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An overall framework for the *E. coli* γ-glutamyltransferase-catalyzed transpeptidation reactions

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ABSTRACT

γ-Glutamyl derivatives of proteinogenic or modified amino acids raise considerable interest as flavor enhancers or biologically active compounds. However, their supply, on a large scale and at reasonable costs, remains challenging. Enzymatic synthesis has been recognized as a possible affordable alternative with respect to both isolation procedures from natural sources, burdened by low-yield and by the requirement of massive amount of starting material, and chemical synthesis, inconvenient because of the need of protection/deprotection steps. The *E. coli* γ-glutamyltransferase (Ec-GGT) has already been proposed as a biocatalyst for the synthesis of various γ-glutamyl derivatives. However, enzymatic syntheses using this enzyme usually provide the desired products in limited yield. Hydrolysis and autotranspeptidation of the donor substrate have been identified as the side reactions affecting the final yield of the catalytic process. In addition, experimental conditions need to be specifically adjusted for each acceptor substrate. Substrate specificity and the fine characterization of the activities exerted by the enzyme over time has so far escaped rationalization. In this work, reactions catalyzed by Ec-GGT between the γ-glutamyl donor glutamine and several representative acceptor amino acids have been finely analyzed with the identification of single reaction products over time. This approach allowed to rationalize the effect of donor/acceptor molar ratio on the outcome of the transpeptidation reaction and on the distribution of the different byproducts, inferring a general scheme for Ec-GGT-catalyzed reactions. The propensity to react of the different acceptor substrates is in agreement with recent findings obtained using model substrates and further supported by x-ray crystallography and will contribute to characterize the still elusive acceptor binding site of the enzyme.

1. Introduction

γ-Glutamyl peptides are compounds in which a glutamic acid residue is linked to another amino acid or to a short peptide through an amide bond involving its γ-carboxyl group. They are naturally found in several plants of food interest [1–3] and several of them are involved in the genesis of kokumi taste sensations [4–8]. Kokumi sensations are related to mouthfulness, thickness, roundness, balance and continuity of the perception of taste and are mediated by the activation of a calcium-sensing receptor on the surface of taste cells [9,10]. Kokumi compounds are produced also during food fermentation and aging and are often recognized as essential in contributing to the typical taste of those food [11–18]. In addition, interesting biological activities have been associated to some γ-glutamyl derivatives [19–22]. Some γ-glutamyl compounds have been recognized to also act as umami compounds, by directly activating the umami taste receptors [23,24]. All these features, together with their usually low perception threshold, make γ-glutamyl derivatives interesting flavor enhancers and potential tools in programs aimed at salt and fats-intake reduction [25–29]. However, the applicative exploitation of γ-glutamyl derivatives is hampered by difficulties connected to their quantitative production at a reasonable cost. In addition to requiring large amounts of starting material, extraction of γ-glutamyl derivatives from natural sources is laborious and time consuming. Yields are usually low and erratic, as their content in vegetables depends on seasonal variation, development stage of the plants and storage conditions [30–32]. Chemical synthesis is also non-

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economical, due to the need of protection-deprotection steps [8,33], although some strategies have been developed to reduce the number of the protective groups required in the process [34,35].

Conversely, enzymatic approaches for the synthesis of γ-glutamyl derivatives have long been investigated since they represent selective and mild methods, able to avoid the need of protective groups and the use of environmentally dangerous organic solvents. The enzymes currently exploited for the synthesis of γ-glutamyl derivatives are glutaminases (E.C. 3.5.1.2) and γ-glutamyltransferases (GGTs, E.C. 2.3.2.2) [22,36–43].

γ-Glutamyltransferases catalyze the transfer of a γ-glutamyl moiety from a donor compound to an acceptor substrate [44,45]. The donor compound is itself a γ-glutamyl derivative, usually glutathione, though microbial GGTs are also able to use glutamine, far less expensive [46,47]. GGTs belong to the *N*-terminal nucleophile superfamily (Ntn) [48]. They are expressed as a single polypeptide chain, then converted into the active form of the enzyme by an autocatalytic, proteolytic cleavage affording a heterodimeric structure composed by a large and a small subunit [49,50]. The conserved *N*-terminal threonine residue of the small subunit is the catalytically active residue, involved in both enzyme maturation and catalysis [51]. The catalytic reaction proceeds through the formation of a γ-glutamyl-enzyme intermediate, in which the γ-glutamyl moiety is temporarily bound into the active site of the enzyme through an ester bond involving the conserved threonine residue at the *N*-terminus of the small subunit [52]. The γ-glutamyl-enzyme intermediate is then resolved by the nucleophilic attack of an acceptor substrate [53]. In case the acceptor substrate is an amino acid, a short peptide or an amine, a transpeptidation reaction occurs. In case the donor compound itself acts as acceptor, an autotranspeptidation product appears, if the acceptor is water from the solvent, the donor is hydrolyzed. Besides the desired γ-glutamyl compound, two common byproducts are thus found in GGT-catalyzed transpeptidation reactions: glutamic acid, arising from hydrolysis, and γ-glutamyl derivatives of the donor, obtained by autotranspeptidation. To some extent, the hydrolysis/transpeptidation ratio can be modulated by varying the pH of the reaction mixture, with basic pH favoring transpeptidation [53–55].

