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pH-Dependent hydrolase, glutaminase, transpeptidase and autotranspeptidase activities of *Bacillus subtilis* γ-glutamyltransferase

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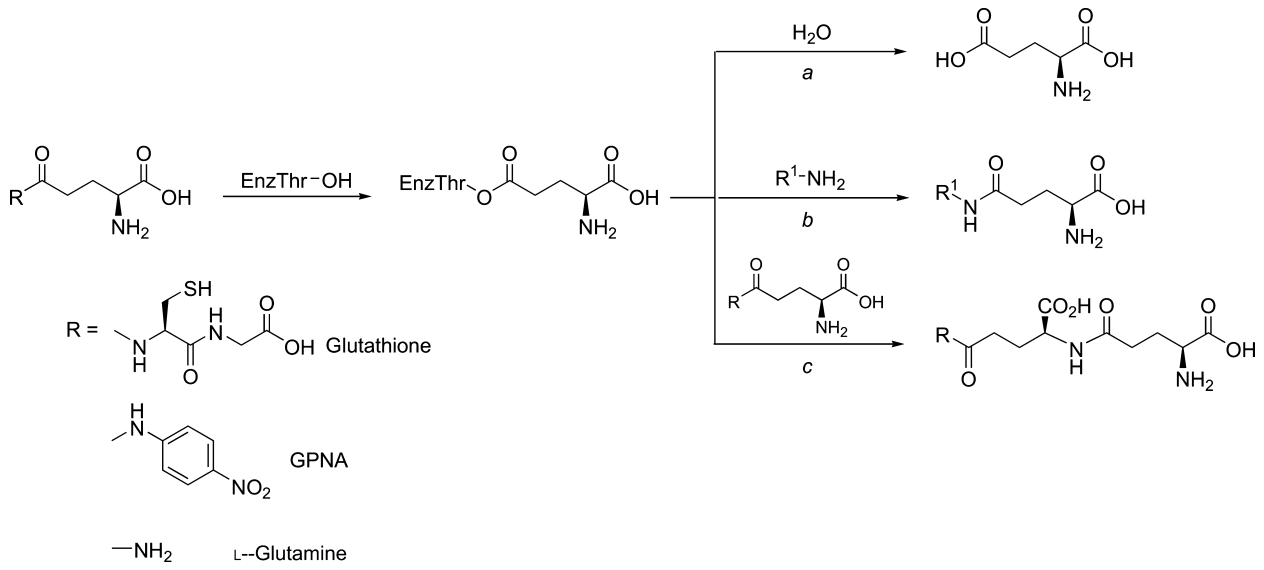
γ-Glutamyltransferases (γ-GTs) are heterodimeric enzymes that catalyze the transfer of a γ-glutamyl group from a donor species to an acceptor molecule in a transpeptidation reaction through the formation of an intermediate γ-glutamyl enzyme. In our search for a γ-GT from a generally recognized as safe microorganism suitable for the production of γ-glutamyl derivatives with flavor-enhancing properties intended for human use, we cloned and overexpressed the γ-GT from *Bacillus subtilis*. In this study, we report the behavior of *B. subtilis* γ-GT in reactions involving glutamine as the donor compound and various acceptor amino acids. The common thread emerging from our results is a strong dependence of the hydrolase, transpeptidase and autotranspeptidase activities of *B. subtilis* γ-GT on pH, also in relation to the pK_a of the acceptor amino acids. Glutamine, commonly referred to as a poor acceptor molecule, undergoes rapid autotranspeptidation at elevated pH, affording oligomeric species, in which up to four γ-glutamyl moieties are linked to a single glutamine. Moreover, we found that D-glutamine is also recognized both as a donor and as an acceptor substrate. Our results prove that the *B. subtilis* γ-GT-catalyzed transpeptidation reaction is feasible, and the observed activities of γ-GT from *B. subtilis* could be interpreted in relation to the known ability of the enzyme to process the polymeric material γ-polyglutamic acid.

Introduction

γ-Glutamyltransferases (γ-GTs, also known as γ-glutamyltranspeptidases; EC 2.3.2.2) [1] are heterodimeric enzymes that are widely distributed, from bacteria [2–5] to plants [6] and mammals [7], belonging to the N-terminal nucleophile hydrolase superfamily [8]. The two subunits originate from autocatalytic cleavage of a single peptide chain [9].

γ-GTs cleave the amide bond involving the γ-carboxyl group of glutamic acid in a γ-glutamyl derivative, usually glutathione, affording a γ-glutamyl enzyme intermediate through the oxygen atom of the conserved, catalytically active N-terminal threonine of the small subunit [10]. The γ-glutamyl moiety can then be transferred to an acceptor nucleophile. If the nucleophile is a water molecule, hydrolysis of the γ-glutamyl enzyme intermediate occurs, and the net result is the release of free glutamic acid (Scheme 1, path a) [11]; if the nucleophile is the amino group of an amino acid or of a small peptide, a transpeptidation reaction ensues, with the formation of a new γ-glutamyl compound

Abbreviations
DbsCl, dabsyl chloride; GPNA, γ-glutamyl-*p*-nitroanilide; γ-GT, γ-glutamyltransferase; γ-PGA, poly-γ-glutamic acid.



Scheme 1. γ-GT-catalyzed reactions. Path a: hydrolysis. Path b: transpeptidation. Path c: autotranspeptidation.

(Scheme 1, path b) [12–15]. In the presence of a high concentration of the γ-glutamyl donor, an autotranspeptidation reaction is also possible, in which the same compound behaves both as the donor and as the acceptor molecule (Scheme 1, path c) [11].

Whereas the biological significance of mammalian γ-GT in glutathione metabolism [7] is well defined, the physiological roles of bacterial γ-GTs are often less clear. Bacterial γ-GTs are soluble proteins found in the periplasmic [3] or extracellular [4] space.

Among the bacterial γ-GTs, those from *Bacillus* species have attracted interest [16–19], owing to some peculiar features.

The gene encoding γ-GT in *Bacillus subtilis* was identified in 1996 [20]. It encodes a 587-residue protein, carrying a 28-residue N-terminal portion signaling the extracellular localization. It shares 41% sequence homology with *Escherichia coli* γ-GT.

Comparison of the amino acid sequence of *B. subtilis* γ-GT with that of other known bacterial and mammalian γ-GTs showed that it lacks the lid loop covering the glutamate-binding site, and has an extra sequence at the C-terminus of the large subunit [21].

