Appl Biochem Biotechnol (2009) 159:614–622
DOI 10.1007/s12010-008-8493-8

Recombinant Glutamine Synthetase (GS) from *C. glutamicum* Existed as Both Hexamers & Dedocamers and C-terminal His-tag Enhanced Inclusion Bodies Formation in *E. coli*

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Received: 24 September 2008 / Accepted: 15 December 2008 /
Published online: 16 January 2009

Abstract In order to investigate the effect of his-tag on glutamine synthetase (GS, EC 6.3.1.2) from *Corynebacterium glutamicum*, recombinant *Escherichia coli* strains over-expressing GSIM, HGSIM (GS fused with N-terminal his-tag), GSIMH (GS fused with C-terminal his-tag), and HGSIMH (GS fused with N-terminal & C-terminal his-tags) were constructed, respectively. Under similar expression conditions, GSIM and HGSIM were partially solubly expressed; no soluble GSIMH and HGSIMH were observed, based on the result of SDS-PAGE. Gel filtration of purified soluble HGSIM showed that hexamers and dedocamers coexisted in the quaternary structure of GS from *C. glutamicum*. Combined this result with the analysis of two GS crystal structure models, we hypothesized that C-terminal residues participated in GS folding after translation on ribosome. After the folding process, C-terminal residues were released again and exposed to solvent. Fused C-terminal his-tag interrupted the GS to fold into its correct conformation so that inclusion bodies formed.

Keywords *Corynebacterium glutamicum* · Glutamine synthetase · His-tag · Inclusion bodies · Quaternary structure

Introduction

L-Glutamine is of importance as one of the most used pharmaceutical and food additives. Fermentation is still the main approach to produce glutamine. In recent years, however, enzymatic synthesis has come to rival direct fermentation as a means of glutamine

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production. The key enzyme responsible for L-glutamine synthesis is glutamine synthetase (GS), which catalyzes the ATP-dependent condensation of glutamine with ammonium [1].

$$\text{glutamate} + NH_3 + \text{ATP} \xrightarrow{\text{GS}} \text{glutamine} + \text{ADP} + \text{Pi}$$

Our group is also interested in enzymatic synthesis of glutamine and intends to construct recombinant *Escherichia coli* strains for glutamine production. Besides this, characterization of GS also draws our attention, aiming at finding the bottlenecks of catalysis for further genetic modification to improve GS performance.

The prerequisite of GS characterization is purification. A widely used procedure nowadays for fast purification of soluble proteins is the construction and use of his-tag fusion proteins [2]. Many examples showed that the proteins fused with 6 or 10 consecutive histidine residues can be effectively purified via immobilized metal affinity chromatography (IMAC) [3, 4, 5]. However, several reports also indicated that tags fused to recombinant proteins may also affect their properties significantly, including expression level, solution properties, protein crystallization, etc. [6]. In this work, in order to select suitable recombinant GS for fast purification and further GS characterization, we constructed several recombinant *E. coli* strains for GS overexpression and investigated the effects of his-tag positions to recombinant GS.

Materials and Methods

Strains, Plasmids, and Genes

Escherichia coli TOP10 and *E. coli* BL21(DE3) were purchased from Tiangen (Tiangen Biotech, Beijing, China). The plasmid pET-28a was purchased from Novagen (Wisconsin, USA).

Construction of Recombinant Plasmids and Strains

The mutant *glnA* genes without his-tag or with tags in different terminals were amplified by polymerase chain reaction (PCR) using the primer pairs in Table 1 and the recombinant plasmid pET-28a-GSIM(*Nde*I/*Hind*III) preserved by our lab was used as the template [7]. The PCR products were digested by *Nco*I/*Hind*III and then ligated to expression vector pET-28a, respectively, and the products of ligation reaction were transformated to competent cell of *E. coli* BL21(DE3). After transformation, four recombinant *E. coli* BL21(DE3) strains were finally obtained: *E. coli*-pET28a-GSIM (expressing GS without his-tag), *E. coli*-pET28a-HGSIM (expressing GS with N-terminal his-tag), *E. coli*-pET28a-GSIMH (expressing GS with C-terminal his-tag), and *E. coli*-pET28a-HGSIMH (expressing GS with N-terminal & C-terminal his-tags).

