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High-level secretory production of recombinant single-chain variable fragment (scFv) in *Corynebacterium glutamicum*

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Abstract We describe the development of a new secretory production system for the enhanced production of a single-chain variable fragment (scFv) against the anthrax toxin in *Corynebacterium glutamicum*. For efficient secretory production of the antibody fragment, the following components were examined: (1) signal peptides, (2) codon usage of antibody fragment, (3) promoters, (4) 5′ untranslated region (5′ UTR) sequence, and (5) transcriptional terminator. Among all the systems examined, the use of a codon-optimized gene sequence, a Sec-dependent PorB signal peptide, and a fully synthetic H36 promoter, allowed the highest production of antibody fragments in a culture medium. For large-scale production, fed-batch cultivations were also conducted in a 5-L lab-scale bioreactor. When cells were cultivated in semi-defined media, cells could grow up to an OD_{600} of 179 for 32 h and an antibody fragment concentration as high as 68 mg/L could be obtained in a culture medium with high purity. From the culture medium, the secreted antibody was successfully purified using a simple purification procedure, with correct binding activity confirmed by enzyme-linked immunosorbent assay. To the best of our knowledge, this is the first report of a fed-batch cultivation for antibody fragment production in *C. glutamicum*.

Keywords *Corynebacterium glutamicum* · Secretion · Antibody fragment · Fed-batch cultivation

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Introduction

Antibody and antibody fragments are currently the most valuable proteins that could be employed as therapeutic, diagnostic, and research tools, and they have the largest worldwide market in the field of pharmaceutical proteins (Buss et al. 2012; Jeong et al. 2011). Techniques in antibody and antibody fragment production have been extensively explored to address the need for recombinant proteins, to lower its production costs, and to shorten the production process (Lee et al. 2013). In terms of the production of antibody fragment and any other recombinant proteins, the choice of production host is one of the most important issues that is closely related to production cost. In the case of a full-length antibody, mammalian cells are often the preferred host because its secreted immunoglobulins (IgGs) harbor a glycosylated Fc region (Buss et al. 2012; Hong and Kim 2002; Jeong et al. 2011). However, antibody fragments such as single-chain variable fragment (scFv) and antigen-binding fragment (Fab), which do not require glycosylation but have a huge potential in medical, diagnostic, and biotechnological applications, have been produced in many bacteria and yeasts (Jeong et al. 2011; Dammeyer et al. 2011). For several decades, *Escherichia coli* has been utilized as the workhorse for the production of antibody fragments owing to its several beneficial properties such as rapid growth, wide spectrum of applications to genetic engineering, and well-documented genetic backgrounds (Chen 2012; Jang and Jeong 2013; Katsuda et al. 2012). However, *E. coli* is a gram-negative bacterium, and therefore, antibody fragments are generally secreted into the periplasmic space for oxidative folding, which in turn requires a time-consuming and labor-intensive purification process for antibody production (Huang et al. 2012; Persson and Lester 2004). To overcome the limitations related to the use of *E. coli*, identification of a new microbial host that offers efficient production and economic purification of antibody fragments is warranted.

Corynebacterium glutamicum is a gram-positive, non-sporulating, and nonpathogenic bacterium that has been traditionally used for the industrial production of various L-amino acids, nucleic acids, antibiotics, and other biochemicals (Lv et al. 2012). In addition, *C. glutamicum* is also an attractive host for the production of heterologous proteins such as industrial enzymes and pharmaceutical proteins owing to several features that are deemed optimal for protein production: (1) It is a gram-positive bacterium with a single cellular membrane that allows direct secretion of target proteins into the culture medium; (2) it produces a small amount of endogenous proteins into the culture medium, further simplifying the downstream process of protein purification compared with other host systems; (3) extracellular hydrolytic enzyme activity is not detectable, which improves the stability and production of secreted proteins in the culture medium (Vertès 2013); and (4) it is an endotoxin-free, generally recognized as safe (GRAS) strain, compared to non-GRAS bacteria such as *E. coli*. In addition, the fermentation conditions using this host are well established for large-scale production of various biomolecules, including recombinant proteins. Compared to other bacterial hosts (particularly *E. coli*), *C. glutamicum* also has several disadvantages: (1) much lower transformation efficiency, (2) only a few expression systems, and (3) relatively slow growth rate compared to *E. coli*. But, in the last decade, there was a big progress in developments of gene manipulation tools and expression systems (Pátek and Nešvera 2013; Yim et al. 2013), and information about cell physiology and metabolism has been accumulated more and more which is very useful for cell engineering toward rapid cell growth and enhanced production of various biomolecules (Kind et al. 2010; Wendisch et al. 2006). Based on those progress and its ideal features, *C. glutamicum* became a competitive host for production of various biomolecules. To date, *C. glutamicum* has been used in the production of recombinant proteins like epidermal growth factor, subtilisin-like serine protease, transglutaminase, and green fluorescent proteins, all of which resulted in high yields and efficiency (Date et al. 2006; Kikuchi et al. 2003; Teramoto et al. 2011; Watanabe et al. 2013). However, *Corynebacterium* spp. have been minimally used in the production of recombinant antibody fragments. Among several formats of antibody fragments, scFv consisting of variable heavy and light chains via flexible linkers has many beneficial properties including small size, easy folding, high solubility, easy conjugation with other cytotoxic drugs, no glycosylation, and cost-effective production in bacterial hosts (Ahmad et al. 2012). Therefore, it has been considered as one of the most important pharmaceutical proteins, and its application to diagnostic systems also increases more and more due to high specificity and affinity. But, although there are many successful reports for the production of antibody fragments in bacterial host including *E. coli*, *Bacillus* sp., and *Pseudomonas* sp., scFv is still considered as difficult-to-produce pharmaceutical protein due to its low solubility and poor folding (Dammeyer et al. 2011; Lee and Jeong 2013; Westers et al. 2004). Expression of the murine antibody fragment (scFv) against progesterone has been reported in *Corynebacterium pseudodiphtheriticum*, which is closely related to *C. glutamicum* (Sundaram et al. 2008). Reports on the production of antibody fragments in *C. glutamicum* are limited, which includes our recent reports (An et al. 2013; Yim et al. 2013) and the patent of Ajinomoto Co. (Matsuda et al. 2013). In these reports, the antibody fragments were examined just as model proteins for secretory production in flask cultivation, with very low production yield. Further extensive engineering of expression systems and optimization of the culture conditions are required for efficient production and purification toward large-scale protein production.