The absence of the lid loop is a structural feature shared by γ-GTs from other *Bacillus* species, such as *Bacillus licheniformis* [22] and also by the γ-GTs from *Geobacillus thermodenitrificans* [23], *Deinococcus radiodurans*, and *Thermus thermophilus* [24]. CapD from *Bacillus anthracis* [25] is another member of the N-terminal nucleophile hydrolase superfamily lacking the lid loop. It shares sequence similarity with both mammalian and bacterial γ-GTs, and catalyzes the breakdown of the γ-glutamyl bond of poly-D-glutamic acid, transferring the polymeric chains to the peptidoglycan cell wall, thus forming the covalently bound poly-D-glutamic acid capsule of the bacterial cells [26,27].

Even though the involvement of γ-GT in the biosynthesis of poly-γ-glutamic acid (γ-PGA) produced and secreted by *B. subtilis* (natto) was initially suggested [28], it is more likely to be involved in γ-PGA hydrolysis for energy supply under carbon-limiting conditions [29] rather than in its biosynthesis.

As *B. subtilis* γ-GT showed transpeptidase activity towards some acceptor amino acids [4], its use as a biocatalyst for the enzymatic synthesis of γ-glutamyl compounds was proposed.

This possibility was, indeed, sometimes suggested by analytical results, and only for a few γ-GTs from the genus *Bacillus* it was experimentally demonstrated at a preparative level [30,31]. When γ-GT-catalyzed reactions were carried out at a preparative level, the reaction conditions, such as pH optimum, temperature, and reactant molar ratio, usually had to be optimized for each acceptor substrate in an *ad hoc* way, despite the fact that the specificities for acceptor substrates have been previously determined with standardized methods.

The transpeptidation activities of γ-GTs were usually evaluated at fixed pH (8.0 or 8.5) in the presence of a large excess of the acceptor compounds, up to ~ 70-fold with respect to the donor, taking the release of the chromogenic *p*-nitroaniline from γ-glutamyl-*p*-nitroanilide (GPNA), used as the donor substrate, as a

measure of the enzyme-catalyzed reaction. The acceptor propensities of the tested amino acids were also evaluated with respect to the hydrolysis reaction (i.e. the same reaction carried out in the absence of an acceptor substrate), taking advantage of the faster transpeptidation reaction in the presence of a good acceptor compound with respect to the hydrolysis of the donor [32]. Moreover, the ability of a compound to act as an acceptor was evaluated with respect to glycylglycine, considered to be the best acceptor.

The aim of this study was to investigate the behavior of *B. subtilis* γ-GT in transpeptidation reactions, in view of its possible use as a biocatalyst for preparative purposes.

γ-Glutamyl derivatives of both unmodified and modified amino acids are naturally occurring flavor enhancers found in many foods such as cheese [33], commonly eaten vegetables [34], mushrooms [35] and seasoning plants used in cuisine worldwide [36–38].

We reported recently the chemoenzymatic synthesis of γ-glutamyl derivatives of methionine and S-substituted cysteines as examples of flavor enhancers found in plants of the genus *Allium* [39]. The synthesis relied on the use of an enzyme of mammalian origin, so the obtained products were not suitable for human use.

In continuing our work, we sought a biocatalyst appropriate for food processing, and the γ-GT derived from *B. subtilis* seemed to be a good choice, being this organism generally recognized as safe (GRAS). In this study, the donor and the acceptor substrates were used in the enzyme-catalyzed reactions in equimolar amounts, usually at concentrations of 100 mM. This approach gave us the opportunity to gain some insights into the enzyme activity, and to find new and unexpected behaviors with respect to those previously reported [16–19,40].

Results and Discussion

Enzyme production and purification

Recombinant *B. subtilis* γ-GT was purified from *E. coli* as a N-terminal His-tagged protein (Fig. S1). Its enzymatic activity, expressed as μmoles of *p*-nitroaniline liberated through the transpeptidation reaction with GPNA as the donor and glycylglycine as the acceptor at pH 8.5 and 22 °C [32–41], was calculated to be 18.33 $U \cdot mL^{-1}$ enzyme solution. The protein content, measured with the method of Bradford [42], was found to be 34.4 $mg \cdot mL^{-1}$. The specific activity was therefore 529 $mU \cdot mg^{-1}$ protein. The enzyme was stored at −20 °C in aliquots, and diluted with appropriate buffer prior to use.

pH-activity profile

pH-rate profiles for the hydrolase and the transpeptidase activities were determined with the standard method, with GPNA as the donor and glycylglycine as the acceptor [13,32,41]. We found that the hydrolase activity reached a maximum at a pH of ~ 9, and then decreased, giving a bell-shaped curve. In contrast, in the presence of the acceptor glycylglycine, the activity steadily increased up to a pH of ~ 10, and showed a marked decrease only at the more elevated pH value tested (Fig. 1).

Mammalian γ-GT showed, instead, bell-shaped curves for the transpeptidation reaction [43], whereas the hydrolase activity was usually lower and much less sensitive to pH changes [44]. Moreover, the transpeptidation reaction catalyzed by mammalian γ-GT is up to 180-fold faster than hydrolysis [45], whereas our results indicate, for *B. subtilis* γ-GT, a two-fold increase in transpeptidase activity with respect to hydrolysis at pH 10. In this respect, *B. subtilis* γ-GT appears to be more similar to *Helicobacter pylori* γ-GT [46], which showed only a modest prevalence of the transpeptidase activity over the hydrolase activity.

pH-dependent behavior of *B. subtilis* γ-GT towards glutamine

Starting from the assumption that the known glutaminase activity of *B. subtilis* γ-GT [4] can represent a source of undesired glutamic acid byproduct in the transpeptidation reactions, we started our investigation

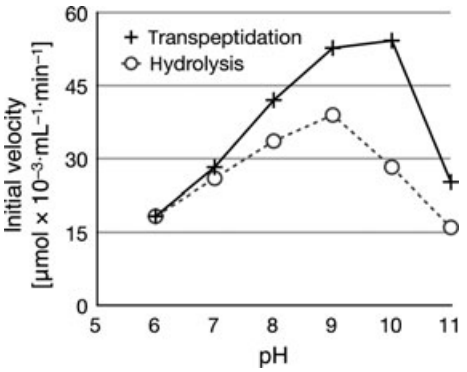


Fig. 1. pH-activity profile for *B. subtilis* γ-GT-catalyzed hydrolysis and transpeptidation reactions. Enzyme (43.6 mU) was added to a 0.25 mM solution of GPNA alone (hydrolysis) or in the presence of 20 mM glycylglycine as the acceptor (transpeptidation) at 22 °C. The amount of released *p*-nitroaniline was monitored spectrophotometrically at 410 nm for 2 min. The concentrations of released *p*-nitroaniline were calculated by use of a calibration curve.

with a study of the behavior of the enzyme towards glutamine at different pH values.