Culture condition and Expression of the glnA gene

The recombinant *E. coli* strains harboring the different *glnA* genes were cultured at 37 °C in LB medium containing 50 μg/ml kanamycin. When the biomass exceeded 0.5(OD600), the temperature was shifted to 30 °C. The recombinant glutamine synthetases (GSIM, HGSIM, GSIMH, and HGSIMH) could be overexpressed under the induction of 4 g/L α-lactose for 16 h.

Table 1 Primers used for gene amplification.

Name	Length	Sequence(5′→3′)
Primer-Ga	22	gatc*ccatgg*cgtttgaaaccc *Nco*I
Primer-Gb	32	gatc*aagctt*ttagcagtcaaagtacaattcg *Hind*III
Primer-HGa	45	gatc*ccatgg*ga*caccaccaccaccaccaccac*atggcgtttgaaacc *Nco*I 6×His
Primer-HGb	32	gatc*aagctt*ttagcagtcaaagtacaattcg *Hind*III
Primer-GHa	22	gatc*ccatgg*cgtttgaaaccc *Nco*I
Primer-GHb	52	gatc*aagctt*tta*gtggtggtggtggtggtg*gcagtcaaagtacaattcgaa *Hind*III 6×His
Primer-HGHa	45	gatc*ccatgg*ga*caccaccaccaccaccac*atggcgtttgaaacc *Nco*I 6×His
Primer-HGHb	52	gatc*aagctt*tta*gtggtggtggtggtggtg*gcagtcaaagtacaattcgaa *Hind*III 6×His

Gel Electrophoresis of the Recombinant GS

SDS-PAGE (12.5%) was carried out according to the method of Laemmli. Middle-molecular-weight protein standards (Beijing HT-Biotech, Beijing, China) were used as markers. Gels were stained with Coomassie brilliant blue R-250.

Enzyme and Protein Assays

Transferase activity assay was performed as described in the reference [8]. During the assay, GS solutions were diluted properly by 40 mM Tris–HCl, pH 7.0, to fit the standard curve. Two replications were run for the activity assay.

Protein concentrations were determined by Bradford method using bovine serum albumin (BSA) as the standard.

Purification of HGSIM

Escherichia coli-pET-28a-HGSIM cells were harvested by centrifugation from 800 ml culture (18,000 g for 8 min at 4 °C) using a centrifuger (KUBOTA 6930, Japan). The cell peppets were washed with a cold solution of 50 mM imidazole-HCl, pH 7.0, and resuspened in 80 ml imidazole-HCl, pH 7.0. The cells were disrupted ultrasonically at 4 °C for 10 min with 3 s pulses at 200 W by a cell sonicator (SCIENTZ JY92-II, China). Cell debris was removed by centrifugation (18,000 g for 10 min at 4 °C), resulting in the cell-free crude extract.

The following GS purification procedures were all performed on ÄKTA Explorer (GE, USA). The buffer of cell-free crude extract was changed to binding buffer (20 mM Tris, 10 mM imidazole and 500 mM NaCl, pH 7.4), through gel filtration column (HiTrap™, Desalting, GE, USA). The protein solution was then loaded at a flow rate of 2 ml/min to metal affinity (Ni^{2+}-IMAC) column (HiTrap™, Chelating HP), which was pre-equilibrated with binding buffer. The unbound protein was washed with binding buffer until the A_{280} of the washed buffer reached the baseline (approximately after eluted with 10–15 column