Herein, we developed a gene expression system for the secretory production of antibody fragments in *C. glutamicum*. As a model antibody fragment, M18 scFv, which has high affinity (K_D=~35 pM) against anthrax toxin PA, was chosen (Harvey et al. 2004). For efficient gene expression and secretory production of M18 scFv into the culture medium, several factors such as signal peptides, promoters, codon usage of scFv gene, 5′ untranslated region (5′ UTR), and transcription terminator sequences were examined. Finally, for large-scale protein production, fed-batch cultivations were also performed in a lab-scale (5 L) bioreactor, and M18 scFv was purified with high purity from the culture supernatant.

Materials and methods

Bacterial strain and plasmid manipulation

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue was used as the host for gene cloning and plasmid maintenance, and *C. glutamicum* ATCC 13032 was used as the main host for production of antibody fragments. A polymerase chain reaction (PCR) was performed using the C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) with PrimeSTAR HS polymerase (Takara Bio Inc., Shiga, Japan). The nucleotide sequences of all primers used in this study are listed in Table 2. The original M18 scFv gene against the anthrax toxin PA with the FLAG tag (774 bp) was PCR-amplified from pMoPac16-M18 (Harvey et al. 2004) by using the primers, M18-F and M18-R. After digestion with *Bam*HI and *Not*I, the PCR product was cloned in the same restriction enzyme sites of pCES-Trc (Yim et al. 2013), thereby yielding pCESTrcM. For secretory production of the antibody fragment, the TorA signal peptide gene (117 bp) and PorB signal peptide gene (81 bp) were PCR-amplified from *E. coli* XL1-Blue and *C. glutamicum* ATCC 13032 chromosomes by using the primer pairs, TorA-F and TorA-R

Table 1 Bacterial strains and plasmids used in this study

	Relevant characteristics	Reference or source
Strain		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]	Stratagene[a]
C. glutamicum	Wild type	ATCC 13032
Plasmids		
pCES208	*E. coli–C. glutamicum* shuttle vector, Km^r	Park et al. (2008)
pCES-Trc	pCES208 derivative, P_{trc}	Yim et al. (2013)
pTrc99A	*trc* promoter, *rrnB*T1T2 terminator	Lab stock
pMoPac16-M18	*lac* promoter, M18 scFv gene (wt), Cm^r	Harvey et al. (2004)
pCES-H36-GFP	pCES208 derivative, P_{H36}, eGFP	Yim et al. (2013)
pCESTrcM	pCES208 derivative, P_{trc}, M18 scFv gene (wt)	This study
pTrcM0	pCES208 derivative, P_{trc}, TorA signal sequence, M18 scFv gene (wt)	This study
pTrcM1	pCES208 derivative, P_{trc}, PorB signal sequence, M18 scFv gene (wt)	This study
pTrcM2	pCES208 derivative, P_{trc}, PorB signal sequence, M18 scFv gene (opt)	This study
pTrcM3	pCES208 derivative, P_{trc}, *tpi* SD, PorB signal sequence, M18 scFv gene (opt)	This study
pTrcM4	pCES208 derivative, P_{trc}, *tpi* SD, PorB signal sequence, M18 scFv gene (opt), *rrnB*T1T2 terminator	This study
pSodM2	pCES208 derivative, P_{sod}, PorB signal sequence, M18 scFv gene (opt)	This study
pSodM3	pCES208 derivative, P_{sod}, *tpi* SD, PorB signal sequence, M18 scFv gene (opt)	This study
pSodM4	pCES208 derivative, P_{sod}, *tpi* SD, PorB signal sequence, M18 scFv gene (opt), *rrnB*T1T2 terminator	This study
pH36M2	pCES208 derivative, P_{H36}, PorB signal sequence, codon-optimized M18 scFv gene (opt)	This study

[a] Stratagene Cloning System, La Jolla, CA, USA

and PorB-F and PorB-R, respectively. Both PCR products were digested with *Bam*HI and *Pst*I and then cloned into pCESTrcM to yield pTrcM0 and pTrcM1, respectively.

The codon-optimized M18 scFv gene was synthesized by GenScript Co. (Piscataway, NJ, USA). For cloning into the gene expression system, the codon-optimized gene was PCR-amplified using the primers, M18opt-F and M18opt-R. After digestion with *Pst*I and *Not*I, the digested PCR product was cloned into pTrcM1 to yield pTrcM2. For introduction of the triosphosphate isomerase Shine–Dalgarno sequence (*tpi* SD) of *C. glutamicum* ATCC 13032, the *trc* promoter with *tpi* SD sequence was PCR-amplified from the pTrc99a with the primers, tpiSD-F and tpiSD-R, and after digestion with *Kpn*I and *Bam*HI, the PCR product was cloned into pTrcM2 to yield pTrcM3. The *rrnB*T1T2 terminator sequence (350 bp) was PCR-amplified from pTrc99a by using the primers, rrnBTer-F and rrnBTer-R, and after digestion with *Not*I, the PCR product was cloned into pTrcM3 to yield pTrcM4.

Table 2 List of oligonucleotides used in this study

No.	Oligonucleotides sequence[a]
M18-F	5′-GCA**ggatcc**TAG**ctgcag**ATGGATATTCAGATGACACAGACTAC
M18-R	5′-CG**gcggccgc**TTATTTGTCATCGTCATCTTTATAATCCGAGGAGACGGTGACTGA
M18opt-F	5′-GCA**ctgcag**AAA**catatg**ATGGACATTCAGATGACCCAGACCAC
M18opt-R	5′-CG**gcggccgc**TTATCACTTATCATCGTCGTCCTTGTAGTCGGAAGACACGGTAA
TorA-F	5′-GA**ggatcc**ATGAACAATAACGATCTCTTTCAGG
TorA-R	5′-GAT**ctgcag**CGCAGTCGCACGTC
PorB-F	5′-GA**ggatcc**ATGAAGCTTTCACACCGCAT
PorB-R	5′-GTG**ctgcag**TGCGGAAGCAGGTGC
rrnBTer-F	5′-GA**gcggccgc**TGCCTGGCGGCAGTA
rrnBTer-R	5′-GA**gcggccgc**AAAAGGCCATCCGTCAGGAT
Psod-F	5′-AT**ggtaccgtcgac**AAGCGCCTCATCAGCG
Psod-R	5′-T**ggatcc**GGGTAAAAAATCCTTTCGTAGGTTTCC
PH36-F	5′-AT**ggtacc**TCTATCTGGTGCCC
PH36-R	5′-T**ggatcc**CATGCTACTCCTACC