Despite the above-mentioned advantages of the enzymatic approach, the synthesis of γ-glutamyl derivatives catalyzed by GGTs is usually plagued by low yields caused either by the competing hydrolysis reaction, which also involves the newly formed product, and/or by the autotranspeptidation reaction, which subtracts donor substrate [20,56–59]. For the latter observation, in reactions catalyzed by *E. coli* GGT much better yields of γ-glutamyl products were obtained by using D-glutamine as the donor substrate [36,60]. Indeed, *E. coli* GGT shows a strong preference for L-acceptor amino acids, while still recognizing D-glutamine as donor [46].

Also GGTs from various *Bacillus* sp. have been proposed as biocatalysts for preparative synthesis of γ-glutamyl derivatives [58,59,61–64]. In addition to hydrolysis and autotranspeptidation, GGTs from *Bacillus* spp. are also able to catalyze the formation of polyglutamylated compounds [55], further lowering product yields and increasing the complexity of the reaction mixture, thereby hampering product purification.

In our ongoing search for a simple, convenient and straightforward method for the synthesis of γ-glutamyl derivatives with flavor-enhancing properties [35,65,66], our attention was turned to GGT from *E. coli* (Ec-GGT). Ec-GGT was proposed as a biocatalyst for the enzymatic synthesis of several γ-glutamyl derivatives since a long time [36,37,67–70]. However, its use requires the heuristic optimization of reaction conditions as the rules governing the enzyme's activity seem to have thus far escaped rationalization. This prompted us to undertake a systematic and comprehensive study of the transpeptidase activity of Ec-GGT which is reported in this work.

2. Results and discussion

The activity of Ec-GGT towards selected acceptor substrates was preliminarily tested following the liberation of *p*-nitroaniline through the standard spectrophotometric assay based on the use of γ-glutamyl-*p*-nitroanilide. The faster reaction rate observed in the presence of compounds carrying a nucleophilic amino group is usually taken as a measure of the ability of the nucleophile to act as acceptor substrate. In the absence of acceptor, the enzyme-mediated liberation of *p*-nitroaniline is ascribed to hydrolysis, a slower reaction compared to transpeptidation. Glycylglycine is one of the best acceptors and is commonly used as reference substrate for GGT [71]. The activity of the enzyme was tested towards different acceptor amino acids, spanning over a broad range of activities in the reaction catalyzed by Ec-GGT [46] (Table 1). Arginine revealed to be the best acceptor substrate, with an activity very close to that recorded using the reference glycylglycine. High activity (72%) was also recorded using methionine. Phenylalanine showed intermediate activity (ca 43%), while isoleucine proved to be a rather poor acceptor (ca 17%). These results, summarized in Table 1, paralleled the order of activities reported in the literature [46] with discrepancies ascribable to the different experimental conditions. In addition, *S*-allylcysteine (SAC) was also tested, as the corresponding γ-glutamyl derivative represents an interesting synthetic target due to its kokumi properties [4]. Its propensity to act as an acceptor was slightly lower than that of methionine (60%).

Although the above assay is routinely used for assessing GGT activity in a simple and fast way, the application of the same conditions for preparative purposes is unfeasible because of the use of the expensive and scarcely soluble γ-glutamyl-*p*-nitroanilide as donor compound and the excess of acceptor substrate required to minimize spurious liberation of *p*-nitroaniline through hydrolysis side reaction. Taking advantage of the glutaminase activity of bacterial GGTs [46,47,72], glutamine is the donor substrate of choice for preparative purposes, although some other derivatives have occasionally been proposed, especially in whole-cell approaches [70,73–75].

In order to reproduce the conditions of synthetic, preparative procedures, the compounds listed in Table 1 were tested as acceptor substrates in Ec-GGT-catalyzed reactions using glutamine as the donor. Ec-GGT was preliminary tested at three different concentrations, namely 0.05, 0.25 and 0.5 U/mL. Ec-GGT concentration of 0.25 U/mL was selected as it ensured both consumption of the donor glutamine and the achievement of a near-equilibrium state (vide infra) within a reasonable time frame. Using more enzyme (0.5 U/mL), reactions were too fast to allow a careful monitoring of the initial formation of the various products, while with 0.05 U/mL the γ-glutamyl derivatives of the acceptor

Table 1
Ec-GGT activity towards selected acceptor substrates relative to GlyGly. The liberation of *p*-nitroaniline from a mixture comprising γ-glutamyl-*p*-nitroanilide (1 mM), the acceptor (100 mM) in TRIS.HCl buffer at pH 8.5 and the enzyme in a final volume of 2 mL was continuously monitored at 410 nm for three minutes. Concentration of *p*-nitroaniline was estimated through a calibration curve; the slope of the resulting curves in their initial, linear range, were taken as the measure of the activity. Activities are expressed as percentages relative to the activity measured in the presence of GlyGly taken as 100. Data are average values ± standard deviation of at least three independent measurements.

