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Novel substrate specificity of glutathione synthesis enzymes from *Streptococcus agalactiae* and *Clostridium acetobutylicum*

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Abstract

Glutathione (GSH) is synthesized by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS) in living organisms. Recently, bifunctional fusion protein, termed γ-GCS–GS catalyzing both γ-GCS and GS reactions from gram-positive firmicutes *Streptococcus agalactiae* has been reported. We revealed that in the γ-GCS activity, *S. agalactiae* γ-GCS–GS had different substrate specificities from those of *Escherichia coli* γ-GCS. Furthermore, *S. agalactiae* γ-GCS–GS synthesized several kinds of γ-glutamyltripeptide, γ-Glu-X_{aa}-Gly from free three amino acids. In *Clostridium acetobutylicum*, the genes encoding γ-GCS and putative GS were found to be immediately adjacent by BLAST search, and had amino acid sequence homology with *S. agalactiae* γ-GCS–GS, respectively. We confirmed that the proteins expressed from each gene showed γ-GCS and GS activity, respectively. *C. acetobutylicum* GS had broad substrate specificities and synthesized several kinds of γ-glutamyltripeptide, γ-Glu-Cys-X_{aa}. Whereas the substrate specificities of γ-GCS domain protein and GS domain protein of *S. agalactiae* γ-GCS–GS were the same as those of *S. agalactiae* γ-GCS–GS.

Keywords: Glutathione; γ-Glutamylpeptide; γ-Glutamylcysteine synthetase; Glutathione synthetase; *Streptococcus agalactiae*; *Clostridium acetobutylicum*

Glutathione (GSH, γ-glutamyl-cysteinyl-glycine) is the predominant non-protein thiol compound in living organisms. In bacteria it plays various important roles in many metabolic processes, such as protection against reactive oxygen toxicity [1,2].

γ-Glutamyl compounds are very attractive, because (i) compounds that have low solubility in water become much more soluble with γ-glutamylation [3]; (ii) γ-glutamyl linkage is resistant to peptidases in serum, and some γ-glutamyl compounds can possibly be used as prodrugs specific for the organs that express γ-glutamyltranspeptidase (GGT) (EC 2.3.2.2) [4]; and (iii) some γ-glutamyl compounds taste good and can be used as food additives [5,6]. Using GGT, the efficient methods for synthesizing various γ-glutamyl compounds were reported [7,8]. The yields of γ-glutamyl compounds with some γ-glutamyl acceptor were not very high. This was mainly because non-negligible amounts of by-products, such as γ-glutamylglutamine and γ-glutamyl-glutamyl compound, were formed. Therefore, the development of more effective γ-glutamyl compound synthesis and the explosion of more effective enzyme were required.

Glutathione is synthesized in a sequential two-step ATP-dependent biosynthesis pathway by two peptide bond-forming enzymes [9–11]. First, γ-glutamate-cysteine synthetase (γ-GCS, EC 6.3.2.2) ligates L-glutamate to L-cysteine with γ-glutamyl bond, forming the dipeptide γ-glutamylcysteine (γ-Glu-Cys), according to

L-glutamate + L-cysteine + ATP
→ L-γ-glutamyl-L-cysteine + ADP + Pi
Reaction 1

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Second, glutathione synthetase (GS, EC 6.3.2.3) ligates γ-Glu-Cys to glycine with α-peptide bond, forming the tripeptide GSH, according to

L-γ-glutamyl-L-cysteine + glycine + ATP
→ GSH + ADP + Pi
Reaction 2

γ-GCS and GS have been isolated and characterized from several gram-negative prokaryotes and from numerous eukaryotes including mammals, plants, yeast, and protozoa. Glutathione synthesis is relatively uncommon among the gram-positive bacteria. However, several gram-positive bacteria such as Streptococci, enterococci, and clostridiale, collectively classified as firmicutes, have been found to accumulate glutathione. Recently, Janowiak and Griffith [12] reported that glutathione synthesis occurs atypically in *Streptococcus agalactiae* and they identified the functional gene encoding for a bifunctional protein catalyzing both γ-GCS and GS reaction. The isolated protein was the first enzyme of GSH synthesis to be identified in a gram-positive organism and was the first bifunctional γ-glutamylcysteine synthetase-GSH synthetase (γ-GCS–GS) to be reported in any species. The N-terminal 518 amino acid sequence of γ-GCS–GS showed 32% identity with that of *Escherichia coli* γ-GCS, but interestingly, the C-terminal GS domain of γ-GCS–GS shows no significant homology with known GS. The C-terminal 360–750 amino acid sequences showed slight homology with D-Ala-D-Ala ligase (DdlA), that indicated they contained typical ATP grasp domain. Thereafter, γ-GCS–GS from *Listeria monocytogenes* and *Pasteurella multocida* was reported to possess γ-GCS–GS [13,14]. A BLAST search revealed that in several gram-positive bacteria and a few gram-negative bacteria, γ-GCS–GS-like fusion proteins were existed. In several bacteria, hypothetical proteins that have homology with C-terminal GS domain of γ-GCS–GS were found out because these proteins have the ATP-grasp domain. In gram-positive bacterium *Clostridium acetobutylicum*, the gene encoding a homologous protein with C-terminal GS domain of γ-GCS–GS lay immediately downstream from a gene encoding the γ-GCS exceptionally, not overlapped. In *Clostridium perfringens* and *Clostridium beijerincki*, γ-GCS–GS-like fusion proteins were present. We expected that the gene located downstream from a gene encoding the γ-GCS in *C. acetobutylicum* would be related to GS and that the two adjacent genes would be a pair of genes related to the novel GSH synthesis enzymes.