[a] Restriction enzyme sites are shown in bold

The superoxide dismutase (*sod*) promoter was PCR-amplified from *C. glutamicum* ATCC 13032 chromosome using the primers, Psod-F and Psod-R, and after digestion with *Kpn* I and *Bam* HI, the PCR product was cloned into pTrcM2, pTrcM3, and pTrcM4 to yield pSodM2, pSodM3, and pSodM4, respectively. The H36 synthetic promoter was PCR-amplified from pCES-H36-GFP (Yim et al. 2013) using the primers, PH36-F and PH36-R, and after digestion with *Kpn* I and *Bam* HI, the PCR product was cloned into pTrcM2 to yield pH36M2. The schematic structures of all constructed plasmids are shown in Fig. 1. After plasmid construction in *E. coli*, each plasmid was transformed into *C. glutamicum* ATCC 13032 by electroporation using a Gene Pulser (Bio-Rad). All DNA manipulations, including restriction digestion, ligation, and agarose gel electrophoresis, were carried out using standard procedures (Sambrook and Russell 2001).

Flask cultivation

For plasmid preparation, *E. coli* was cultivated in Luria–Bertani (LB) broth (tryptone, 10 g/L; yeast extract, 5 g/L; and NaCl, 5 g/L) at 37 °C. For antibody production, *C. glutamicum* was cultivated in 100-mL flasks containing 20 mL of brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA) medium at 30 °C for 48 h with shaking at 200 rpm. In all cultivations, kanamycin (Km, 25 μg/mL) was added to the culture medium as the sole antibiotic. The contents of M18 scFv in each flask cultivation were quantified by enzyme-linked immunosorbent assay (ELISA) using a purified M18 scFv as a standard for calibration.

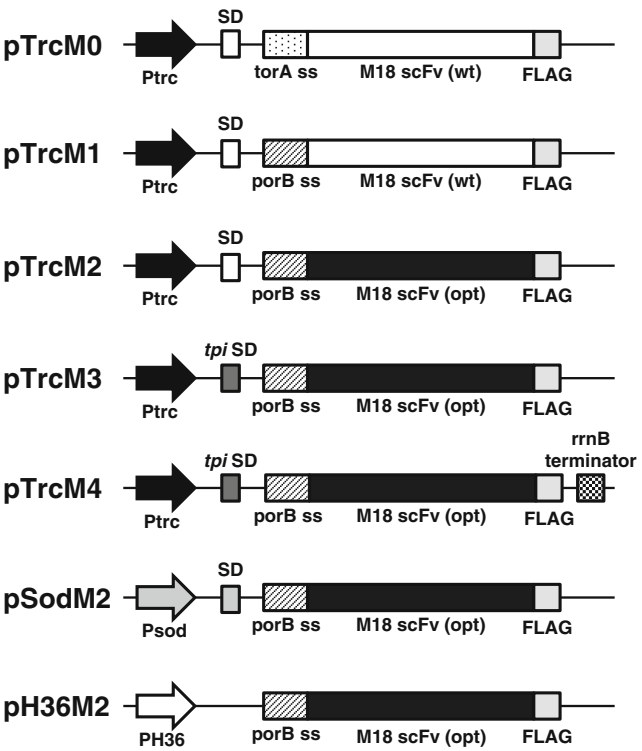

Fig. 1 Schematic diagram of plasmids used for secretory production of M18 scFv in *C. glutamicum*. P_{trc} and P_{H36} indicate *trc* promoter and H36 promoter, respectively. Plasmids pSodM2, pSodM3, and pSodM4 possess the same structure as pTrcM2, pTrcM3, and pTrcM4, respectively, except for the promoter. The *trc* promoter was replaced with the *sod* promoter

Protein preparation

After cell cultivation in shake flasks for 48 h, extracellular proteins were prepared using the acetone precipitation method (Jiang et al. 2004). After centrifugation, the culture supernatant was vigorously mixed with two volumes of cold acetone and incubated at −20 °C for 60 min. The protein samples were then precipitated by centrifugation (13,000 rpm, 30 min, 4 °C), and the pellets were resuspended in phosphate-buffered saline (PBS, 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2). Protein samples were stored at −20 °C until further analysis.

Fed-batch cultivation

As a seed culture, *C. glutamicum* harboring either pSodM2 or pH36M2 was inoculated into 200 mL of defined or semi-defined medium containing 20 g/L glucose in a 1-L baffle flask and cultivated at 30 °C for 20 h with constant shaking at 200 rpm. The defined medium consists of 3 g of K_2HPO_4, 1 g of KH_2PO_4, 2 g of urea, 10 g of $(NH4)_2SO_4$, 2 g of $MgSO_4$, 200 μg of biotin, 5 mg of thiamine, 10 mg of calcium pantothenate, 10 mg of $FeSO_4$, 1 mg of $MnSO_4$, 1 mg of $ZnSO_4$, 200 μg of $CuSO_4$, and 10 mg of $CaCl_2$ per liter with 25 mg/L Km. The semi-defined medium contains yeast extract (2 g/L) and casamino acid (7 g/L) in the same defined medium composition. The seed culture was inoculated into 2 L of fresh defined or semi-defined medium in a 5-L jar bioreactor (BioCNS, Daejeon, Republic of Korea).

Throughout the cultivation, the temperature was maintained at 30 °C. The pH and dissolved oxygen (DO) concentration were controlled at the set points by online monitoring. The DO concentration was maintained at 30 % (*v*/*v*) by automatically increasing the agitation speed up to 1, 200 rpm and then by mixing the pure oxygen through a gas mixer. The pH was maintained at 7.0 by adding 5 N ammonia solution. During the cultivation, glucose concentration was monitored as follows: an aliquot culture was centrifuged, and glucose concentrations from the supernatant were determined using a glucose analyzer (YSI 2700 SELECT™ Biochemistry Analyzer; YSI Life Science, Yellow Springs, OH, USA). To prevent glucose starvation, a glucose solution (90 g in 150 mL) was added to the cultures when the glucose concentration dropped lower than 0.5 % (*w*/*v*). Cell growth was monitored by measuring the optical density at 600 nm

(OD_{600}) with a spectrophotometer (Optizen POP; Mecasys, Daejeon, Republic of Korea). The contents of M18 scFv at each time point were quantified by ELISA using a purified M18 scFv as a standard for calibration.