Acceptor	Relative activity %
GlyGly	100
None	18.4 ± 5.7
Arg	98.5 ± 3.0
Leu	16.7 ± 1.7
Met	72.2 ± 8.7
Phe	42.8 ± 8.7
S-allyl-cysteine	60.3 ± 9.2

substrates did not always reach the maximum concentration within the set time frame (7 h). Ec-GGT-catalyzed reactions were monitored by withdrawing samples at different time points, which were set closer at the beginning of the reaction, to gain a clear picture of the timing of products formation, and more infrequent after 3.5 h, for the evaluation of the distribution of later products. Several compounds were identified and quantified by chromatography (Fig. 1) allowing to draw a detailed profile of each reaction. The time-resolved trend of the reaction between glutamine and *S*-allylcysteine is shown in Fig. 2. A drop in glutamine concentration could be observed within 4 h, mainly leading to the transpeptidation product γ-glutamyl-*S*-allylcysteine and the autotranspeptidation product γ-glutamylglutamine (Fig. 2A). The decrease in concentration of the acceptor substrate *S*-allylcysteine was slower than that of the donor, reaching a minimum between 2.5- and 3-hour, followed by a slow resurgence. At this stage, the autotranspeptidation product γ-glutamylglutamine appears to be used as donor substrate, leading to a decline in its concentration and a consequent accumulation of the desired transpeptidation product γ-glutamyl-*S*-allylcysteine. However, the concentration of γ-glutamyl-*S*-allylcysteine never exceed 32 mM, ca 30% with respect to each of the input substrate and remained fairly constant from 3.5 to 4 h on. Notably, both γ-glutamyl-γ-glutamylglutamine and γ-glutamyl-γ-glutamyl-*S*-allylcysteine were detected in the reaction mixture. The formation of these byproducts was slower than that of the parent γ-glutamyl derivatives and their concentrations declined very slowly after 4 h (Fig. 2B). Only the concentration of glutamic acid produced by hydrolysis rose slowly but steadily throughout the reaction. After one hour, the signal attributable to γ-glutamyl-glutamic acid was also distinguishable and quantifiable in the chromatograms (Fig. 2B). Compounds carrying three γ-glutamyl residues linked to a single acceptor molecule, either glutamine or *S*-allylcysteine, were only detectable at late stages and in very low amount.

The rather low conversion of *S*-allylcysteine into the corresponding γ-glutamyl derivative prompted us to check the behavior of a better acceptor substrate (Table 1), namely arginine. Glutamine and arginine were incubated with Ec-GGT in the same reaction conditions (100 mM donor and acceptor; 0.25 U/mL enzyme and 40 °C). The reaction proceeded similarly (Table 2). Conversion of the acceptor substrate into the corresponding γ-glutamyl derivative was limited to ca 37% after 3 h and was achieved after the transient formation of γ-glutamylglutamine. γ-Glutamyl-γ-glutamyl-arginine was also detected together with a little peak attributable to γ-glutamyl-γ-glutamyl-γ-glutamyl-arginine. Extending experiments to the other acceptors, the formation of nearly equal amounts of γ-glutamyl-glutamine and transpeptidation product within the first hour of reaction, and the formation of γ-glutamyl-γ-glutamyl derivatives revealed to be common traits of the reactions, independently from the nature of the acceptor used (Table 2). Unfortunately, γ-glutamyl-γ-glutamyl-methionine could not be quantified in the related reaction, due to the overlap of its peak with that of a byproduct of the derivatization procedure [76].

From these data it can be concluded that, differently from what could be assumed on the basis of the spectrophotometric assay, the concentration of γ-glutamyl derivatives of all the acceptor substrates under analysis reached a maximum conversion rate ranging from 29 to 43 % within 1.5–2.5 h. Neither the maximum conversion rate, nor the initial rate of formation of the γ-glutamyl derivatives of the various acceptors were in agreement with the activities measured using γ-glutamyl-*p*-nitroanilide as donor compound, which curiously qualitatively matched with the maximum concentrations recorded for the corresponding γ-glutamyl-γ-glutamyl derivatives. A few of the Ec-GGT-catalyzed reactions between glutamine and some of the acceptor compounds used