Even though the pH dependence of the glutaminase activity of *B. subtilis* γ-GT has been already reported [4], we quantitatively monitored the time course of the conversion of the substrate glutamine into glutamic acid with HPLC. Three reactions were carried out with 100 mM solutions of glutamine, at pH 7.4, 8.2, and 9.8. At fixed time intervals (1, 2, 3, 5, 7 and 24 h), 100-μL aliquots were withdrawn from each reaction mixture, derivatized with dabsyl chloride (DbsCl), and analyzed with HPLC for glutamine and glutamic acid contents.

At pH 7.4, a decrease in glutamine concentration in the reaction mixture was observed with time, which paralleled the increase in glutamic acid content (Fig. 2, top). When the pH was increased to 8.2, a new peak in the chromatograms appeared transiently between 1 h and 5 h, which was attributed to γ-glutamylglutamine (Fig. 2, middle). HPLC-ESI-MS experiments confirmed our hypothesis of an autotranspeptidation reaction, attributing to the new peak in the chromatograms a molecular mass of 562, corresponding to the dabsyl derivative of γ-glutamylglutamine (Fig. S2). Further evidence came from the use of an authentic sample of γ-glutamylglutamine, deliberately synthesized and used for the construction of a calibration curve, with the aim of quantitative estimation in the reaction mixtures. The most impressive result was, however, obtained at pH 9.8 (Fig. 2, bottom), in that glutamine disappeared very quickly within 2 h, and a series of small peaks appeared in the chromatograms. The amounts of glutamic acid and γ-glutamylglutamine formed in the same time period were insufficient to explain such a rapid disappearance of glutamine. HPLC-ESI-MS experiments showed that oligomeric species containing up to four γ-glutamyl residues linked to a single glutamine molecule were formed (Fig. 3; Fig. S3).

The same experiment was repeated with D-glutamine, and identical results were obtained (Fig. S4).

In order to assess the role of substrate concentration in the autotranspeptidation reaction, further experiments were carried out with lower glutamine concentrations (25 mM and 50 mM) at pH 9.8 (Fig. S5). The main reaction observed in the mixture containing 25 mM glutamine was hydrolysis (Fig. S5A). However, low amounts of the autotranspeptidation products γ-glutamylglutamine, γ-glutamyl-γ-glutamylglutamine and γ-glutamylglutamic acid were found with HPLC-MS experiments recorded after 1 h of reaction time (Fig. S6). These products were rapidly hydrolyzed, and the glutamic acid concentration in the reaction mixture reached its maximum within 5 h of reaction time.

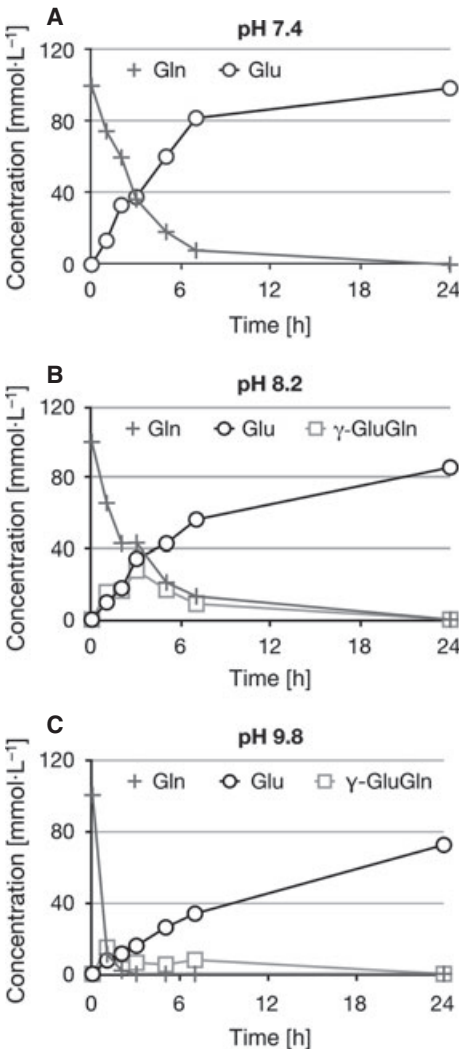


Fig. 2. Time course of the reaction of glutamine in the presence of *B. subtilis* γ-GT at three different pH values. The enzyme (43.6 mU) was added at 22 °C to three solutions of glutamine (100 mM) at pH 7.4 (top), pH 8.2 (middle), and pH 9.8 (bottom). At fixed time intervals, 100-μL aliquots were withdrawn from each reaction mixture, derivatized with DbsCl, and analyzed with HPLC. Calibration curves were constructed by the use of authentic materials, with the aim of monitoring the concentrations of glutamine, glutamic acid, and γ-glutamylglutamine.

When the concentration of the starting glutamine was increased to 50 mM, the autotranspeptidation products were more evident, and their amounts increased for up to 2 h of reaction time (Figs S5B and S7). The peak attributable to the hydrolysis product glutamic acid in the HPLC chromatograms increased slightly more slowly, and reached its maximum at ~ 7 h, owing to the enzyme-catalyzed hydrolysis not only of the starting glutamine, but also of the previously formed autotranspeptidation products.

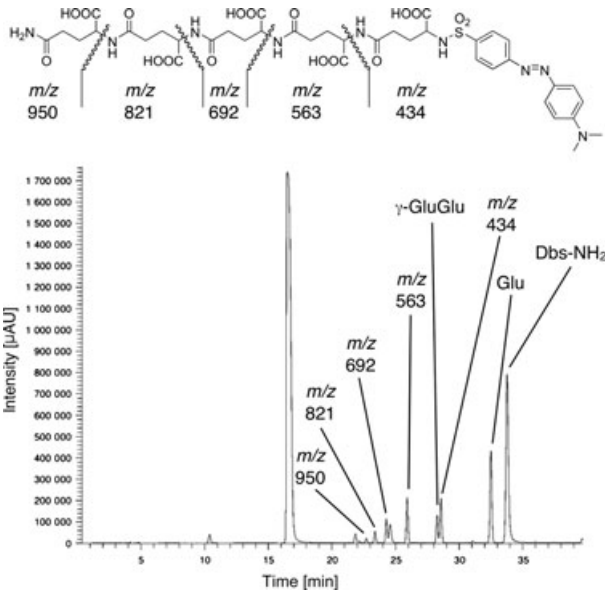


Fig. 3. HPLC chromatogram derived from an HPLC-ESI-MS experiment carried out on the sample obtained by incubating glutamine and *B. subtilis* γ-GT at pH 9.8 after 2 h of reaction at 22 °C. $DbsNH_2$, dabsyl amide.