Purification of antibody fragments

After the cultivation, the culture supernatant was prepared by centrifugation at 5,000×*g* for 30 min at 4 °C. The residual insoluble matters in the culture supernatant were filtered using a 0.45-μm syringe filter (Sartorius Stedim Biotech., Goettingen, Germany). Approximately 20 mL of the filtrated supernatant was dialyzed against an equilibration buffer (50 mM Tris–HCl, pH 7.0), and the antibody fragment was purified by anion-exchange column chromatography by using FPLC (AKTA Purifier 10 FPLC System, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The protein samples were loaded onto a HiTrap Q HP System (5 mL; GE Healthcare, Buckinghamshire, UK) that had been pre-equilibrated with 50 mM Tris–HCl (pH 7.0). Subsequently, the protein was eluted using a linear gradient (0–1.0 M) of NaCl in the same buffer at a rate of 0.5 mL/min. The protein concentration in each fraction was monitored using an UV detector (UPC-900; Amersham Pharmacia Biotech, Piscataway, NJ, USA). After pooling the scFv fractions, NaCl was removed by dialysis using a 7000-MWCO dialysis tube (Pierce Biotechnology, Rockford, IL, USA) against 2 L of PBS for 24 h with three buffer exchanges. After purification of M18 scFv antibody fragment, proteins were quantified using a Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin (BSA) as standard.

Enzyme-linked immunosorbent assay

The binding activity of M18 scFv was determined by ELISA (Harvey et al. 2004). Briefly, 50 μL of the antigen (anthrax toxin PA; 5 μg/mL) dissolved in carbonate–bicarbonate buffer was coated onto each well of a 96-well microarray plate (Thermo Fisher Scientific, Waltham, MA) at 37 °C for 2 h. After washing with PBS-T (0.5 % Tween-20 in PBS) four times, the coated antigens were blocked with 200 μL/well of 5 % (*w*/*v*) BSA (Invitrogen, Carlsbad, CA, USA) solution in PBS for 1 h at room temperature. Serially diluted protein samples from the culture media were added to the coated wells and allowed to bind for 1 h at room temperature. After washing, 50 μL of 1:5,000 diluted horseradish peroxidase-conjugated monoclonal ANTI-FLAG M2 antibody (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in 5 % (*w*/*v*) BSA solution in PBS was added to each well and allowed to react for 1 h. After the washing steps, 50 μL of a TMB peroxidase substrate system (BD, Franklin Lakes, NJ, USA) was added to each well for detection and 50 μL of 2 M H_2SO_4 was added as a stopping agent. The signals were quantified by measuring the absorbance at 450 nm by using a TECAN Infinite M200 Pro ELISA plate reader (Tecan Group Ltd., Männedorf, Switzerland). As a negative control, BSA was coated onto the plate, and all the steps as previously described were performed.

Other analytical methods

All protein samples were analyzed by 12 % (*w*/*v*) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting. After SDS-PAGE, all proteins bands were transferred onto a polyvinyl difluoride membrane (Roche, Penzberg, Germany) for 60 min at 100 mA by using a Bio-Rad transblot apparatus (Bio-Rad). The membrane was incubated with a blocking solution (Tris-buffered saline, 24.7 mM Tris, 137 mM NaCl, 2.7 mM KCl, and 0.5 % Tween-20) with 5 % (*w*/*v*) skim milk for 1.5 h at room temperature. The membrane was then incubated with a horseradish peroxidase (HRP)-conjugated monoclonal ANTI-FLAG M2 antibody (Sigma-Aldrich) for the immunodetection of FLAG-tagged protein. After incubation, each membrane was washed four times with TBS-T for 5 min and the ECL kit (Amersham ECL Prime Western Blotting Detection Reagent; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used for protein detection.

Nucleotide sequence accession number

The nucleotide sequence reported in this study has been deposited in the GenBank data under the accession no. KF525940.

Results

Construction of a basic expression system

There have been several expression systems for secretory production of recombinant proteins in *C. glutamicum*. However, the expression systems constructed previously are not expected to be optimal for production of M18 scFv because production yields of recombinant proteins generally depend on the characteristic of each protein. Therefore, expression systems were constructed newly for optimization of M18 scFv production. The key features of the constructed plasmid for antibody expression are shown in Fig. 1. All expression systems were constructed based on the *E. coli–C. glutamicum* shuttle vector, pCES208 (Park et al. 2008), as previously described in the "Materials and methods" section. The scFv format consists of variable regions of the antibody heavy and light chains (VH and VL, respectively) joined by a triplet of a Gly_4Ser (Gly-Gly-Gly-Gly-Ser) linker. In each construct, FLAG tag (DYKDDDDK) coding sequences were placed downstream of the M18 scFv coding gene to facilitate

the detection of the antibody fragment in western blot and ELISA (Fig. 1).

Comparison of Sec-dependent and Tat-dependent pathway for secretory production of scFv

C. glutamicum has two major secretory pathways: (1) Sec-dependent pathway, which mediates the secretion of unfolded proteins; and (2) Tat-dependent pathway, which mediates the secretion of folded proteins. Both pathways have been widely used for the secretory production of heterologous proteins in *C. glutamicum* (Vertès 2013). To choose the better pathway for the secretory production of antibody fragment, both secretion pathways were examined and compared. Two signal peptides, Porin B (PorB) (An et al. 2013) and TorA (Kikuchi et al. 2009), were employed for Sec-dependent and Tat-dependent secretion, respectively. *C. glutamicum* harboring either pTrcM0 or pTrcM1, in which gene expression was controlled under the constitutive *trc* promoter, was cultivated, and then, the secretory production of M18 scFv in the culture medium was subjected to western blot analysis following SDS-PAGE. When the PorB signal peptide (pTrcM1) was used, the band of M18 scFv was clearly detected in western blotting, but in the case of the TorA signal peptide (pTrcM0), no band of M18 scFv was detected (Fig. 2a). The high secretion yield of M18 scFv through the Sec-dependent pathway (pTrcM1) was also clearly confirmed by ELISA. *C. glutamicum* harboring pTrcM1 showed high signal intensity against specific antigens (anthrax toxin PA), but negligible signal intensity was detected with the culture supernatant of *C. glutamicum* harboring pTrcM0 (Fig. 2b). These results showed that M18 scFv, which were not folded in the cytoplasm, could be secreted into the culture supernatant through a Sec-dependent secretion pathway. In all subsequent studies, the PorB signal peptide was used for the secretory production of M18 scFv.