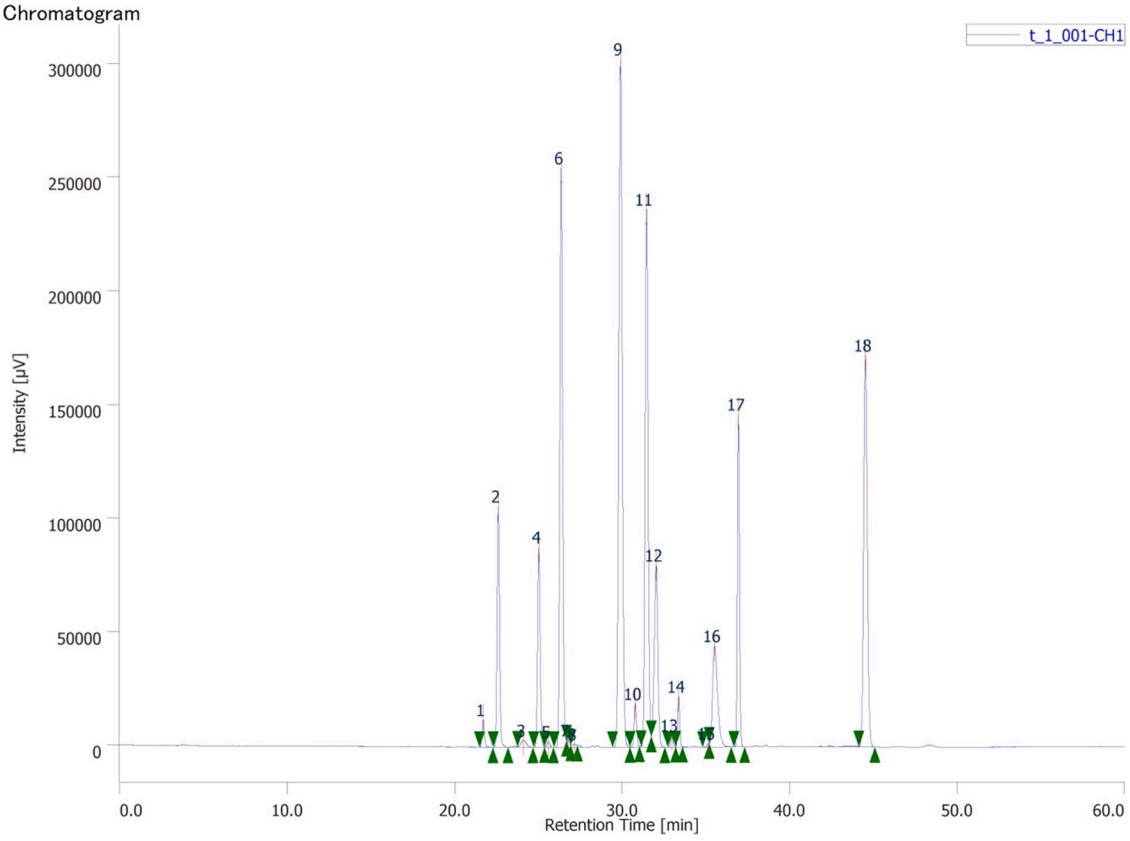


Fig. 1. Chromatogram of the Ec-GGT-catalyzed reactions of glutamine and *S*-allylcysteine (SAC) after 1 h. Glutamine and *S*-allylcysteine were used at 100 mM concentration; Ec-GGT was 0.25 U/mL, pH 10, 40 °C. Peaks attribution: 1) γGlu-γGlu-Gln; 2) γGluGln; 4) Gln; 6) Ser, used as internal standard; 9) excess Sanger's reagent; 10) Glu; 11) 2,4-dinitroaniline, arising from the reaction of Sanger's reagent with ammonia liberated from Gln; 12) derivatization byproduct; 14) γGlu-γGlu-SAC; 16) derivatization byproduct; 17) γGlu-SAC; 18) SAC.

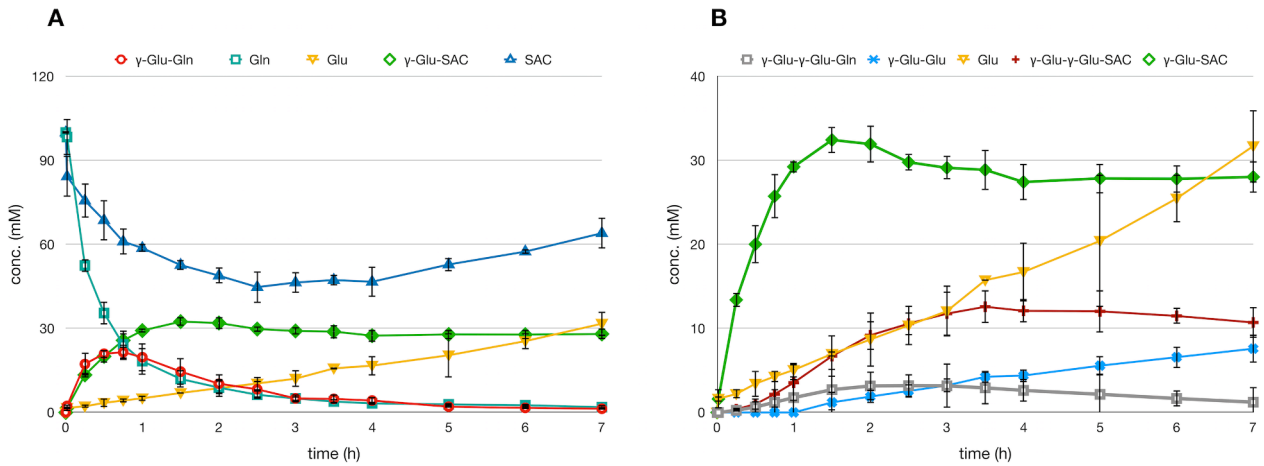


Fig.2. Time course for the Ec-GGT-catalyzed reaction of glutamine and *S*-allylcysteine (SAC). See caption of Fig. 1 for experimental conditions. A) graph of the time-dependent consumption of substrates Gln and SAC and the formation of the main reaction products γ-GluGln, Glu and γ-Glu-SAC. B) Time-dependent formation of γ-GluGlu and the di-glutamylated products γ-Glu_2Gln and γ-Glu_2SAC in comparison with γ-Glu-SAC and Glu.

Table 2
Parameters of Ec-GGT-catalyzed reactions with different acceptor substrates.

