Bacterial γ-glutamyltranspeptidase: Food and medicinal applications

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Received 11 Nov 2019
Accepted 11 Dec 2019

ABSTRACT: γ-Glutamyltranspeptidase (GGT) catalyzes the hydrolysis of γ-glutamyl compounds and the transfer of their γ-glutamyl moiety to another amino acids or peptides. We have proposed several applications of this enzyme, especially in food and medicine, for the wellness of humans. These applications depend on the fact that the pH optima of the hydrolysis and transpeptidase reactions are distinctly different in E. coli GGT. The applications of bacterial GGTs are introduced in this review.

KEYWORDS: glutathione, γ-glutamyl transferase, γ-glutamyl transpeptidase, γ-glutamyl linkage

INTRODUCTION

γ-Glutamyltranspeptidase (GGT; EC 2.3.2.2) is widely used as a marker for hepatic diseases such as cirrhosis and hepatoma during blood tests. There are many studies from different groups on the function and regulation of GGT. However, no one has elucidated the basic enzymatic properties of its (a) active center, (b) three-dimensional structure, and (c) maturation. Since the author first found that GGT is expressed in the periplasm of Escherichia coli by culturing at 20 °C, further studies on bacterial GGT, especially in E. coli, using genetic, biochemical, and structural biological techniques were performed. Among the researchers who studied GGTs from various organisms, our research group was the first to succeed in clarifying all the points (a)–(c) mentioned above. (a) Using a new affinity labeling agent, it was found that the nucleophilic atom in the enzymatic reaction was the oxygen atom of the side chain of N-terminal Thr-391 of the small subunit. (b) The three-dimensional structure of E. coli GGT was determined and it was confirmed that the side-chain oxygen atom of Thr391 was γ-glutamylated. (c) A GGT precursor is translated from a single open reading frame as an inactive single-chain polypeptide that is subsequently processed into an active mature enzyme consisting of two subunits, large and small, via an ester-type intermediate by intramolecular autocatalysis. Further, it was shown that GGT is an enzyme classified in the N-terminal nucleophile hydrolase superfamily. The three-dimensional structure of the precursor GGT was also determined. Comparison of three-dimensional structures of the precursor and mature GGTs revealed that there are no significant changes in the overall structures due to the processing, but that the alterations in the dynamic structure around the active center lead to the formation of a mature active center.

GGT catalyzes the hydrolysis of γ-glutamyl compounds and transfer of their γ-glutamyl moiety to another amino acids or peptides (Fig. 1). The oxygen atom of the side chain of N-terminal Thr-residue of the small subunit attacks the carbonyl carbon of γ-glutamyl compounds to form the γ-glutamyl-enzyme intermediate. If the intermediate is attacked by water and glutamate is released, it is the hydrolysis reaction. If the intermediate is attacked by the amino group of amino acid or peptide to form a new γ-glutamyl compound, it is the transpeptidation reaction. The pH optima for these two reactions are sharply different in E. coli GGT (Fig. 1). Therefore, by adjusting the reaction pH, we can let E. coli GGT catalyze one of the reactions selectively.

In this review, the examples of utilizing GGT activity for food and medicinal applications to benefit human life are presented.

UTILIZATION OF THE HYDROLYSIS ACTIVITY OF GGT TO IMPROVE THE TASTE OF FERMENTED SEASONINGS

If the initial γ-glutamyl compound is glutamine, the hydrolysis reaction is the same as the reaction catalyzed by glutaminase, a very important enzyme
in food industry. Soy sauce and miso (fermented soy beans) are typical Japanese fermented seasonings. In the case of soy sauce, soy proteins are hydrolyzed by proteases and peptidases produced by *Aspergillus oryzae* or *Aspergillus sojae*, releasing amino acids especially glutamate that contributes to the umami taste. Glutamine which is also liberated from soy protein can be further hydrolyzed to glutamate by glutaminase produced by the fungi, leading to the addition of umami taste. However, the soy sauce and miso are fermented in the presence of high salt concentration, in such condition the fungal glutaminase is strongly inhibited and a non-negligible amount of liberated glutamine is chemically converted to pyroglutamate, which cannot give umami taste. Concerning the problem of enzyme inhibition at high salt concentration, we found GGTs from *Bacillus* species are salt-tolerant. GGT from *Bacillus subtilis*, for example, retained 76% of hydrolysis activity even in the presence of 18% salt which is the salt concentration for soy sauce fermentation mixture^7_. Hence, *B. subtilis* GGT, as a salt-tolerant glutaminase was added to the fermentation mixture at the beginning of soy sauce fermentation, and glutamate was continuously monitored. We found that, after 3 months, glutamate concentration of soy sauce with the addition of GGT with salt-tolerant glutaminase activity was obviously higher than that without the enzyme (about 50 mM). Nine out of ten panel members evaluated that soy sauce with the addition of *B. subtilis* GGT had stronger umami and more preferable taste in comparison to that without GGT^8_. We also got similar results on miso fermentation^9_. These results indicate that *B. subtilis* GGT is a useful enzyme to improve the taste of Japanese fermented seasonings, soy sauce and miso.