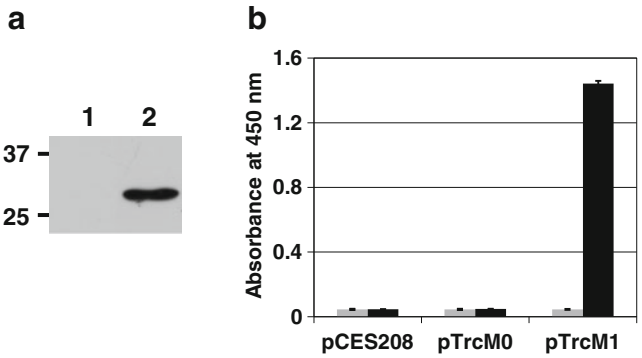

Fig. 2 Comparison of production and binding activity of M18 scFv using the two different Tat- and Sec-dependent pathways: **a** western blot analysis of culture supernatant. *Lane 1*, *C. glutamicum* (pTrcM0); *lane 2*, *C. glutamicum* (pTrcM1). For normalization of the loading samples, the same volume (8 μL) of 30-fold concentrated culture supernatant was loaded on each lane. **b** ELISA analysis of culture supernatant. *Black* and *gray bars* indicate signals from wells coated with the anthrax toxin PA and by using BSA as a negative control, respectively

Codon usage optimization of scFv

Most bacteria have different codon usage, and it is well known that codon usage is highly related to the translation efficiency and should thus be considered for enhanced gene expression (Ermolaeva 2001). The presence of the rare codon in the coding gene causes the early exhaustion of the corresponding tRNAs, and consequently, sufficient amounts of the target protein cannot be produced. *C. glutamicum* is a high G+C-rich organism, and its codon usage is reported to be highly biased that G and C bases are predominant at the third position of codon throughout all genes (Eikmanns 1992; Liu et al. 2010). This strong preference for the codon usage already revealed that codon optimization of heterologous genes would be a useful tool for production of recombinant proteins in *C. glutamicum* (Jo et al. 2007; Kind et al. 2010; Malumbres et al. 1993). When we checked codon usage of the original M18 scFv, eight rare codons with frequencies below 0.05 were detected. Based on the codon preferences of *C. glutamicum*, all codons of M18 scFv were optimized to preferable codons (GenBank accession no. of the codon-optimized M18 scFv gene sequence: KF525940). With the codon-optimized M18 scFv gene under the *trc* promoter (pTrcM2), the expression level was compared to that in the original gene expression system (pTrcM1). We observed that the use of the codon-optimized gene resulted in a slightly higher level (1.2-fold) of gene expression than that of the original gene sequence (Fig. 3a) (Table 3). ELISA analysis also confirmed the higher production of M18 scFv with the codon-optimized gene sequence (Fig. 3b). For all the next expression systems, the codon-optimized M18 scFv gene was used.

5′ UTR and transcription termination

In prokaryotic gene expression, 5′ UTR region and termination of transcription are considered important factors for efficient gene expression. Teramoto et al. (2011) reported that the use of a *tpi* SD combined with a *tac* promoter showed a significantly enhanced translation efficiency compared to that using the original SD sequence in *C. glutamicum*. To examine the effect of the SD sequence on the production of M18 scFv, the original SD sequence in pTrcM2 was exchanged with the *tpi* SD sequence (pTrcM3). In addition, for efficient transcription through rapid turnover of RNA polymerase, a strong transcription terminator (*rrnB*T1T2) was also introduced into the downstream region of the M18 scFv coding gene (pTrcM4). After flask cultivation, the levels of secretory productions of M18 scFv in the culture medium were analyzed by western blot analysis and ELISA. We clearly observed that the exchange of the *tpi* SD sequence resulted in a significantly enhanced production of M18 scFv, but the additional introduction of

Table 3 Summary of production yields from flask cultivations

	pTrcM1	pTrcM2	pTrcM3	pTrcM4	pSodM2	pSodM3	pSodM4	pH36M2
Production yield (mg/L)	0.275	0.330	0.879	0.707	1.67	0.268	0.261	18.12

*rrnB*T1T2 terminator did not show any marked difference in production yield (Fig. 3a). The increase in production yield associated with the use of the *tpi* SD sequence and codon-optimized gene was also confirmed by ELISA. The production yield of M18 scFv in *C. glutamicum* harboring pTrcM3 was approximately 3.2-fold higher than that observed in *C. glutamicum* harboring pTrcM1 and pTrcM2 (Table 3).

Gene expression under the *sod* promoter and H36 synthetic promoter

Promoter strength is an important factor in recombinant gene expression and secretion. In addition to the *trc* promoter, two other promoters, the *sod* promoter of *C. glutamicum* (Neuner and Heinzle 2011) and the H36 synthetic promoter (Yim et al. 2013), which are relatively stronger than the *trc* promoter, were examined. First, for gene expression under the *sod* promoter, *trc* promoters in earlier constructs (pTrc series) were exchanged with the *sod* promoter to yield pSodM2, pSodM3, and pSodM4. After cultivation in flasks, secretory production of M18 scFv into the culture medium was examined by western blot analysis. Compared to the *trc* promoter, the *sod* promoter with the codon-optimized M18 scFv gene (pSodM2) exhibited a relatively higher production yield than that observed in the best system with a *trc* promoter (pTrcM3) (Fig. 4a). Under the *sod* promoter, the effects of 5′ UTR modifications (*tpi* SD sequence) and *rrnB*T1T2 transcription termination were also examined. However, replacement of the SD sequence with the *tpi* SD sequence (pSodM3) resulted in a significant decrease in the expression level (Fig. 4a). The use of *tpi* SD sequence under the *trc* promoter (pTrcM3) resulted in a significant increase of production yield unlike the *sod* promoter. In a previous report (Teramoto et al. 2011), *tpi* SD sequence was also highly effective under the *tac* promoter which has almost the same sequence as the *trc* promoter but very different sequence to the *sod* promoter. It is also well known that both promoter and SD sequence are highly effective on overall gene expression (Mutalik et al. 2013), and our results imply that it is very important to find the optimal combination of promoter and SD sequence in the construction of gene expression systems. In addition, introduction of the *rrnB*T1T2 terminator with the *tpi* SD sequence (pSodM4) did not result in any positive effect on gene expression, but this might be the deleterious effect of the *tpi* SD sequence under the *sod* promoter.