Acceptor	γ-Glu-acceptor [mM] (h)[a]	γ-Glu_2-acceptor [mM][b]	γ-GluGln [mM] (h)[a]	γ-GluGln [mM][b]	Glu [mM][b]
GlyGly	29 ± 0.7 (4)	13 ± 0.2	26 ± 0.4 (0.5)	8 ± 2.2	3 ± 0.3
Arg	37 ± 1.4 (2)	18 ± 3.5	21 ± 1.2 (0.5)	7 ± 3.4	4 ± 1.2
Met	40 ± 1.4 (1.5)	Nd	15 ± 1.2 (0.5)	12 ± 2.5	4 ± 1.5
Phe	37 ± 1.0 (1)	8 ± 2.6	17 ± 2.1 (0.5)	12 ± 3.5	4 ± 1.2
Leu	43 ± 2.5 (1.5)	7 ± 2.3	21 ± 4.4 (0.5)	15 ± 5.0	7 ± 0.5
SAC	32 ± 1.6 (1.5)	7 ± 2.3	21 ± 1.5 (0.75)	15 ± 2.2	7 ± 1.3
SAC[c]	76 ± 2.4 (3)	< 1	<1 (7)	<1	3 ± 0.5

a: maximum concentration attained and peak time (h).
b: concentration at the peak time of the transpeptidation product.
c: D-Gln was used as the donor substrate.

also in this study have already been described in the literature. For example, γ-glutamyl-phenylalanine was synthesized in 2 h using equimolar (200 mM) amounts of glutamine and phenylalanine using 0.2 U/mL Ec-GGT at pH 8.3 and 37 °C, with a conversion rate of 36%, as estimated by HPLC analysis, in good agreement with our results. However, increasing the enzyme concentration to 0.5 U/mL and raising the pH to 10.4, the same Authors obtained a 70% conversion rate, even if the isolated yield was much lower (19%) [77]. In another work, in 7 h incubation, Ec-GGT-catalyzed the synthesis of 3.4 mM γ-glutamyl-leucine at pH 9.5 and 37 °C, using 20 mM glutamine with a 5-fold molar excess leucine, in the presence of 0.08 U/mL Ec-GGT [56]. In the aforementioned cases, it is difficult to relate our results with those from the literature, as the presence of byproducts in the reaction mixtures is often not mentioned. Only for the enzymatic synthesis of γ-glutamyl-taurine, account has been given for both the transient formation of γ-glutamyl-glutamine and the formation of γ-glutamyl-γ-glutamyl-taurine as a byproduct [20].

After a careful examination of the reaction profiles obtained using the different acceptor substrates, our attention was attracted by the fact that the concentrations of the desired transpeptidation products dropped slightly after reaching a maximum and remained nearly constant, decreasing extremely slowly over time. These "quasi-stationary phases" occurred after the concentration of the species able to behave as donor substrates, either glutamine or γ-glutamyl-glutamine, dropped below a certain threshold, usually ca 20 mM. To increase the yield of the desired product, reactions were repeated using a fed-batch approach, restoring the concentration of the donor glutamine when the concentration of the autotranspeptidation product γ-glutamyl-glutamine began to drop.

A similar approach was attempted to improve the conversion of the rather poor acceptor substrate valine into the corresponding γ-glutamyl derivative [56]. In our experiments, the further addition of glutamine resulted primarily in a transient increase of the autotranspeptidation product, as shown in Fig. 3. However, this approach favored the formation of γ-glutamyl-γ-glutamyl-acceptor and not of the simple transpeptidation product. The same behavior occurred for all the tested acceptors and was particularly evident in the reaction with arginine reported in Fig. 3, in which the concentration of γ-glutamyl-γ-glutamyl-arginine, after the further addition of the donor glutamine, nearly equaled that of γ-glutamyl-arginine from 3.5 h on. Only using methionine as the acceptor substrate, a slightly increase in the concentration of the transpeptidation product was observed. Unfortunately, γ-glutamyl-γ-glutamyl-methionine could not be quantified, due to the overlap of the corresponding peak with that of a byproduct of the derivatization procedure. It is also worth to note that the production of glutamic acid was apparently not affected by the further addition of glutamine, as its concentration seemed to raise with the same rate before and after supplementing additional donor substrate. Products distribution seems to

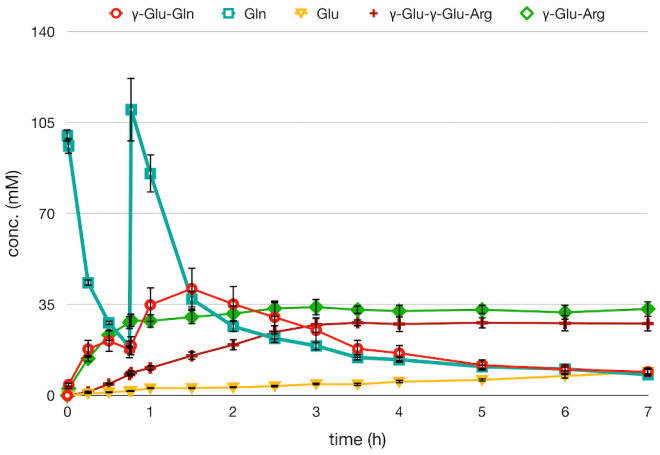


Fig. 3. Effect of the donor substrate glutamine feeding on products distribution. Ec-GGT-catalyzed reaction between Gln and Arg; starting concentration 100 mM; Ec-GGT 0.25 U/mL; pH 10, 40 °C. The concentration of the donor Gln was restored to ca 100 mM after 45 min.

be dependent upon the relative molar ratio of the donor and acceptor substrates, but not upon their concentration. Using glutamine and *S*-allylcysteine in equimolar amounts but at different concentrations, namely 100, 250, and 400 mM, the maximum attainable conversion rate did not significantly improve and occurred with the same timing (Table 3, entries 2, 4 and 5). Lower concentrations of enzyme caused a delay in the reaction time, without affecting the conversion rate (Table 3, entry 1). Shorter reaction time could be attained using higher concentrations of enzyme, but with a sensible decrease in the yield of the desired transpeptidation product (Table 3, entries 3 and 6).