The results obtained in these experiments are, in our opinion, noteworthy for at least three reasons. First, they confirmed the glutaminase activity of *B. subtilis* γ-GT, but they also showed the ability of the enzyme to recognize glutamine as an acceptor substrate in a transpeptidation reaction. This is in apparent conflict with results previously reported, in which the transpeptidase activity towards glutamine was reported to be very low [4]. However, those results were obtained at a lower pH value (8.5), below the pK_a of the glutamine amino group. It was proposed that an alkaline pH is beneficial for the γ-GT-catalyzed transpeptidation reaction, in that it increases the concentration of unprotonated nucleophilic amino groups [44]. In our case, the autotranspeptidation reaction was mainly observed at pH 9.8, which is slightly above 9.1, the pK_a of the amino group of glutamine.

Second, the enzyme can also accept as substrates oligomeric poly-γ-glutamyl derivatives, as shown by the HPLC-ESI-MS experiments. Although the formation of oligomeric derivatives carrying more than two γ-glutamyl residues has already been proposed, and, on the basis of those findings, the involvement of *B. subtilis* γ-GT in the biosynthesis of γ-PGA has been suggested [28], our results constitute the first clear evidence of their presence in a γ-GT-catalyzed reaction in which only glutamine acts as the substrate. When *E. coli* γ-GT was used for synthetic purposes in the presence of a high concentration of glutamine as the donor at elevated pH, only the formation of γ-glutamylglutamine was reported, but no mention of the formation of oligomeric compounds can be found in the literature [47]. In the rat kidney γ-GT-catalyzed reaction of glutamine, the presence of γ-glutamyl-γ-glutamylglutamine was only tentatively supposed [48]. The ability of *B. subtilis* γ-GT to also process oligomeric γ-glutamyl derivatives is ascribable to the possible role of this enzyme in the homeostasis of poly-γ-glutamic acid produced by some strains of *B. subtilis* [49].

Third, the ability of *B. subtilis* γ-GT to accept D-glutamine as an acceptor substrate is another difference from both mammalian and *E. coli* γ-GTs. The latter can indeed accept γ-glutamyl compounds with the D-configuration as donors, but amino acid acceptors have to be in the L-configuration [3,13]. The similar results obtained when L-glutamine and D-glutamine were subjected to the action of *B. subtilis* γ-GT could, once again, be related to the involvement of the enzyme in homeostasis of the extracellular poly-γ-glutamic acid, as this polymeric material is usually formed by L-glutamic acid and D-glutamic acid units [50] in different proportions, depending on the *Bacillus* strain and environmental conditions [51,52].

Time course of the transpeptidation reaction

The time course of the transpeptidation reaction was monitored at pH 8.5 with equimolar amounts of 100 mM glutamine as the donor and glycylglycine as the acceptor. The reaction course monitored with HPLC (Fig. 4) showed that the donor glutamine disappeared within 24 h. In the meantime, the glutamic

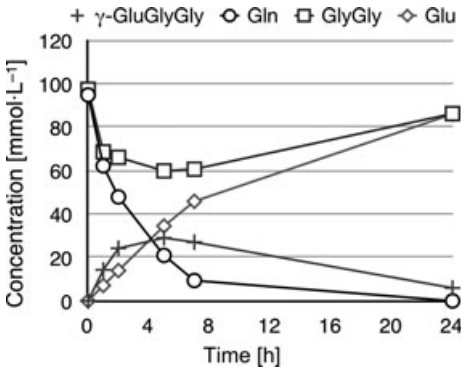


Fig. 4. Time course of the *B. subtilis* γ-GT-catalyzed transpeptidation reaction with glutamine as the donor and glycylglycine as the acceptor at pH 8.5 and 22 °C. Equimolar proportions of donor and acceptor (100 mM) were used, and the concentrations of glutamine, glycylglycine, glutamic acid and γ-glutamylglutamine were monitored with HPLC at time intervals after derivatization of the withdrawn samples with DbsCl.

acid content in the reaction mixture increased. The expected product, γ-glutamylglycylglycine, accumulated during the first 5 h of reaction time, reaching an estimated concentration of 30 mM, and the amount of the starting compound glycylglycine decreased accordingly during the same time period. Other peaks noticed in the chromatogram were attributed to γ-glutamyl-γ-glutamylglycylglycine and γ-glutamylglutamine, by means of an HPLC-MS experiment (Fig. S8). After 5 h of reaction time, the free glycylglycine concentration started to increase, while the transpeptidation product disappeared, owing to irreversible hydrolysis reactions. Because the hydrolysis of the donor glutamine is also irreversible, within 24 h the reaction mixture contained mainly the starting glycylglycine, with a very low concentration of residual γ-glutamylglycylglycine and the irreversibly formed glutamic acid.

Therefore, the hydrolysis and the autotranspeptidation (Fig. 2) of the donor glutamine seem to be competitive reactions with respect to the expected transpeptidation reaction at pH 8.5. Mammalian γ-GTs (e.g. equine kidney γ-GT) show a different behavior, owing to the very low extent of autotranspeptidation and the transpeptidation reaction being favored with respect to hydrolysis at basic pH values [39,53]. Thus, it seems to be more difficult to separate the three enzymatic activities for *B. subtilis* γ-GT than for other γ-GTs.

pH dependence of the transpeptidation and autotranspeptidation reactions

The results described so far showed that the extent of the autotranspeptidation reaction of glutamine was clearly dependent on pH. Moreover, the hydrolase activity of *B. subtilis* γ-GT, unlike the activities of other γ-GTs [12,54], seems not to be inhibited at higher pH values. On the basis of these observations, the transpeptidation reactions between glutamine as the donor and selected acceptor amino acids were evaluated at different pH values, ranging from 7.5 to 11.0. Through this set of experiments, we tried to assess the degree of transpeptidation with respect to the autotranspeptidation reaction. Usually, 100 mM solutions of glutamine and the acceptor amino acid were incubated in the presence of *B. subtilis* γ-GT for 1 h at 22 °C, and 100-μL aliquots of the reaction mixtures were then withdrawn, derivatized with DbsCl, and analyzed with HPLC. Only the reaction with phenylalanine as the acceptor substrate was carried out at 50 mM, for solubility reasons.

Candidate amino acids were selected on the basis of the pK_a of the amino group. Glycylglycine was chosen as being representative of an acceptor with a low pK_a of the nucleophilic amino group (8.2); methionine, phenylalanine and serine were selected as having intermediate, similar pK_a values that are also similar to that of glutamine (9.21, 9.13, 9.15, and 9.13, respectively, for methionine, phenylalanine, serine, and glutamine). Alanine, with a pK_a of the amino group of 9.69, was representative of an acceptor with a slighly higher pK_a.