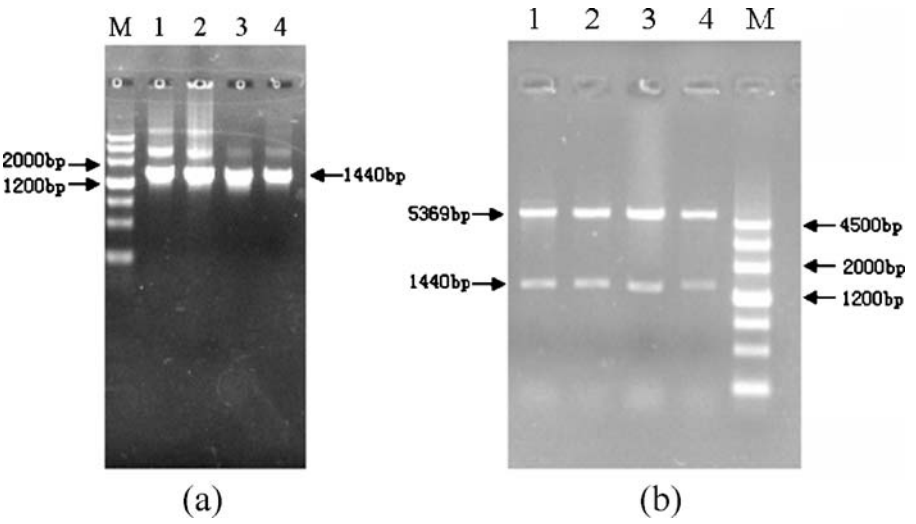

Fig. 1 Results of recombinant plasmids constructions by agarose gel electrophoresis. **a** PCR products from: *1*. Primer-GSIM and Primer-GSIM'; *2*. Primer-HGSIM and Primer-HGSIM'; *3*. Primer-GSIMH and Primer-GSIMH'; *4*. Primer-HGSIMH and Primer-HGSIMH'. **b** Recombinant plasmids after *NcoI*/*Hind*III restriction endonuclease digestion. *1*. pET-28a-GSIM; *2*. pET-28a-HGSIM; *3*. pET-28a-GSIMH; *4*. pET-28a-HGSIMH

volumes). The proteins were eluted with imidazole gradient from 10 mM to 500 mM. The peak fractions collected were further analyzed by GS activity assay and SDS-PAGE. The fraction proved containing purified HGSIM was finally changed into 40 mM Tris–HCl, pH 7.0 through gel filtration column (HiTrap™, Desalting, GE, USA).

Size-exclusion chromatography

Gel filtration was carried out on a Superdex 200 column. The column was calibrated with known weigh protein markers in 40 mM Tris–HCl, 150 mM KCl, pH 7.0 buffer. The marker proteins were thyroglobulin [Stokes radius (Rs) 8.5 nm, molecular weight (Mw) 669 kDa], ferritin (Rs 6.1 nm, Mw 440 kDa), catalase (Rs 5.22, Mw 232 kDa), and albumin (Rs 3.55 nm, Mw 67 kDa). The elution volume (Vm) at the apex of each marker protein was monitored by absorbance at 280 nm. To estimate subunits of HGSIM, the column was

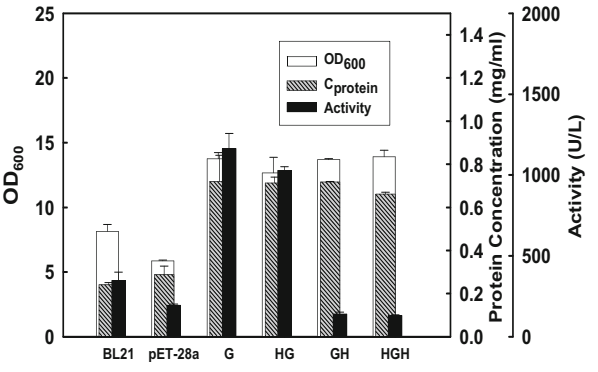

Fig. 2 OD600, soluble protein concentration and soluble GS acitivity after GS overexpression in recombinant *E. coli* strains. (1) BL21, *E. coli* BL21(DE3); (2) pET-28a, *E. coli* BL21(DE3) haboring native plasmid pET-28a; (3) G, *E. coli*-pET28a-GSIM; (4) HG, *E. coli*-pET28a-HGSIM; (5) GH, *E.coli*-pET28a-GSIMH; (6) HGH, *E. coli*-pET28a-HGSIMH

equilibrated with 40 mM Tris–HCl, 150 mM KCl, pH 7.0 buffer. Eight hundred microliters of purified HGSIM was loaded and eluted with the same buffer at a flow rate of 0.5 ml/min.