**UTILIZATION OF THE TRANSPEPTIDATION ACTIVITY OF GGT TO SYNTHESIZE VARIOUS γ-GLUTAMYL COMPOUNDS**

**γ-Glutamylation improves the taste of food**

Theanine (γ-glutamylethylamide) is the major umami component of green tea^10_, a positive correlation between the grade of Japanese green tea and the concentration of theanine was reported^11_. It also decreases blood pressure of spontaneously hypertensive rats^12_ and causes a feeling of relaxation^13_. The enzymatic method to synthesize L-theanine from L-glutamine and ethylamine by GGT was developed^14_.

Basic, aromatic and branched-chain amino acids taste bitter and some of them are essential amino acids. The bitter taste is a problem when we take these amino acids orally. We showed that bitterness of some amino acids was dramatically reduced and a refreshing lemon-like sourness was increased by their γ-glutamylation^15_.

Recently, several reports indicated that γ-glutamyl compounds are kokumi substances^16–19_. Kokumi substances are defined as having a weak taste, but addition of even small amounts of them to the meal enhances their flavor character, such as continuity, mouthfulness, and thickness^20_. Since kokumi substances enhance especially saltiness and sweetness, their additions give similar saltiness and sweetness taste even if the amount of salt and sugar in cooking is reduced. By adding kokumi substances to food, we can ameliorate the taste of diets for patients with diabetes and hypertension, and thereby improve their quality of life. Commercially available kokumi seasonings in Japan usually contain various combinations of broths, yeast extracts, Maillard-reacted peptides, and protein hydrolysates. Glutathione
(γ-glutamylcysteinylglycine) has been known as a kokumi substance. However, since glutathione is categorized as a pharmaceutical compound by the Ministry of Health, Labor and Welfare of Japan (MHLW), pure glutathione is not allowed to be used as a food additive in Japan. That is why yeast extracts are usually included in kokumi seasonings because of their high content of glutathione. Recently, γ-glutamylvalylglycine has been listed in food additive category by the MHLW and commercialized as a kokumi seasoning. We have shown that valylglycine is an ideal γ-glutamyl acceptor for E. coli GGT. Although γ-glutamylvalylglycine is the strongest kokumi substance known so far, kokumi seasoning does not necessarily have to be pure substance. Protein hydrolysates are commercially available for food manufacturers and widely added to processed food to increase the complexity of umami taste in Japan. Therefore, we developed a new method to produce kokumi seasoning by γ-glutamylation of protein hydrolysates made by bacterial protease.

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lated to 7-ACA by glutaryl-7-ACA acylase. D-Amino acid oxidase is detected in various microorganisms, but glutaryl-7-ACA acylase (cephalosporin acylase) is only found in limited bacterial species. The only difference between the glutaryl moiety and γ-glutamyl moiety is the existence of an α amino group. E. coli GGT is very likely to cleave γ-glutamyl-7-ACA to glutamate and 7-ACA. However, it is not able to cleave glutaryl-7-ACA. Comparison of the amino acid sequences of class IV cephalosporin acylases (glutaryl-7-ACA acylases) with those of GGTs revealed their surprisingly high similarity. Asp-433 of E. coli GGT is one of the residues that are completely conserved in GGTs, but not in class IV cephalosporin acylases. The corresponding residue of human GGT was suggested to interact with the α amino group of the γ-glutamyl moiety of the substrate, and the corresponding residue of class IV cephalosporin acylases is Asn. Therefore, a mutant E. coli GGT with D433N mutation was made and it was found to have glutaryl-7-ACA acylase activity, albeit very weak. In the meantime, the three-dimensional structure of E. coli GGT was determined and more rational substitutions of amino acid residues became available. Effective screening method for glutaryl-7-ACA acylase activity using glutaryl-α-naphthylamide was also developed. Liberated α-naphthylamide makes a diazo compound of a deep red color with Fast Garnet GBC sulfate salt, which allows screening of a mutant with high glutaryl acylase activity against whitish background (Fig. 2). The \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) values of the best mutant were 18- and 50-fold higher than D433N mutant, respectively. Since we found B. subtilis GGT has inherent glutaryl-7-ACA acylase activity and we also determined its three-dimensional structure, mutations were introduced into B. subtilis GGT and eventually the enzyme catalytic efficiency \( k_{\text{cat}}/K_m \) became higher than class IV cephalosporin acylase from Pseudomonas sp. V22. Our mutation work on GGTs thus made the enzymes exert the acylase activity capable of making cephalosporin antibiotic.

**FUTURE PERSPECTIVE**

The cost of enzyme preparation is the biggest barrier to the practical application of substance production by enzymatic methods. The cost of enzyme purification and the difficulty of recovering and reusing the enzyme after the reactions hinder the spreading of the enzymatic synthesis. Currently, immobilization of an enzyme is one of the widely performed methods. GGTs from Bacillus species have also been immobilized, but the authors provide data of the total enzymatic activity, which implies both transpeptidation and hydrolysis activities. Unlike E. coli GGT, GGTs from Bacillus species have pH optima of both activities at basic pH. Therefore, without evaluations of the transpeptidation and hy-
Acknowledgements: I would like to express sincere gratitude to my mentor Emeritus Professor Hidehiko Kumagai for his guidance, encouragement, and continuous support. Thanks also go to all of my co-workers and students who were involved in this study. The Ministry of Education, Science and Technology, Japan and many foundations are appreciated for financial supports.

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