On the basis of our results, a general picture of Ec-GGT behavior as a biocatalyst can be drawn (Scheme 1). The donor glutamine **1** reacts irreversibly with Ec-GGT forming a γ-glutamyl-enzyme intermediate **2** with liberation of ammonia. γ-Glutamyl-enzyme intermediate plays a pivotal role in the GGT-catalyzed reactions and can be resolved by the attack of a nucleophilic species. If the nucleophile is another glutamine molecule, the autotranspeptidation product γ-glutamyl-glutamine **3** is produced; if the nucleophile is the acceptor substrate **4**, the transpeptidation product **5** ensues, while if the nucleophile is a water molecule, hydrolysis occurs with liberation of glutamic acid **6**. As glutamic acid is not able to act as a donor substrate [46], hydrolysis is an irreversible reaction. On the contrary, both compounds **3** and **5** are recognized by the enzyme and can give again the γ-glutamyl-enzyme intermediate. When the concentration of the autotranspeptidation and transpeptidation products **3** and **5** rises, those products start to compete as acceptors with both glutamine **1** and compound **4**, leading to γ-glutamyl-γ-glutamyl derivatives **7** and **8**, respectively. As the reaction proceeds, the concentration of glutamine **1** decreases irreversibly and the formation of the γ-glutamyl-enzyme intermediate **2** is ensured by the reverse reaction of compounds **3**, **5**, **7** and **8**. Upon reaction of **3**, glutamine is liberated and it can be assumed that it is consumed faster, due to the preference of Ec-GGT for donor substrates with small molecular size [78]. This leads to the transient formation of both **3** and **7**. At later stages, **5** and **8** are the main substrates able to afford γ-glutamyl-enzyme intermediate. This explains both the slight decrease in the concentration of **5** observed during the time-course reactions and the small rise in the concentration of the acceptor **4** (Fig. 2). The fairly constant concentration of the transpeptidation product **5** is due to its continuous re-formation, thanks to the presence of a relatively high amount of acceptor **4**. Usually, **5** reaches a concentration high enough to compete with **4** as acceptor, affording the γ-glutamyl-γ-glutamyl derivative **8**. The slow decrease of **5** and **8** over time, noticed from 3.5 − 4 h on, is then due to the small fraction of γ-glutamyl-enzyme intermediate that undergoes irreversible hydrolysis.

Table 3
Effect of substrates concentration on the Ec-GGT-catalyzed reaction between Gln and SAC. All experiments were carried out at pH 10 and 40 °C and were monitored by HPLC.

Entry	GGT U/mL	donor/acceptor molar ratio	Gln [mM][a]	SAC [mM][a]	γ-Glu-SAC conv. % (h)[b]	γ-Glu_2-SAC conv. %[c]
1	0.05	1:1	100	100	34 ± 0.9 (6)	4 ± 1.5
2	0.25	1:1	100	100	33 ± 1.5 (1.5)	7 ± 0.7
3	0.50	1:1	100	100	20 ± 1.0 (0.5)	2 ± 0.8
4	0.25	1:1	250	250	30 ± 0.5 (1.5)	14 ± 1.2
5	0.25	1:1	400	400	30 ± 0.6 (1.5)	11 ± 1.3
6	0.50	1:1	400	400	27 ± 1.0 (0.5)	10 ± 1.2
7	0.25	1:3	100	300	88 ± 2.6 (2)	15 ± 1.8

a: initial concentration.
b: maximum conversion attained and peak time (h).
c: conversion of the transpeptidation product at the peak time.

Taking this general scheme as a guideline, the outcomes of the various Ec-GGT-catalyzed reactions towards the different acceptor substrates were compared.

The rate of formation of the transpeptidation products in the presence of the different acceptors did not show any relationship with the activities measured through spectrophotometric assays (Table 1). Based on HPLC-monitored experiments, the high γ-glutamyl-methionine yield demonstrates that methionine is a good acceptor, efficiently competing with glutamine (Table2). Also, the rates of formation of glutamic acid were different in the reactions of the various acceptors, with no relationship with the activities measured through the spectrophotometric assay. In addition, glutamic acid concentration is quite low in the initial stages of all reactions. It raises steadily and becomes appreciable in the late stages (from 3.5 to 4 h on), when the transpeptidation products are the only substrates still present in substantial concentrations in the mixture. Despite the differences in product formation rates, the rates of consumption of the donor glutamine revealed to be very similar in the presence of the various acceptor substrates (Fig. 4).