Glycylglycine was early recognized as a very good acceptor for γ-GT-catalyzed transpeptidation reactions [11]; it therefore became the substrate of choice and the reference compound for such studies. This was attributable to the low pK_a (8.2) of its nucleophilic amino group with respect to those of other amino acids [44]. The low pK_a of the glycylglycine amino group ensures that a large proportion of deprotonated, reactive nucleophilic species is present, even at those pH values at which the amino groups of standard amino acids are mainly in the protonated, unreactive form.

In the transpeptidation reactions with glycylglycine as the acceptor substrate, the product concentration appeared to be appreciable even between pH 7.5 and pH 9.0; after that, it increased with increasing pH (Fig. 5). However γ-glutamylglutamine, derived from the autotranspeptidation reaction, was also always noted in the reaction mixtures, albeit in lower concentrations than those of the desired transpeptidation product (data not shown). As the pH of the reaction mixtures approximated the pK_a of the donor glutamine amino group, the concentration of the residual glutamine after 1 h of reaction time decreased, owing to the

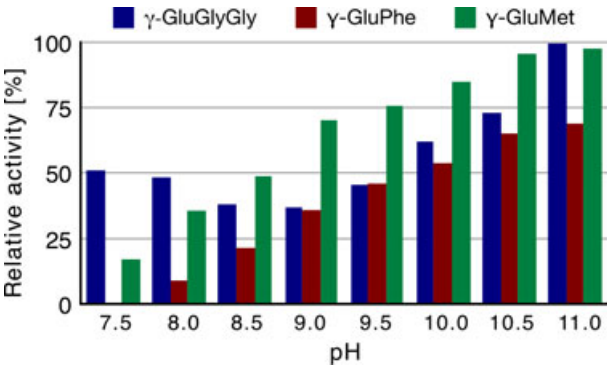


Fig. 5. pH dependence of the transpeptidation reaction for selected acceptors. Equimolar amounts (100 mM) of the donor glutamine and the candidate acceptor were incubated for 1 h at different pH values in the presence of a fixed amount of *B. subtilis* γ-GT, and 100-μL samples of each reaction mixture were then withdrawn, derivatized with DbsCl, and analyzed with HPLC. Results are relative to the maximum concentration of transpeptidation product obtained (glycylglycine as the acceptor and pH 11).

autotranspeptidation reaction. Usually, glutamine was hardly detectable after 1 h in reactions carried out at pH 9.5 and above. HPLC-MS experiments on the reaction at pH 10.5 demonstrated that, as well as being present in the expected autotranspeptidation product, the γ-glutamyl moiety derived from the starting glutamine also ended up in γ-glutamyl-γ-glutamylglycylglycine and γ-glutamyl-γ-glutamyl-γ-glutamylglycylglycine. Species formed by two γ-glutamyl residues bound to a single glutamine molecule were also identified (Fig. S9).

It is of note that the pH-activity profile obtained with glutamine as the donor by monitoring the formation of the transpeptidation product by means of HPLC is different from that obtained with GPNA as the donor substrate and monitoring of the reaction spectrophotometrically (Figs 1 and 5). When GPNA was used as the donor, liberation of *p*-nitroaniline, and hence only formation of the γ-glutamyl enzyme intermediate (Scheme 1), is detected. As stated above, at elevated pH, autotranspeptidation and poly-glutamylation reactions occurred, so the rate of release of *p*-nitroaniline was high, owing to the rapid enzyme turnover, which may also depend on autotranspeptidation and hydrolysis reactions in addition to the expected transpeptidation.

Reactions in which phenylalanine and methionine were used as acceptors showed low activity at the lower pH values, with a steady increase in transpeptidation product concentration with pH (Fig. 5).

Taking the reaction with methionine as an example, HPLC-MS analysis of the derivatized reaction mixture after 1 h of reaction at pH 8.0 confirmed the presence of the unreacted methionine and glutamine as the major components (Fig. S10). The hydrolysis product glutamic acid, the expected γ-glutamylmethionine and the autotranspeptidation product γ-glutamylglutamine were present in similar, quite low, concentrations. After 1 h of reaction at pH 10.5, on the other hand, the peak of the transpeptidation product γ-glutamylmethionine was the most prominent among those of the newly formed compounds (Fig. S11). As reported previously, glutamine was present in very low concentrations, as was glutamic acid. No peaks attributable to poly(γ-glutamyl)glutamine were detected with HPLC-MS, although low amounts of compounds formed by up to three γ-glutamyl moieties bound to a single methionine were identified.

Phenylalanine behaved similarly, and its activity as an acceptor substrate was lower than that of methionine (Figs. 5, S12 and S13).

On the basis of these results, it can be concluded that methionine and phenylalanine compete effectively with glutamine as acceptor substrates, provided that the pH of the reaction medium is maintained above the pK_a of their nucleophilic amino groups.

Moreover, in the presence of a good nucleophile, the hydrolysis reaction is minimized, as demonstrated by the low amount of glutamic acid.

Serine was also chosen as having a pK_a of the amino group similar to that of glutamine; however, its behavior in *B. subtilis* γ-GT-catalyzed reactions was different. It showed a very low propensity to act as an acceptor as compared with methionine and phenylalanine. The transpeptidation product γ-glutamylserine was evident only at relatively high pH values and, more interestingly, the donor substrate glutamine did not disappear (Figs S14 and S15).

Similar results were obtained with alanine as the acceptor substrate. In this case, HPLC-MS of the reaction at pH 10.5 showed the presence of only unreacted starting materials and very low amounts of transpeptidation and autotranspeptidation products (Fig. S17).

The low propensity of serine and alanine to act as acceptor substrates in γ-GT-catalyzed transpeptidation reactions has already been established for *B. subtilis* γ-GT [4] and several related enzymes [17,19,28], but this cannot explain the lack of the autotranspeptidation reaction involving glutamine. Such a behavior could be indicative of an inhibitory activity of a component of the reaction mixture towards the enzyme. Whether the inhibitory activity is attributable to the amino acids themselves, or arises from the newly formed γ-glutamyl derivatives, is currently under investigation in our laboratories. It is worth noting that inhibition phenomena have been reported for alanine in *E. coli* γ-GT-catalyzed reactions [3], and inhibitory activity towards rat kidney γ-GT was observed for a number of amino acids [14]. However, serine and alanine have been reported to be good acceptors in transpeptidation reactions catalyzed by γ-GTs of mammalian origin [13], albeit serine was recognized as a competitive inhibitor of human γ-GT [11,45], and a serine–borate complex is considered to be a transition state analog inhibitor for this enzyme [55].