Results and Discussion

Construction of Recombinant Plasmids

Four different target products of *glnA* genes were amplified by PCR using the primer pairs in Table 1 from the template pET-28a-GSIM(*Nde*I/*Hind*III) (Fig. 1a). Additionally, four target recombinant plasmids were finally obtained by ligating PCR products into the *Nco*I/

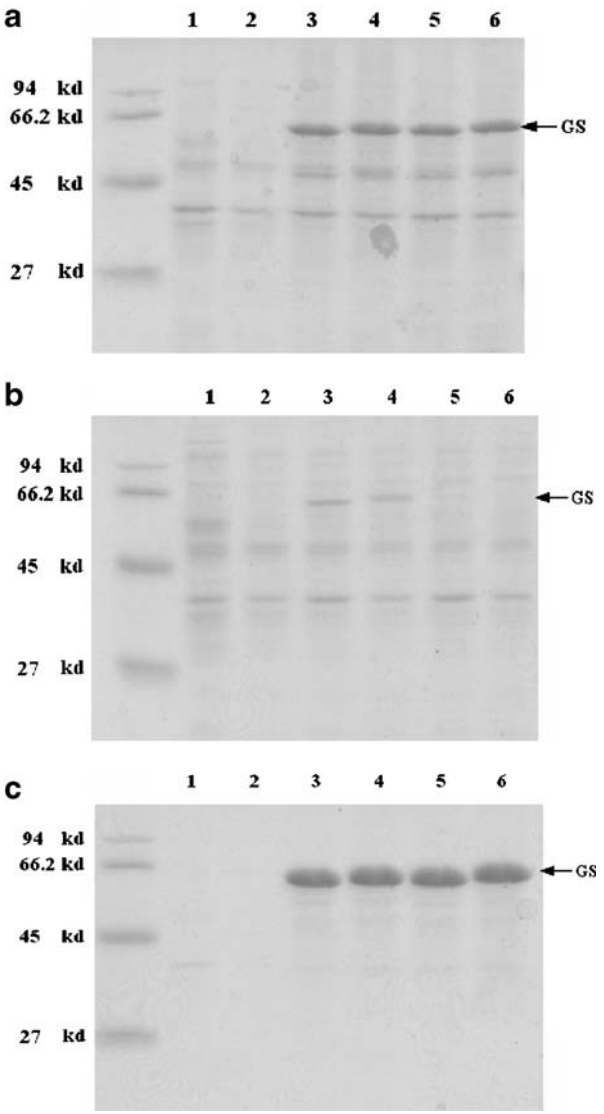

Fig. 3 SDS-PAGE (12.5%) analysis of GS expression in recombinant *E .coli* strains. **a** Whole-cell lysates; **b** cell-free crude extracts; **c** Cell debris dissolved in 8M urea. *Lane 1*, *E. coli* BL21(DE3); *lane 2*, *E. coli* BL21 (DE3) haboring native plasmid pET-28a; *lane 3*, *E. coli*-pET28a-GSIM; *lane 4*, *E. coli*-pET28a-HGSIM; *lane 5*, *E. coli*-pET28a-GSIMH; *lane 6*, *E. coli*-pET28a-HGSIMH

*Hind*III sites of the plasmid pET-28a (Fig. 1b), which were further proved by sequencing analysis(data not shown). The constructs were transformed into *E. coli* BL21(DE3) for recombinant GS overexpression.

Expression of recombinant GS in *E. coli*

Recombinant GS were overexpressed under the inducing of α-lactose at 30 °C for 16 h. Four recombinant *E. coli* strains harboring *glnA* showed similar cell densities and soluble proteins concentrations (Fig. 2). However, GS activities of cell-free crude extracts were different among them. Both the crude extracts containing GSIM and HGSIM had activities above 1,000 U/L, while both the crude extracts containing GSIMH and HGSIMH only had activities below 200 U/L.