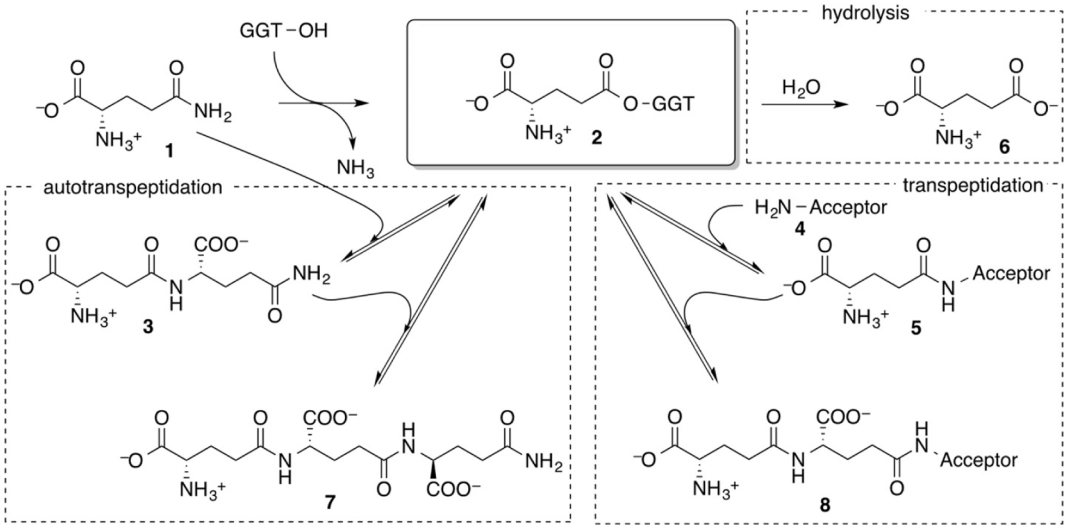
Taken together, all these observations shed a special light on the use of Ec-GGT as a biocatalyst for preparative purposes. Indeed, from a synthetic point of view, hydrolysis seems not to be the most detrimental side reaction catalyzed by Ec-GGT. Also, autotranspeptidation might not represent a problem *per se*, because the γ-glutamyl-glutamine initially formed becomes a donor substrate at a later stage of reaction. Instead, the formation of γ-glutamyl$_{(n)}$-acceptor appears to be the main cause of low yields attainable through Ec-GGT-catalyzed transpeptidation.

It is known that Ec-GGT can accept D-glutamine as the donor substrate, but not as an acceptor. Using D-glutamine, the yield in the single transpeptidation product improves [36,60]. This has been attributed to the lack of competing autotranspeptidation reactions. An alternative hypothesis is that the real advantage in the use of D-glutamine relies in the inability of the transpeptidation product to compete with the acceptor substrate, thus abolishing further transpeptidation that would lead to γ-glutamyl$_{(n)}$-acceptor product. To test this hypothesis, reactions were carried out using D-glutamine as a substrate. In this case, the yield of γ-D-glutamyl-SAC was estimated to reach 76% after three hours, being glutamic acid the main byproduct detected in 3% amount (Table 2). Indeed, γ-D-Glu-D-Gln and γ-D-Glu_2SAC were estimated to be <1% each.

Furthermore, with the aim to increase the yield of the transpeptidation products, an excess SAC was used as acceptor substrate, to circumvent competition by newly formed γ-glutamyl-SAC, thus limiting the formation of γ-Glu_2-SAC. When SAC was used in three-fold molar excess with respect to the donor glutamine, the yield of γ-GluSAC increased to 88% within 2 h (Table 3, entry 7). At a one-millimole preparative level, the reaction afforded γ-GluSAC in 60% isolated yield after ion exchange column chromatography, being the HPLC-estimated conversion measured at the end of the reaction 75% ca.

3. Conclusions

The moderate conversion rates obtained using the various acceptor substrates, the similar trends of transpeptidation and autotranspeptidation in the initial stages of the reactions with various acceptor substrates, the similar tendency to accept γ-glutamyl-derivatives as acceptor substrates leading to γ-glutamyl-γ-glutamyl compounds and the stereoselectivity of Ec-GGT for L acceptor amino acids, suggest that the enzyme recognizes the acceptor substrates mainly through their α-amino acidic moieties. The nature of the side chain of the acceptor amino acids seems to be scarcely relevant in affecting their ability to act as acceptor substrates, at least in our experimental conditions. This conclusion is consistent with the results obtained in inactivation studies carried out using phosphonate diesters as mechanism-based inhibitors [79]. As acceptor substrates are thought to bind where the cysteinyl-glycine portion of glutathione is accommodated during hydrolysis [80], phosphonate diesters mimicking glutathione were used as inhibitors. Ec-GGT revealed indeed to be inhibited in a



Scheme 1. Representation of the possible reactions catalyzed by Ec-GGT.

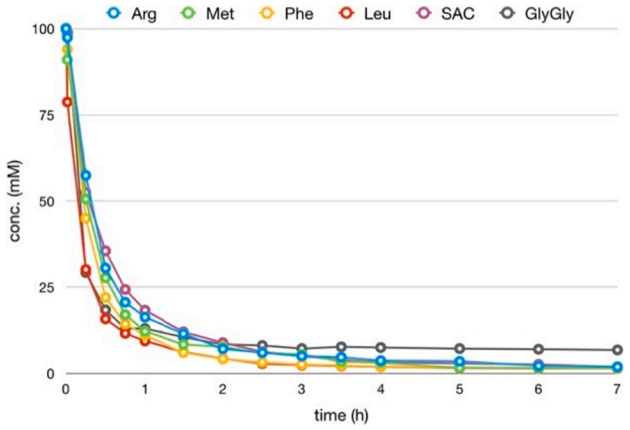


Fig. 4. Time-dependent consumption of the donor glutamine in the presence of various acceptor substrates. Reaction conditions: glutamine 100 mM; acceptor amino acid 100 mM, Ec-GGT 0.25 U/mL; pH 10; t = 40 °C.

concentration- and time-dependent manner. However, inhibition appeared to be related solely to the intrinsic chemical reactivity of the phosphonate diesters (pK_a of the leaving group), irrespective of their chemical structures. Based on these data, a rather undefined acceptor binding site has been postulated for Ec-GGT, which indeed shows a relaxed substrate specificity. This assumption is further supported by x-ray crystallography [81]. The apparent different activities measured spectrophotometrically in the presence of the various acceptor amino acids could then be related to other factors than the recognition of the amino acid side chains by the enzyme. For instance, it is known that some acceptor substrates can compete for the donor binding site in mammalian GGTs [80,82]. If this is the case, the high concentrations of acceptor substrates used in spectrophotometric assay reactions with respect to those employed for HPLC monitoring might amplify the differences observed. This and other hypotheses are currently under consideration, to rationally interpret our reproducible findings.