Next, the fully synthetic H36 promoter was examined. Recently, we isolated 20 novel promoters from the synthetic promoter library in *C. glutamicum*, and among them, the H36 promoter exhibited the highest promoter strength (Yim et al. 2013). One important feature of the H36 promoter is that it produces leaderless mRNA transcripts, in which transcription and translation starting positions are identical, and thus, the SD sequence is not required for translation. The expression of the codon-optimized M18 scFv gene under the H36 promoter (pH36M2) was examined, and we observed a significant increase in the secretory production yield of M18 scFv into the culture supernatant (Fig. 4a). The secretory production yields of M18 scFv in *sod* and H36 promoter-employing expression systems were also evaluated by ELISA, and similar results as those observed from western blot analysis were obtained (Fig. 4b). Finally, the use of the H36 promoter resulted in 20.6- and 10.9-fold increases in production yield compared with the *trc* promoter (pTrcM3) and *sod* promoter (pSodM2), respectively (Table 3).

Enhanced production of M18 scFv by fed-batch cultivation

To achieve large-scale production of M18 scFv, fed-batch cultivations with *C. glutamicum* harboring either pSodM2 or pH36M2 were carried out in a lab-scale (5-L) bioreactor system. Cells were cultivated in either defined or semi-defined media, and nutrient feeding solutions (90 g in each feeding) were fed according to the level of glucose in the media. In defined media, *C. glutamicum* harboring pSodM2 grew up to an OD_{600} of 116

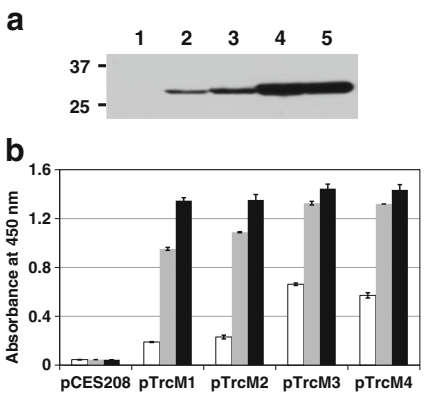

Fig. 3 Optimization of expression systems controlled by the *trc* promoter. **a** Western blot analysis of the culture supernatant. *Lane 1*, pCES208; *lane 2*, pTrcM1; *lane 3*, pTrcM2; *lane 4*, pTrcM3; *lane 5*, pTrcM4. For normalization of the loading samples, the same volume (8 μL) of 30-fold concentrated culture supernatant was loaded on each lane. **b** ELISA analysis of the culture supernatant. *White*, *gray*, and *black bars* indicate signals from 10, 50, and 100 μL of the culture supernatant, respectively

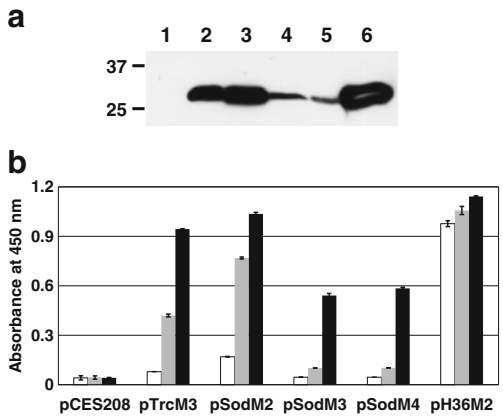

Fig. 4 Analysis of gene expression under the *sod* promoter and H36 promoter. **a** Western blot analysis of the culture supernatant. *Lane 1*, *C. glutamicum* (pCES208); *lane 2*, *C. glutamicum* (pTrcM3); *lane 3*, *C. glutamicum* (pSodM2); *lane 4*, *C. glutamicum* (pSodM3); *lane 5*, *C. glutamicum* (pSodM4); *lane 6*, *C. glutamicum* (pH36M2). For normalization of the loading samples, the same volume (8 μL) of 30-fold concentrated culture supernatant was loaded on each lane. **b** ELISA analysis of the culture supernatant. *White*, *gray*, and *black bars* indicate signals from 4, 20, and 100 μL of the culture supernatant, respectively

for 21 h and then cell density decreased gradually (Fig. 5a). In the exponential growth phase, a specific growth rate (μ) was approximately 0.111 h^{-1}. At 9 h after inoculation, M18 scFv was produced and its quantity continuously increased. The maximum concentration of M18 scFv (approx. 3.9 mg/L) in the culture medium was achieved at 21 h, after which its content decreased gradually. In contrast, *C. glutamicum* harboring pH36M2 grew up to an OD_{600} of 115 for 27 h, with a specific growth rate (μ) of 0.102 h^{-1} in the exponential growth phase (Fig. 5b). The maximum concentration of M18 scFv in the culture supernatant was approximately 9 mg/L at 25 h and then decreased gradually.

Next, in semi-defined media that additionally contains 2 g/L of yeast extract and 7 g/L of casamino acid in defined media, *C. glutamicum* harboring pSodM2 grew up to an OD_{600} of 176.4 for 22 h and then the cell density decreased gradually (Fig. 6a). In the exponential growth phase, a specific growth rate (μ) was approximately 0.136 h^{-1}, and the maximum concentration of M18 scFv in the culture supernatant (approximately 4.4 mg/L) was obtained at 24 h. In contrast, *C. glutamicum* harboring pH36M2 grew up to an OD_{600} of 179 for 32 h, with a specific growth rate (μ) of 0.129 h^{-1} in the exponential growth phase (Fig. 6b). The maximum concentration of M18 scFv in the culture supernatant was approximately 68 mg/L at 34 h. Among the four different cultivations, the last case (H36 promoter and semi-defined medium) also showed the highest volumetric productivity (2.0 mg M18 scFv/L/h). The results of the fed-batch cultivations are summarized in Table 4.