The proteins of the whole cell lysates, cell-free crude extracts, and inclusion bodies were further analyzed by SDS-PAGE. The results suggested that the four recombinant strains had similar whole-cell expression level of GS (Fig. 3a), and most GS existed as inclusion bodies (Fig. 3c). SDS-PAGE of cell-free crude extracts showed that soluble GSIM and HGSIM were partially expressed; no soluble GSIMH and HGSIMH were observed (Fig. 3b). This indicated the GS activities loss in the cell-free crude extracts containing GSIMH

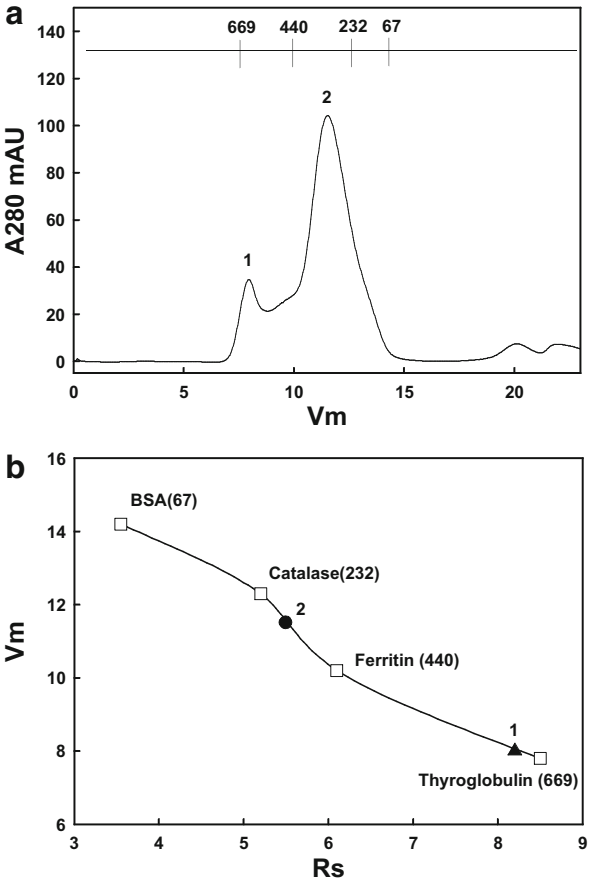

Fig. 4 GS subunits estimation by gel filtration. **a** Elution profile of purified HGSIM from gel filtration column Superdex 200; **b** molecular weights estimation of purified HGSIM by comparison with protein standards. The molecular weights of the protein standards are shown in parentheses in kDa

and HGSIMH were caused by C-terminal his-tag, which enhanced GS insoluble expression.

It was noticed that crude extracts of *E. coli*-pET28a-GSIMH and *E. coli*-pET28a-HGSIMH showed activity around 135 U/L, similar to crude extracts of *E. coli* BL21 and *E. coli*-pET28a (Fig. 2). The activity could be owed to endogenous soluble GS of *E. coli* or soluble GSIMH and HGSIMH in crude extracts, but they only existed in a tiny amount and could not be detected by SDS-PAGE (Fig. 3b).

Subunits Determination

N-terminal his-tag facilitated fast purification of HGSIM by Ni^{2+}-IMAC. Gel filtration of the purified HGSIM was carried out on Superdex 200 to estimate subunits number in GS quaternary structure. Elution profile indicated that two forms of GS existed in the purified HGSIM (Fig. 4a). The calculated monomer molecular weight of HGSIM was 54 kDa. By comparison with soluble protein standards and by referring to X-ray structural studies [9, 10], we estimated that soluble HGSIM could existed as hexamers and dedocamers. Fractions containing hexamers and dedocamers underwent activity assay. Both exhibited GS activity, with hexamers 1.31 U/mg and dedocamers 1.47 U/mg.

Mechanism of inclusion bodies formation

In the past two decades, several crystal structures of the GS in Gram-negative bacteria, as well as that from *Mycobacterium tuberculosis*, have been elucidated in Eisenberg's laboratory [11]. GS from *Salmonella typhimurium*, which had homology 50.84% to GS