Use of *B. subtilis* γ-GT as a biocatalyst

The results described so far suggest that the *B. subtilis* γ-GT-catalyzed transpeptidation reaction between glutamine as the donor and an acceptor amino acid is feasible, provided that the pH of the reaction medium is basic enough to ensure a certain concentration of a deprotonated, nucleophilic amino group of the acceptor molecule. It was also anticipated that, at the required basic pH, the donor glutamine would disappear rapidly from the reaction mixture, owing to the

autotranspeptidation reaction affording γ-glutamylglutamine as the major byproduct, together with poly-glutamylated glutamines in lower concentrations.

The *B. subtilis* γ-GT-catalyzed synthesis of γ-glutamylmethionine [33], a known naturally occurring flavor enhancer, was chosen in order to test this possibility. γ-GT-catalyzed synthesis of γ-glutamylmethionine was carried out at an analytical level (1 mL of a 100 mM solution of glutamine and methionine), and monitored with HPLC. The results are shown in Fig. 6. The concentration of glutamine dropped rapidly, whereas the decrease in the methionine concentration was accompanied by a parallel increase in γ-glutamylmethionine up to a calculated concentration of ~ 50 mM (50% yield) within the first hour of reaction time, after which their concentrations remained fairly constant for up to ~ 2 h. The amounts of both glutamic acid and γ-glutamylglutamine remained low throughout the reaction. Substantial amounts of glutamic acid and hydrolysis of the formed product γ-glutamylmethionine became evident only after 24 h of reaction time (data not shown).

The constant concentration of the transpeptidation product even after the disappearance of the donor substrate glutamine can be explained, in our opinion, by the early formation of γ-glutamylglutamine and other poly-glutamylated species, which became able to function as γ-glutamyl donors in later reaction stages. In these circumstances, a complex equilibrium arises, in which the autotranspeptidation and poly-γ-glutamylation reactions of glutamine, occurring mainly at the beginning of the reaction, reduce the molar concentrations of the donor species, so that the acceptor substrate methionine will be in molar excess with respect to the donors. The transfer of the γ-glutamyl moieties from the newly formed donor species to methionine therefore becomes the preferred reaction path up to 2 h of reaction time.

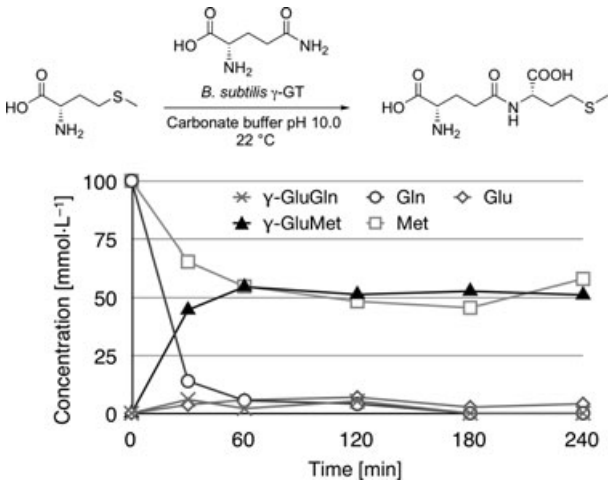


Fig. 6. Time course of the *B. subtilis* γ-GT-catalyzed transpeptidation reaction of glutamine (donor) and methionine (acceptor) at pH 10 and 22 °C to give γ-glutamylmethionine. The formation of glutamic acid was also monitored. Reaction conditions: equimolar amounts of glutamine and methionine (100 mM) in sodium carbonate/hydrogencarbonate buffer, pH 10.0, 22 °C, *B. subtilis* γ-GT 4.36 mU·mL^{-1}.

The reaction was repeated at at a 1-mmol preparative level with 10 mL of starting solution. After 1.5 h of reaction time, the enzyme was inactivated, and the products were isolated with ion exchange chromatography. γ-Glutamylmethionine was obtained in a yield of 45%. From the reaction mixture, unreacted methionine (~ 40%) and low amounts of glutamic acid and γ-glutamylglutamine (< 10% each) were also isolated and identified with NMR and MS.

Conclusions

Our results offer an unprecedented overview of the activities exerted by *B. subtilis* γ-GT from the viewpoint of the qualitative and quantitative determination of the products formed in the enzyme-catalyzed reactions under various experimental conditions.

The common thread emerging in our investigation was the strong influence of pH in modulating enzyme activity. When the pH was increased beyond the pK_a value of the nucleophilic amino group, glutamine, which is commonly considered to be a poor acceptor in γ-GT-catalyzed transpeptidation reactions, underwent a rapid autotranspeptidation reaction, affording not only γ-glutamylglutamine, but also oligomeric species, in which up to four γ-glutamyl residues were linked to a single glutamine molecule. This behavior was also seen with other known acceptor peptides or amino acids. Poly-glutamylation products were indeed found when glycylglycine, methionine and phenylalanine were used, provided that the pH of the reaction mixtures was basic enough to ensure the presence of a deprotonated, nucleophilic amino group in a reasonable concentration.

This finding, together with the observation that D-glutamine is also recognized both as a donor and as an acceptor substrate, leads us to assume the involvement of *B. subtilis* γ-GT in the homeostasis of extracellular γ-PGA, supporting the hypothesis that the enzyme acts *in vivo* as an exo-hydrolase towards γ-PGA [49]. The liberated glutamic acid is then used by the organism as a source of carbon and nitrogen.

In this context, some structural features of the enzyme appear to gain significance. The lack of the lid loop can

be related to the molecular sizes of the physiological substrates. γ-GTs that accept only discrete, low molecular mass γ-glutamyl derivatives (e.g. glutathione) as substrates possess the lid loop, whereas, in enzymes involved in the homeostasis or remodeling of large polymers composed of a huge number of γ-glutamyl residues, the lid loop seems to be absent, as in some strains of *B. subtilis* γ-GT, e.g. natto, or capD from *B. anthracis*, even if these enzymes have different physiological roles. The significance of the lid loop, although not completely understood, could therefore be related to substrate selection [56], although, at the current state of knowledge, its involvement in the modulation of the hydrolase/transpeptidase activity of the enzyme cannot be ruled out (Calvio C, Morelli CF, unpublished).

Although the involvement of γ-GT in the biosynthesis of poly-γ-glutamic acid has been proposed [28], its catalytic properties do not support this view. Glutamic acid is indeed unable to act as a γ-glutamyl donor, and the autotranspeptidase activity of *B. subtilis* γ-GT towards glutamine becomes appreciable at high substrate concentrations and rather high pH values (~ 10). The discovery of a multienzymatic system able to synthetize γ-PGA [57,58] therefore points towards a hydrolytic function of *B. subtilis* γ-GT in the homeostasis of γ-PGA.