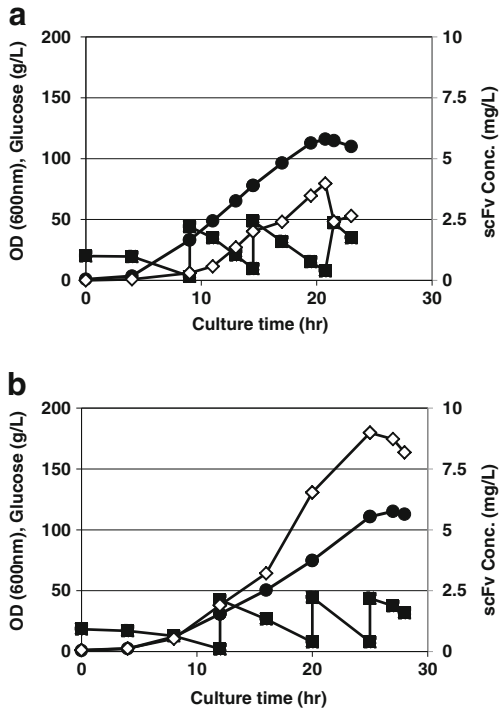

Fig. 5 Profiles of cell growth, glucose concentration, and M18 scFv production yield during fed-batch cultivations of **a** *C. glutamicum* (pSodM2) and **b** *C. glutamicum* (pH36M2) in defined media. *Closed circles* indicate cell density (OD_{600}), *closed squares* indicate glucose concentration (grams per liter), and *open diamonds* indicate M18 scFv concentration in the culture supernatant

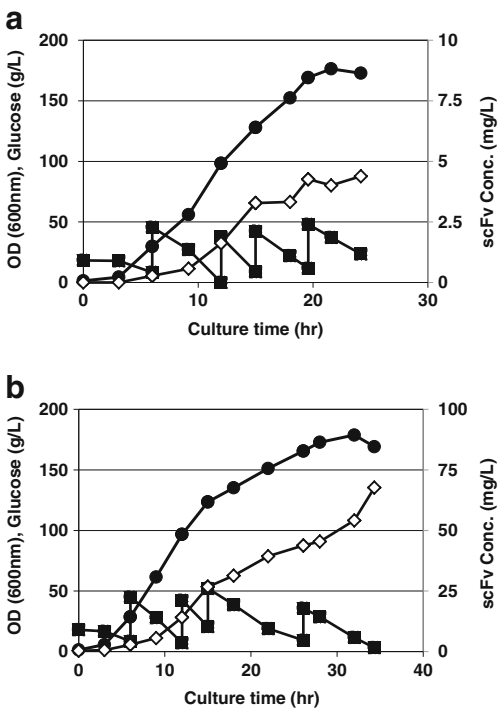

Fig. 6 Profiles of cell growth, glucose concentration, and M18 scFv production yield during fed-batch cultivations of **a** *C. glutamicum* (pSodM2) and **b** *C. glutamicum* (pH36M2) in semi-defined media. *Closed circles* indicate cell density (OD_{600}), *closed squares* indicate glucose concentration (grams per liter), and *open diamonds* indicate M18 scFv concentration in the culture supernatant

Table 4 Summary of results from fed-batch fermentations

Medium	Plasmid	Specific cell growth rate (h^{-1})	Max. cell density (OD_{600})	Max. production yield (mg/L)	Productivity (mg/L/h)
Defined	pSodM2	0.111	116	3.9	0.188
	pH36M2	0.102	115	9	0.360
Semi-defined	pSodM2	0.136	176.4	4.4	0.191
	pH36M2	0.129	179	68	2.0

Purification and activity of purified scFv

After fed-batch cultivation with *C. glutamicum* harboring pH36M2 in semi-defined media, the culture supernatant was collected by centrifugation and M18 scFv was purified as described in the "Materials and methods" section. From a 1-L culture broth, approximately 8.2 mg of M18 scFv could be purified with high purity (>99 %) and recovery yield (ca. 12 %) (Fig. 7a). Using a purified sample, the N-terminal sequence was determined using the Edman degradation method, and the five amino acid sequences (LQKHM) were perfectly matched with the sequences of the first six amino acids immediately after PorB signal peptide, which indicates that the PorB signal peptide was correctly removed during secretion into the culture medium. To assess whether the secreted and purified M18 scFv had acquired correct binding activity, the binding of the purified M18 scFv to its cognate antigen (anthrax toxin) was measured by ELISA using plates coated with the anthrax toxin or BSA as the control. As shown in Fig. 7b, the purified M18 scFv has a high binding affinity, as well as high specificity for the antigen.

Discussion

After its discovery in 1956, *C. glutamicum* has been extensively used as a microbial factory for the production of amino acids and various chemicals owing to its optimal features for large-scale production. These features have also increased its biotechnological potential for the production of various pharmaceutical proteins. However, compared to *E. coli*, information on *C. glutamicum*, as well as tools for its recombinant gene expression, is limited. In this study, various components such as signal peptides, codon usage, UTR sequences (including SD sequences, transcription terminations, and different promoters), and their effect on the production of antibody fragments were examined to construct the optimized gene expression system. Among all the systems examined, we observed that the use of a codon-optimized gene sequence, a Sec-dependent PorB signal peptide, and a fully synthetic H36 promoter, allowed the highest production of M18 scFv into a culture medium. Particularly, among the examined components, we found that modification of the 5′ UTR sequence resulted in a highly significant effect on overall gene expression. Under the *trc* promoter, replacement of the original 5′ UTR sequence of the *trc* promoter with the 5′ UTR sequence of the *tpi* (*tpi* SD) significantly improved M18 scFv gene expression, which was similar to previous results wherein the *tpi* SD sequence was introduced under the *tac* promoter (Teramoto et al. 2011). In general, the effect of the 5′ UTR sequence on gene expression is closely related to the secondary structure of the mRNA transcript, and mRNA transcripts with the higher free energy (ΔG°) exhibit better translation efficiency and higher gene expression (Pfleger et al. 2006). Using the RNAstructure software (ver. 5.5, http://rna.urmc.rochester.edu/RNAstructure.html), the secondary structures of the 5′ end region (+1 to 100) of the mRNA transcripts in gene expression systems with original and *tpi* SD sequences were determined. However, we could not find any significant differences in free energy in both predicted structures (data not shown). We think this was not primarily related to the promoters because the mRNA transcripts produced under both the *sod* and *trc* promoters were the same. The exact cause of this deleterious effect thus remains unclear, but these results strongly indicate the importance of the 5′ UTR sequence in the construction of a gene expression system, and thus, it is necessary to identify the best combination of the promoter and the 5′ UTR sequence for efficient gene expression in *C. glutamicum* which generally requires the extensive cloning

