presence of glycylglycine. *p*-Nitroaniline concentrations were estimated through a calibration curve.

Hydrolase and transpeptidase activities

Hydrolase and transpeptidase activities were evaluated with the same procedure for enzyme activity assay, in the presence and in the absence of glycylglycine as the acceptor, respectively.

Enzyme activity towards different acceptor amino acids

Measurements were carried out in the same conditions of enzyme activity assay, by substituting GlyGly by the proper acceptor amino acid.

Pre-column derivatization procedure with Sanger's reagent

Pre-colums derivatization was carried out as previously described.[18c, 22] Briefly, standard solutions for calibration curve or aliquots of reaction mixture to be analyzed (20 μL) were diluted 1:20 with water. The diluted solution (100 μL) was transferred into a pyrex tube equipped with a perforated screw cap fitted with a forcible sealing septum. 5 mM L-Serine (50 μL) was added as the internal standard and the resulting solution was diluted with borate buffer at pH 8.5 (350 μL). 10 mM 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) solution in acetone (500 μL) was then added and the mixture was shaken and heated at 70°C for 45 minutes in the dark. A needle was introduced into the septum and heating was continued for further 10 minutes, in order to evaporate most of the acetone. The tube was finally cooled under running water and 200 μL of the resulting mixture was diluted 1:1 with 0.1% TFA solution, affording the sample for HPLC analysis.

Enzyme-catalyzed reactions at analytical level. General procedure

Solution containing glutamine, the acceptor amino acid and the enzyme at the proper concentrations in sodium carbonate/sodium hydrogencarbonate buffer at the right pH was stirred at the given temperature for 6 hours. At fixed time points, 20 μL aliquots were withdrawn, derivatized as described and analyzed by HPLC.

EcoGGT—Y444F catalyzed synthesis of γ-glutamyl derivatives. General procedure

L-glutamine (219 mg, 1.5 mmol) and the proper acceptor amino acid (1.5 or 4.5 mmol) were dissolved in water and the pH was adjusted to 10 with 1M NaOH. EcoGGT-Y444F was added (0.4 U/mL); final reaction volume was 15 mL. The solution was stirred at 21-23 °C for 4 hours. Reaction was checked at 2 and 4 hours reaction time by HPLC after pre-column derivatization, as described. The mixture was charged onto a pad of Dowex 1 x 8 ion exchange resin in the acetate form and the pad was eluted with water (3 column volumes) and then with a scalar gradient of acetic acid solutions (0.5, 1.0, 1.5, and 2.0 M, three column volumes each). Eluate was collected in fractions; fractions were combined on the basis of TLC analysis (silica gel, staining reagent: ninhydrin) and freeze-dried.

By applying this procedure, the following compounds were synthesized:

γ-L-glutamyl-L-leucine obtained from a 100 mM solution of L-glutamine and L-leucine in 32% isolated yield; estimated yield by HPLC was 44%. An additional amount of product was recovered as a 1 : 0.78 mol mixture (^{1}H NMR-based) with g-L-glutamyl-L-glutamine (40 mg). ^{1}H NMR as in ref. [8f].

γ-L-glutamyl-L-phenylalanine obtained from a 100 mM solution of L-glutamine and L-phenylalanine in 35% isolated yield; estimated yield by HPLC was 39%. ^{1}H NMR as in ref. [8e].

γ-L-glutamyl-L-methionine obtained from a 100 mM solution of L-glutamine and L-methionine in 34% isolated yield; estimated yield by HPLC was 38%. ^{1}H NMR as in ref. [27].

γ-L-glutamyl-*S*-allyl-L-cysteine (equimolar amount) obtained from a 100 mM solution of L-glutamine and S-allyl-L-cysteine in 21% isolated yield; estimated yield by HPLC was 27%. ^{1}H NMR as in ref. [27].

γ-L-glutamyl-*S*-allyl-L-cysteine (3-fold excess acceptor substrate) obtained from a solution of 100 mM L-glutamine and 300 mM *S*-allyl-L-cysteine in

51% isolated yield; estimated yield by HPLC was 57%. ^{1}H NMR as in ref. [27].

EcoGGT—T413L catalyzed synthesis of γ-glutamyl derivatives. General procedure

L-glutamine (100 or 200 mM, 1 eq) and the proper acceptor amino acid (1 or 3 eq.) were dissolved in water and the pH was adjusted to 10 with 1M NaOH. EcoGGT-T413L was added (0.4 U/mL); final reaction volume was 5 mL. The solution was stirred at 21-23 °C for 2 hours. Reaction was checked at the end of the reaction time by HPLC after pre-column derivatization, as described. The mixture was charged onto a pad of Dowex 1 x 8 ion exchange resin in the acetate form and the pad was eluted with water (3 column volumes) and then with a scalar gradient of acetic acid solutions (0.5, 1.0, 1.5, and 2.0 M, three column volumes each). Eluate was collected in fractions; fractions were combined on the basis of TLC analysis (silica gel, staining reagent: ninhydrin) and freeze-dried.

γ-L-glutamyl-L-methionine (equimolar amount) obtained from a 100 mM solution of L-glutamine and L-methionine in 23% isolated yield; estimated yield by HPLC was 35%

b) γ-L-glutamyl-L-methionine (3-fold excess acceptor substrate) obtained from a 200 mM solution of L-glutamine and 600 mM L-methionine in 47% isolated yield; estimated yield by HPLC was 49%.

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Keywords: Biocatalysis • Mutagenesis • Amino acids • γ-glutamyltransferases • taste-active compounds

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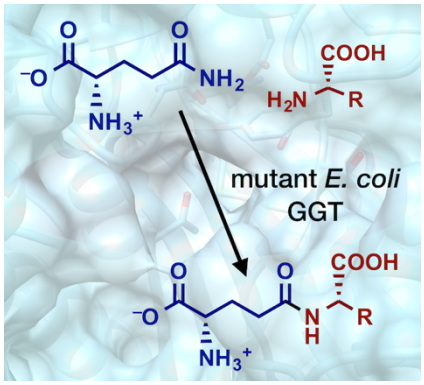

Two residues were identified inside the active site of *E. coli* γ-glutamyltransferase, putatively involved in acceptor substrate binding. Point-mutation of these residues afforded two mutant enzymes with altered catalytic properties. Mutant T413L showed a very promising transpeptidation-to-hydrolysis ratio up to 20 : 1. The two mutants were tested as biocatalysts for the enzymatic synthesis of γ-glutamyl dipeptides with flavor-enhancer properties.