4. Experimental

4.1. General

L-Glutamine, L-glutamic acid, L-serine, glycilglycine, L-arginine, L-methionine, L-phenylalanine, L-leucine, L-glutamic acid 5-(*p*-nitroanilide) and 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) were from Sigma Aldrich (Darmstadt, Germany) and were used as received. *S*-allylcysteine and γ-glutamyl-*S*-allylcisteine were prepared as previously described [35]. HPLC-grade solvents were from Aldrich.

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: UV lamp (λ 254 nm), 4.5% w/v $CeSO_4/(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ solution or 5% w/v ninhydrin solution in ethanol, followed by heating at 150 °C ca.

HPLC analyses were carried out using a 250 × 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 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 1 × 8 resin 200–400 mesh (Aldrich, Darmstadt, Germany) in the acetate form.

^1H NMR and ^{13}C NMR spectra were acquired at 400.13 MHz and 100.61 MHz, respectively, on a Bruker Advance 400 spectrometer (Bruker, Karlsruhe, Germany) equipped with a TOPSPIN software package. ^{13}C signal multiplicities were based on attached proton test experiments (APT) and attributions were based on HSQC (Hetero Single Quantum Correlation) and HMBC (Hetero Multiple Bond Correlation) experiments. Chemical shifts are given in ppm (δ) and are referenced to solvent signal (δ_H D_2O 4.79 ppm) or to TSP (3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt) as external standard (δ_{Me} 0.00 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).

4.2. Cloning of E. Coli GGT

GGT from *E. coli* was obtained from the wild type *ggt* gene from strain BL21(DE3) genome (accession number CP001509) as previously described [78].

4.3. Enzyme activity assays

4.3.1. Measurement of enzymatic activity

To a 1.980 mL-solution containing 1 mM GPNA and 100 mM GlyGly in 0.1 M TRIS buffer pH 8.5, 20 µl of the purified enzyme was added. The release of *p*-nitroaniline was continuously monitored at 410 nm recording data every 10 s for 3 min. One enzyme unit was defined as the amount of enzyme that liberates 1 µmol mL^{-1} min^{-1} of *p*-nitroaniline. *p*-Nitroaniline concentrations were estimated through a calibration curve.

4.3.2. Hydrolase and transpeptidase activities

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

4.3.3. Enzyme activity towards different acceptor amino acids

Measurements were carried out in the same conditions as before, by substituting GlyGly with the proper acceptor amino acid.

4.4. Pre-column derivatization procedure with Sanger's reagent

Pre-colums derivatization was carried out as already described [66].

Briefly, standard solutions for calibration curve or aliquots of the reaction mixture (20 µl) were diluted 1:20 with water. 100 µl of the diluted solution was transferred into a Pyrex tube equipped with a perforated screw cap fitted with a forcible sealing septum. 50 µl of 5 mM L-Serine in water was added as the internal standard, followed by 350 µl borate buffer at pH 8.5. The mixture was shaken and 500 µl 10 mM 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) solution in acetone was added. The tube was sealed and heated at 70 °C for 45 min in the dark. A needle was introduced into the septum and heating was continued for further 10 min, to evaporate acetone. The tube was cooled under running water and 200 µl of the resulting mixture was diluted 1:1 with 0.1% TFA solution before HPLC analysis.

4.5. Ec-GGT-catalyzed reactions at analytical level. General procedure

To a 100 mM solution of glutamine and acceptor amino acid dissolved in 0.1 M sodium carbonate/sodium hydrogencarbonate buffer at pH 10, Ec-GGT was added to a final concentration of either 0.05, 0.25 or 0.5 U/mL. The mixture was stirred in a thermostated water bath at 40 °C. At fixed time points, 20 µl aliquots were withdrawn, derivatized as described and analyzed by HPLC.

4.6. Ec-GGT-catalyzed synthesis of γ-glutamyl-*S*-allyl-L-cysteine

L-glutamine (146 mg, 1 mmol) and *S*-allyl-L-cysteine (484 mg, 3 mmol) were dissolved in water and the pH was adjusted to 10 with 1 M NaOH before enzyme addition (0.25 U/mL); final reaction volume was 10 mL. The solution was stirred at 40 °C for 2 h. 20 µl reaction mixture was withdrawn, derivatized as described and analyzed by HPLC. The mixture was loaded onto a pad of Dowex 1 × 8 ion exchange resin in the acetate form and the pad was eluted with water (5 column volumes) and then with a scalar gradient of acetic acid solution (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 and freeze-dried. γ-Glutamyl-*S*-allyl-L-cysteine was obtained as a white solid (174 mg, 60% isolated yield; conversion estimated by HPLC analysis was 75%).

^{1}H NMR in agreement with that previously reported [2].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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