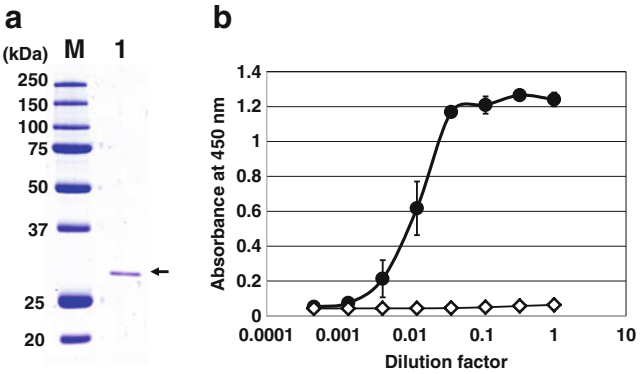

Fig. 7 Purification of M18 scFv produced from fed-batch cultivation of *C. glutamicum* (pH36M2) in semi-defined media. **a** SDS-PAGE analysis of purified M18 scFv. *Lane M*, molecular weight size markers (kilodalton); *lane 1*, the elute from anion-exchange column chromatography. The *arrow* indicates the purified M18 scFv. **b** ELISA of purified M18 scFv. Signals were obtained from wells coated with anthrax toxin PA (*closed circles*) or BSA as a negative control (*open diamonds*). In the *X*-axis, 1 indicates 1 μg of M18 scFv

works. In this aspect, the use of a H36 synthetic promoter is strongly recommended because the H36 synthetic promoter produces a leaderless mRNA transcript, and hence, the effect of 5′ UTR sequence for the construction of a gene expression system does not need to be considered. As demonstrated in flask cultivations (Fig. 4) and large-scale fed-batch cultivations (Figs. 5 and 6), the elevated gene expression in *C. glutamicum* can be achieved just by inserting the target gene into the downstream region of the H36 promoter.

For large-scale protein production, fed-batch cultivations were performed with *C. glutamicum* harboring pSodM2 and pH36M2, and similar to that observed during flask cultivation, the use of H36 synthetic promoter also resulted in a significantly enhanced production (68 mg/L) of M18 scFv into the culture supernatant compared to that using the *sod* promoter. We also examined two different media: defined and semi-defined media. When cells were cultivated in semi-defined media, both cultures exhibited higher maximum cell density, cell growth rates, and production yields compared to those in defined media (Table 3). In fed-batch cultivation, cell growth to high cell density and protein production require many resources, and the inefficient supplementation of nutrients can be one limiting factor in the production of recombinant proteins and cell growth during fed-batch cultivation. Our results indicate that supplementation of more resources from the semi-defined media can be used for the enhanced production of M18 scFv and much higher cell growth. In both defined and semi-defined media, *C. glutamicum* harboring the pH36M2 showed slightly lower cell growth rates compared to that using pSodM2, and this might be attributable to the higher secretory production of scFv under the stronger H36 promoter. However, in all cultivations, any serious problems involving cell growth were not observed during the secretory production of scFv. In addition, when we analyzed the cytoplasmic fractions by western blot analysis, M18 scFv (premature form) was not detected (data not shown). All these results indicate that the use of the Sec-dependent PorB signal peptide and synthetic promoter (H36 promoters) allowed the efficient secretion of M18 scFv into the culture medium.

Although we successfully performed the secretory production of antibody fragments in *C. glutamicum*, the production yields were relatively lower than those obtained by secretory production of other recombinant proteins in *C. glutamicum* (An et al. 2013; Teramoto et al. 2011; Watanabe et al. 2013; Yim et al. 2013). To increase the secretory production yield, host cell engineering and optimization of environmental conditions also need to be considered. Recently, Teramoto et al. (2011) reported that the secretory production of green fluorescent protein (GFP) through the Tat-dependent pathway could be drastically increased through the addition of calcium chloride in the medium. More recently, Watanabe et al. (2013) reported the effect of *sigB* inactivation on the secretory production yield. The use of *sigB*-deficient *C. glutamicum* resulted in the significant increase in the secretory production of α-amylase and GFP compared to those using wild-type cells. Protein production in bacteria may cause a physiological change in the host. Recent progress in proteomics can also provide new insights to the physiological changes occurring in host cells during the production and secretion of recombinant proteins (Aldor et al. 2005). Strategies in host cell engineering based on proteomics and optimization of culture conditions would also be useful in the preparative scale production of antibody fragments in *C. glutamicum*. Recently, we also reported the secretory production of the same M18 scFv in the periplasm of *E. coli* strain (Lee and Jeong 2013). Although a little higher production yield (89.8 mg/L) could be obtained in fed-batch cultivation of *E. coli*, the current strategy for scFv production in *C. glutamicum* provided several promising results compared to those by *E. coli*: (1) Unlike production in *E. coli*, M18 scFv could be steadily accumulated in culture medium without significant degradation by proteolytic activities throughout the whole fed-batch cultivation. (2) M18 scFv was produced in culture medium so the purification step could be much easier and simpler than the *E. coli* system in which M18 scFv was produced in periplasm. Whole cells did not need to be disrupted but removed just by centrifugation and the M18 scFv could be purified simply by single column chromatography with high purity (>99 %) as shown in Fig. 7. (3) Also, we did not need to use the expensive IPTG for gene expression in *C. glutamicum*. Based on these observation, it is clear that more economic production can be achieved using *C. glutamicum* host than *E. coli* host even though we did not do economic evaluation for production of M18 scFv in both hosts.

In conclusion, we have developed an expression system for the efficient secretory production of antibody fragments in *C. glutamicum*. Using the synthetic H36 promoter, PorB signal peptide, and codon-optimized sequence, we could achieve the high-level secretory production of M18 scFv into the culture supernatant. In addition, through fed-batch cultivation, its large-scale production was successfully demonstrated in which almost 200 mg of M18 scFv was produced in one fed-batch cultivation (final volume 2.8 L). We also successfully demonstrated the purification of the functional M18 scFv from the culture medium by single ion-exchange column chromatography. To the best of our knowledge, this is the first report on the production of antibody fragments using fed-batch cultivation of *C. glutamicum*. This study has shown that *C. glutamicum* has a great potential for the secretory production of recombinant proteins into cultures, and large-scale production through fed-batch cultivations could be easily achieved. We believe that our strategy has shown that *C. glutamicum* may be used as a potential host for the cost-effective production of various therapeutic proteins, as well as antibody fragments in an industrial scale.

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