Because of the transpeptidase activity, *B. subtilis* γ-GT could be used as a biocatalyst for the enzymatic synthesis of γ-glutamyl derivatives, avoiding the protection/deprotection steps required with chemical approaches. The glutaminase activity allows glutamine to be used as the donor compound. A possible drawback is the formation of poly-γ-glutamylglutamine through the autotranspeptidation reaction. In a preliminary, unoptimized experiment, we obtained the desired product in moderate yield, but adjustment of reaction conditions (temperature, time, and reactant molar ratio) could lead to improved results.

The search for suitable reaction conditions and for a readily available donor compound other than glutamine, in order to minimize autotranspeptidation side reactions, is currently being pursued in our laboratories.

Experimental procedures

General

L-Glutamine, D-glutamine, L-methionine, L-phenylalanine, L-serine, L-alanine, L-glutamic acid, *N*-phtaloyl-L-glutamic acid, phtalic anhydride, GPNA, DbsCl and anhydrous dimethylformamide were from Aldrich, and were used as received.

Analytical TLC was performed on Silica gel 60 F_{254} precoated aluminum sheets (Merck, Darmstadt, Germany). Plates were 9 cm high; mixtures were spotted 1.5 cm from the inferior edge, and plates were eluted with n-BuOH/AcOH/water (4 : 1 : 1) to a line drawn 7 cm from the start. Components were detected by inspection under a UV lamp (254 nm) in the case of chromophoric substances, and by spraying a 1% ninhydrin solution in ethanol followed by heating at ~ 150 °C.

Ion exchange chromatography was carried out with Dowex 1 × 8 resin 200–400 mesh (Aldrich) in the acetate form. The resin was regenerated with standard methods, and equilibrated by passing five volumes of 0.5 M acetic acid through it prior to use.

Analytical HPLC was performed on a Waters 600 (Millipore) instrument equipped with a Hewlett-Packard diode array detector Series 1050 (Palo Alto, CA, USA) at 436 nm, with a 250 × 4.60-mm Gemini RP C18 column (5 μm) (Phenomenex, Torrance, CA, USA). Eluents: 0.1% aqueous solution of trifluoroacetic acid (solvent A) and acetonitrile/solvent A (80 : 20) (solvent B). Elution gradient: 0–5-min isocratic elution with solvent A/solvent B (80 : 20); 5–35-min linear gradient up to solvent A/solvent B of 20 : 80; 35–45-min isocratic -elution with solvent A/solvent B (20 : 80). Flow rate: 1.5 $mL \cdot min^{-1}$.

^{1}H-NMR and ^{13}C-NMR spectra were acquired at 400.13 MHz and 100.61 MHz, respectively, in D_2O or in dimethylsulfoxide d_6 on a Bruker Advance 400 spectrometer (Bruker, Karlsruhe, Germany) interfaced with a workstation running a Windows operating system and equipped with a TOPSPIN software package. ^{13}C-signal multiplicities were based on attached proton test experiments. Chemical shifts are given in p.p.m. (δ), and are referenced to 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (δ_{Me} 0.00 p.p.m.) as an external standard, or to dimethylsulfoxide signal as an internal standard (δ_H dimethylsulfoxide 2.50 p.p.m.; δ_C dimethylsulfoxide 39.52 p.p.m.). Spectral analyses were carried out with INMR READER software (www.inmr.net, last access September, 2013) on an Apple computer.

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).

HPLC-MS was performed with a Thermo Finnigan Surveyor LC pump equipped with a Thermo Finnigan Surveyor photodiode array detector and interfaced with the ESI ThermoFinnigan LCQ Advantage spectrometer.

Enzyme production and purification

For enzyme production and purification and for the synthesis of reference compounds, see Doc. S1.

Enzyme aliquots (60 μL) were diluted to 500 μL with appropriate buffer prior to use.

Enzyme activity assay

One unit of *B. subtilis* γ-GT was defined as the amount of enzyme that liberates 1 μmole of *p*-nitroaniline per minute from GPNA in the presence of the acceptor glycylglycine. To a solution of GPNA (0.25 mM) and glycylglycine (2.0 mM) in Tris buffer at pH 8.5 and 22 °C, *B. subtilis* γ-GT was added (10 μL; final volume of 2.0 mL), and the release of *p*-nitroaniline was monitored for 4 min at 410 nm by means of a UV spectrophotometer. The amount of released *p*-nitroaniline was determined by use of a calibration curve obtained with freshly recrystallized, pure *p*-nitroaniline. Experiments were carried out in triplicate.

pH-activity profile

The pH-activity profile was determined at 22 °C with solutions at different pH values from 6.0 to 11.0 (final volume of 2.0 mL), containing 0.25 mM GPNA alone (hydrolysis), and in the presence of 2.0 mM glycylglycine (transpeptidation). Reactions were initiated by the addition of 10 μL of enzyme solution (21.9 mU). Initial velocities were measured by monitoring the release of *p*-nitroaniline at 410 nm for 2 min, with the absorbance being read every 10 s. The amount of released *p*-nitroaniline was calculated from a calibration curve obtained with freshly recrystallized, pure *p*-nitroaniline. Experiments were carried out in duplicate.

Precolumn derivatization procedure for HPLC analysis

The method of Keillor [59] was used, with some modifications. A 10 mM solution was prepared by dissolving 32.4 mg of DbsCl in 7–8 mL of dry acetone. The solution was filtered through a Millipore membrane into a 10-mL volumetric flask, and the volume was adjusted with dry acetone. The solution was transferred into a dark-glass, screw-capped bottle, and stored at 4 °C until use. In the precolumn derivatization procedure, the DbsCl was used in at least two-fold excess with respect to the material to be derivatized, taking into account that the initial reaction mixtures were 100 mM in both the donor glutamine and the acceptor. Reaction times were carefully observed. To a 100-μL aliquot of the mixture to be derivatized, 100 μL of a 50 mM solution of the internal standard L-leucine dissolved in a 100 mM hydrogencarbonate buffer at pH 8.2 was added. The final volume was adjusted to 1.0 mL by adding 800 μL of buffer. From this solution, a 100-μL aliquot was withdrawn and diluted with 400 μL of buffer in a glass vial equipped with a screw cap with a rubber septum. To the 500 μL of aqueous solution, 500 μL of DbsCl in acetone was added; the vial was then tightly sealed and placed in a preheated water bath at 70 °C for 10 min, during which time complete dissolution was achieved. After this time, a needle was introduced through the rubber septum, and heating was continued for a further 5 min, in order to evaporate most of the acetone. The vial was cooled with current water, and 500 μL of the resulting orange–red solution was diluted with 500 μL of a 0.1% aqueous solution of trifluoroacetic acid and used for HPLC analysis. Derivatized samples are stable for months, and can be stored at –20 °C.