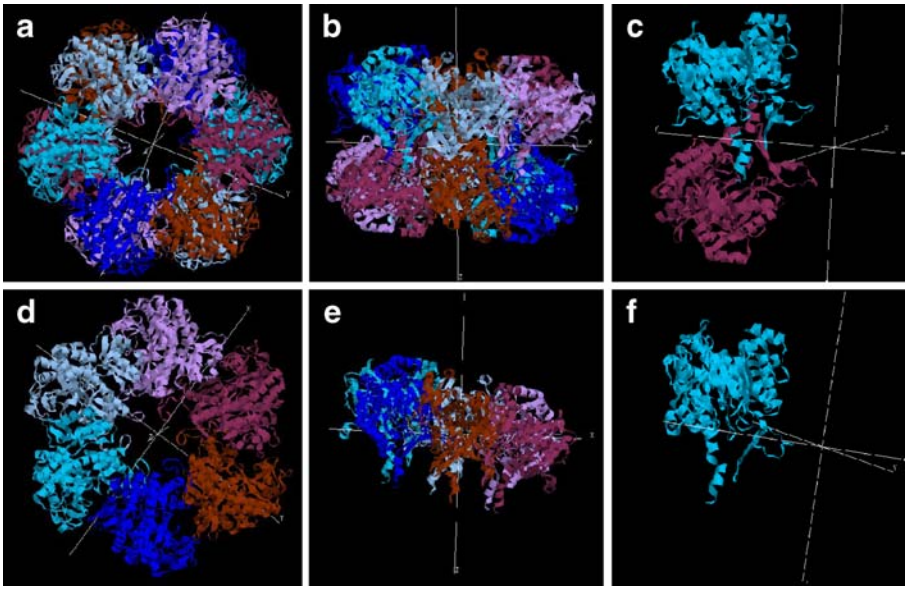

Fig. 5 Crystal structures of two GS molecules. GS from *S. typhimurium* (PDB no.1FPY) is a dedocamer molecule composed of two hexameric rings. The figures were generated with the program RasMol Version 2.7.4.2. **a** Top view; **b** front view. C-terminal of one subunit in a hexamic ring inserts into the subunit in the opposite ring (**c**). GS from *M. tuberculosis* (PDB no. 2BVC) is a hexamer molecule composed by one ring. **d** Top view; **e** front view. C-terminal of one subunit is exposed to solvent (**f**)

from *Corynebacterium glutamicum*, was a dedocamers molecule formed from two face-to-face hexameric rings of subunits (Fig. 5a, b). Formation of the dedocamers required up-to-down subunit contacts at face-to-face rings. Such nonconvalent contacts involved GS C-terminal, which had several hydrophobic residues. In fact, C-terminal residues of one subunit were inserted into the subunit on the other side (Fig. 5c) [9]. GS from *M. tuberculosis*, which had higher homology to GS from *C. glutamicum* (70.08%), was a hexamers molecule (Fig. 5d, e). This suggested that C-terminal residues could be exposed to solvent (Fig. 5f). Previous results of gel filtration on Superdex 200 have already proved both hexamers and dedocamers could exist in soluble GS from *C. glutamicum*. This suggested C-terminal residues involving subunits interactions should be mild and trivial to the correct conformation of active site so that hexamers and dedocamers of GS with almost equal activity were in equilibrium in solution. Fused C-terminal his-tag might interfere with this equilibrium, but it would hardly interrupt GS thermodynamically stable conformation.

Inclusion bodies formation was not only a thermodynamic problem, but also connected with the kinetic process of protein folding [12]. Mechanisms of protein folding were widely discussed. Nowadays, the hypothesis generally accepted was that a protein had already folded into its correct conformation before they were released from ribosomes [13, 14]. This implied C-terminal residues could not participate in protein folding because they still bounded to ribosomes. In the work, however, results were contradictory. Fused C-terminal his-tag enhanced GS inclusion bodies formation, so that soluble GSIMH and HGSIMH could not be detected by SDS-PAGE. This indicated C-terminal residues should participate in GS folding, and six extra hydrophilic histidine residues fused to C-terminal interrupted this process. What is more, GS crystal structures also implied that after folding into the active form, GS should release its C-terminal residues and exposed them to the solvent. From the discussion above, we could conclude impact of C-terminal his-tag to GS inclusion bodies formation was kinetic rather than thermodynamic.

Acknowledgements This work is supported by grants from the National Natural Science Foundation of China (No. 20506012) and the National Key Fundamental Research and Development Project of China (973 Project) (No. 2003CB716007). We give special thanks to Prof. Jiawei Wu and her student Dr. Zhihao Jiao (Department of Biological Science and Technology, Tsinghua University) for providing the Superdex 200 column for gel filtration.

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