The previous reports provided the insight into various properties of γ-GCS–GS [12–14]. However, their substrate specificities were not investigated, in detail. In this study, we first comprehensively elucidated substrate specificity of *S. agalactiae* γ-GCS–GS and revealed various γ-glutamylpeptides that could be synthesized. Moreover, each domain protein of *S. agalactiae* γ-GCS–GS was expressed separately and the substrate specificities of the separated domain proteins and of *C. acetobutylicum* γ-GCS and GS were clarified.

Materials and methods

Materials. Genomic DNA from *S. agalactiae* 2603 V/R (ATCC number BAA-611(D), GenBank Accession No. AE009948) was obtained from the American Type Culture Collection. *E. coli* K12 W3110 strain (GenBank Accession No. AP009048) and *C. acetobutylicum* NBRC13948 (GenBank Accession No. AE001437) were obtained from NITE Biological Resource Center (NBRC). *E. coli* BL-21λ(DE3), JM109, and plasmid pET21a(+), pET21d(+), and pET30Xa/LIC were purchased from Novagen. The KOD-plus-PCR kit was purchased from TOYOBO. HisTrap™ HP was purchased from Amersham Pharmacia Biotech (Buckinghamshirc, England). DeterminerL IP was purchased from Kyowa medex. *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alanine amide (FDNP-Ala-NH_2) as derivatizing reagent for HPLC analysis was purchased from Tokyo Chemical Industry Co. Ltd. All other chemicals used were commercially available and of chemically pure grade.

Construction of plasmid for expression of S. agalactiae γ-GCS–GS. The γ-GCS–GS gene (*sag1821*) was amplified from *S. agalactiae* genomic DNA (ATCC BAA-611(D)) by PCR using the primer 5′-GGCATTCC ATATGATTATCGATCGACTG-3′ and 5′-GGCAAGCTTTAATTCT GGGAACAGTTTAG-3′. The resulting PCR product was digested with *Nde*I and *Hin*dIII, and was ligated into pET21a(+) digested with *Nde*I and *Hin*dIII. The resulting plasmid was introduced into *E. coli* JM109 and was extracted.

Expression and purification of S. agalactiae (His)$_6$-tagged γ-GCS–GS. *Escherichia coli* BL-21λ(DE3) was transformed with the extracted plasmid and was cultivated in Luria–Bertani medium (10 g/L Bacto tryptone, 5 g/L yeast extract, and 10 g/L NaCl) containing 50 μg/ml of ampicillin at 30 °C for 16 h with vigorous shaking. The culture broth was transferred to fresh Luria–Bertani medium and incubated at 37 °C. In 3 h after the transfer, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; final concentration) was added, cultivation was continued for 18 h. Cells were harvested by centrifugation. Cell pellet was suspended in 50 mM Tris–HCl buffer (pH 7.4) and disrupted by sonication at 4 °C. Cellular debris was removed by centrifugation (10,000 rpm, 30 min, 4 °C), and the supernatant was subjected to further purification using HisTrap, following the instruction of the manufacturer. Protein concentration was determined using Coomassie Protein Assay Reagent (PIERCE) with bovine serum albumin as a standard.

Construction of plasmid for expression of C. acetobutylicum γ-GCS and GS. The *C. acetobutylicum cac1539* gene was amplified from the *C. acetobutylicum NBRC13948* genome by PCR using primer 5′-CCGAATTC CCATGGATTGTTTTCCT-3′ and 5′-ACGCGGATCCGCTTCAG CATATTC-3′. The resulting PCR product was digested with *Nco*I and *Bam*HI, and was ligated into pET21d(+) digested with *Nco*I and *Bam*HI. The *cac1540* gene was amplified by using primer 5′-CGCGCCATG GATGAAGATTATACAAAAC-3′ and 5′-GCGCCTCGAGGTATC CAAGTAAATCTAA-3′. The resulting PCR product was digested with *Nco*I and *Xho*I, and was ligated into pET21d(+) digested with *Nco*I and *Xho*I. Each plasmid was introduced into *E. coli* JM109 and was extracted. The resulting plasmid was introduced into *E. coli* BL-21λ(DE3). Each crude homogenate was purified by HisTrap, as described in "Expression and purification of *S. agalactiae* $(His)_6$-tagged γ-GCS–GS".

Construction of plasmid for expression of E. coli γ-GCS and GS. In *E. coli*, γ-GCS and GS are encoded by the genes *gshA* and *gshB*, respectively. The *gshA* gene was amplified from the *E. coli* K12 W3110 genome by PCR using primer 5′-ATATCCATGGTCCCGGACGTATCACA GGC-3′ and 5′-TATACTCGAGGGCGTGTTTTTCCAGCCACA-3′. The resulting PCR product was digested with *Nco*I and *Xho*I, and was ligated into pET21d(+) digested with *Nco*I and *Xho*I. The *gshB* gene was amplified by using primer 5′-GGTATTGAGGGTCGCATGAT CAAGCTCGG-3′ and 5′-AGAGGAGAGTTAGAGCCTTACTGCT GCTGTAAACG-3′. pET30Xa/LIC vector kit (Novagen) was used for the plasmid construction. Each plasmid was introduced into *E. coli* JM109 and was extracted. The resulting plasmid was introduced into *E. coli* BL-21λ(DE3). Each crude homogenate was purified by HisTrap, as described in "Expression and purification of *S. agalactiae* $(His)_6$-tagged γ-GCS–GS".