Time course of the γ-GT-catalyzed reaction of glutamine at three pH values

Calibration curves were constructed for glutamine, glutamic acid and γ-glutamylglutamine with increasing concentrations of authentic materials in a sodium carbonate/hydrogencarbonate buffer at pH 8.5. Aliquots of 100 μL of each solution were derivatized in the presence of 100 μL of 50 mM L-leucine as the internal standard, as described above, and the obtained samples were analyzed with HPLC.

To three solutions of glutamine (L-glutamine or D-glutamine, 1 mL, 100 mM) at three different pH values (7.4, phosphate buffer; 8.2 and 9.8, sodium carbonate/hydrogencarbonate buffers) in a 2-mL Eppendorf tube, *B. subtilis* γ-GT (50 μL, 0.109 U) was added. The Eppendorf tube was sealed and shaken briefly by means of a vortex apparatus. Reactions were carried out at 22 °C for 24 h, and, at fixed time intervals (0, 1, 2, 3, 5, 7 and 24 h), 100-μL aliquots were withdrawn from each mixture, derivatized as described, and analyzed for glutamine, glutamic acid and γ-glutamylglutamine content by use of the corresponding calibration curves.

Time course of the transpeptidation reaction between glutamine donor and glycylglycine acceptor

Calibration curves were constructed for glutamine, glutamic acid, glycylglycine, and γ-glutamylglycylglycine, with increasing concentrations of authentic materials in a sodium carbonate/hydrogencarbonate buffer at pH 8.5. Aliquots of 100 μL of each solution were derivatized in the presence of 100 μL of 50 mM L-leucine as the internal standard, as described above, and the obtained samples were analyzed with HPLC.

The transpeptidation reaction was carried out with 1 mL of a solution of glutamine (100 mM) and glycylglycine (100 mM) in sodium carbonate/hydrogencarbonate buffer at pH 8.5 in a 2-mL Eppendorf tube. The reaction was initiated by the addition of *B. subtilis* γ-GT (50 μL, 0.109 U). After addition of the enzyme, the Eppendorf tube was sealed and shaken briefly with a vortex apparatus. The reaction was allowed to proceed at 22 °C for 24 h. At time intervals (0, 1, 2, 3, 5, 7 and 24 h), 100 μL of the reaction mixture was withdrawn, derivatized as described previously,

and analyzed for glutamine, glutamic acid, glycylglycine and γ-glutamylglycylglycine content by use of the corresponding calibration curves.

pH dependence of the transpeptidation reactions of selected acceptor amino acids

Eight stock solutions (50 mL each) of glutamine (100 mM) and the candidate amino acid (100 mM) were prepared with sodium hydrogencarbonate or sodium carbonate/hydrogencarbonate solutions (0.1 M in different proportions, pH 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0). The pH of each solution was carefully adjusted with 1 M or 0.1 M NaOH. One milliliter of each stock solution was then transferred into an Eppendorf tube, and the reaction was initiated by addition of the enzyme (50 μL, 0.109 U; total final volume of 1.05 mL). After 1 h of reaction at 20 °C, 100 μL of each reaction mixture was withdrawn, derivatized with DbsCl in the presence of the internal standard as described, and analyzed with HPLC. The amounts of the transpeptidation products in the reaction mixtures were estimated on the basis of response factors calculated with respect to the internal standard and determined by the use of synthetic, pure samples. Experiments were carried out in triplicate.

B. subtilis γ-GT-catalyzed transpeptidation reaction at a preparative level

Reaction course

To a solution (1 mL) of glutamine and methionine (100 mM) in sodium carbonate/hydrogencarbonate buffer (pH 10.0), the enzyme was added (50 μL, 0.109 U). The mixture was shaken briefly in a vortex apparatus, and the reaction was allowed to proceed at 22 °C. At time intervals (1, 2, 3, 5 and 24 h), 100 μL of the mixture was withdrawn, derivatized as described, and analyzed with HPLC.

Synthesis of γ-glutamylmethionine at a preparative level

To a solution of glutamine (146 mg, 1 mmol) and methionine (149 mg, 1 mmol) in sodium carbonate/hydrogencarbonate buffer (pH 10.0; final volume of 10 mL) (100 mM), 60 μL of undiluted enzyme solution (1.09 U) was added, and the reaction mixture was shaken by means of an orbital shaker at 22 °C for 1.5 h. The enzyme was inactivated by warming the mixture in a preheated water bath at 70 °C for 15 min. The mixture was lyophilized, and the residue was taken up in water; the pH was then adjusted to 9.5 with 0.5 M NaOH, and the solution was charged onto a column packed with Dowex 1 × 8 200–400 mesh ion exchange resin in the acetate form. The column was eluted with water and with a scalar gradient of AcOH solutions (0.5, 1.0, 1.5 and 2.0 M, three column volumes each). Fractions were collected on the basis of TLC analysis, and lyophilized. Four fractions were obtained: unreacted methionine (76 mg), eluted with 0.5 M AcOH; glutamic acid (12 mg), eluted with 1.5 M AcOH; γ-glutamylmethionine (117 mg, 42% yield, eluted between 1.5 and 2.0 M AcOH; and γ-glutamylglutamine (23 mg), eluted with 2.0 M AcOH.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Doc. S1. Supplementary experimental procedures.
Fig. S1. His-tagged *B. subtilis* γ-GT purification.
Fig. S2 HPLC-MS of sample at pH 8.2 after 2 h of reaction time.
Fig. S3. HPLC-MS of sample at pH 9.8 after 2 h of reaction time.
Fig. S4. HPLC-MS of sample at pH 9.8 after 2 h of reaction time (D-glutamine).
Fig. S5. Time course of the reactions.

Fig. S6. HPLC-MS of sample from the reaction of 25 mM glutamine after 1 h of reaction time.
Fig. S7. HPLC-MS of sample from the reaction of 50 mM glutamine after 2 h of reaction time.
Fig. S8. HPLC-MS of sample at 2 h of reaction time.
Fig. S9. γ-GT-catalyzed reaction between glutamine and glycylglycine at pH 10.5.
Fig. S10. γ-GT-catalyzed reaction between glutamine and methionine at pH 8.0.
Fig. S11. γ-GT-catalyzed reaction between glutamine and methionine at pH 10.5.
Fig. S12 γ-GT-catalyzed reaction between glutamine and phenylalanine at pH 8.0.
Fig. S13. γ-GT-catalyzed reaction between glutamine and phenylalanine at pH 10.5.
Fig. S14. γ-GT-catalyzed reaction between glutamine and serine at pH 8.5.
Fig. S15. γ-GT-catalyzed reaction between glutamine and serine at pH 10.5.
Fig. S16. γ-GT-catalyzed reaction between glutamine and alanine at pH 8.5.
Fig. S17. γ-GT-catalyzed reaction between glutamine and alanine at pH 10.5.