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Development of a New Platform for Secretory Production of Recombinant Proteins in *Corynebacterium glutamicum*

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ABSTRACT: *Corynebacterium glutamicum*, which has been for long an industrial producer of various L-amino acids, nucleic acids, and vitamins, is now also regarded as a potential host for the secretory production of recombinant proteins. To harness its potential as an industrial platform for recombinant protein production, the development of an efficient secretion system is necessary. Particularly, regarding protein production in large-scale bioreactors, it would be appropriate to develop a secretory expression system that is specialized for high cell density cultivation conditions. Here we isolated a new signal peptide that mediates the efficient secretion of recombinant proteins under high cell density cultivation conditions. The secretome of *C. glutamicum* ATCC 13032 under high cell density cultivation conditions was initially investigated, and one major protein was identified as a hypothetical protein encoded by *cg1514*. Novel secretory production systems were then developed using the Cg1514 signal peptide and its own promoter. Efficient protein secretion was demonstrated using three protein models: endoxylanase, α-amylase, and camelid antibody fragment (VHH). For large-scale production, fed-batch cultivations were also conducted and high yields were successfully achieved—as high as 1.07 g/L (endoxylanase), 782.6 mg/L (α-amylase), and 1.57 g/L (VHH)—in the extracellular medium. From the culture media, all model proteins could be simply purified by one-step column chromatography with high purities and recovery yields. To the best of our knowledge, this is the first report of the development of an efficient secretory expression system by secretome analysis under high cell density cultivation conditions in *C. glutamicum*.

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KEYWORDS: *Corynebacterium glutamicum*; secretome; Cg1514; secretion; fed-batch cultivation

Introduction

Corynebacterium glutamicum is a gram-positive, non-pathogenic, and non-sporulating bacterium that is considered an industrial workhorse for the production of various L-amino acids, nucleic acids, and vitamins (Diesveld et al., 2009; Lv et al., 2012). Recently, this microorganism has attracted attention as a potential cell factory for the production of recombinant proteins since it exhibits numerous ideal features for protein secretion (An et al., 2013; Vertes, 2013). First, it has a single cellular membrane as a gram-positive bacterium, which allows proteins to be secreted into the extracellular medium by simply crossing a single membrane barrier. Second, *C. glutamicum* secretes only a few endogenous proteins into the culture medium, which allows for the simpler purification of target proteins in downstream process compared to the protein production and purification processes of other bacterial hosts such as *Escherichia coli*. Third, secreted proteins from *C. glutamicum* can be kept stable because extracellular protease activity is rarely detectable. Moreover, *C. glutamicum* is an endotoxin-free and generally recognized as safe (GRAS) organism that is suitable for the production of therapeutic proteins. With all these advantages, *C. glutamicum* has great potential to become very efficient industrial platform for recombinant protein production.

A signal peptide is a short peptide present at the N-terminus of newly synthesized proteins that are destined to be secreted. In most hosts, signal peptides play a key role in directing proteins to secretion channels; therefore, the choice of signal peptide has a significant impact on the overall yield of protein secretion (Ng and Sarkar, 2013; Tuteja, 2005). In *C. glutamicum*, two well-known

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signal peptides, PS1 and PS2, have been used for the secretory production of recombinant proteins (Date et al., 2003, 2006; Joliff et al., 1992). However, the use of both signal peptides does not always ensure successful secretion of various target proteins. Efforts have therefore been made to isolate new signal peptides which would improve the protein production yield. From the various sources of endogenous and heterologous secretory proteins, their signal peptides have been used for construction of secretory production systems such as TorA signal peptide from *E. coli* (Kikuchi et al., 2009), PhoD signal peptide from *Bacillus subtilis* (Meissner et al., 2007) and Porin B (PorB) signal peptide from *C. glutamicum* ATCC 13032 (An et al., 2013; Yim et al., 2013). Using those signal peptides, various recombinant proteins were successfully produced into culture medium and significant production yields (615–846 mg/L) were achieved. The recent development of genome-wide techniques has enabled extensive and systematic analysis of protein secretion in *C. glutamicum* and as a result, new signal peptides have been isolated. Through the genomic analysis of *C. glutamicum* R with the aid of signal peptide identifying software, 405 protein candidates with signal peptides and 108 signal peptides with the ability to secrete α-amylase were identified (Watanabe et al., 2009). Analysis of *C. glutamicum* R strain revealed two secreted proteins encoded by *cgR_1176* (Gene ID: 4994404) and *cgR_2070* (Gene ID: 4992673) and their signal peptides were successfully used to secrete α-amylase into extracellular medium (Suzuki et al., 2009). However, those secretome analyses were done in flask cultivations and the development of secretory expression systems in those approaches cannot ensure the efficient secretion of recombinant proteins in high cell density cultivation, which is the industrially relevant production condition. During high cell density cultivation, cells are under highly stressful conditions and can exhibit different characteristics compared to relatively mild flask cultivation conditions (Shojaosadati et al., 2008). Therefore, an extensive secretome analysis in *C. glutamicum* under high cell density cultivation (higher than 100 of $OD_{600\,nm}$ value in general) is necessary to develop a new potential platform for the secretory production of recombinant proteins.

In this study, we sought to isolate a new potential signal peptide based on secretome analysis of *C. glutamicum* during fed-batch cultivation in order to develop an efficient secretory production system in *C. glutamicum*. From secretome analysis, one major protein, Cg1514, was isolated and its signal peptide was characterized. The potential of the isolated signal peptide toward secretory production of recombinant proteins in *C. glutamicum* was successfully demonstrated with three recombinant proteins: endoxylanase (XynA), α-amylase (AmyA), and camelid antibody fragment (VHH).

Materials and Methods

Bacterial Strain and Plasmid Manipulation

The bacterial strains and plasmids used in this study are listed in Table I. *E. coli* XL1-Blue was used as a host for gene cloning and plasmid maintenance, and *C. glutamicum* ATCC 13032 was used as a main host for protein production. A polymerase chain reaction (PCR) was performed using the C1000^{TM} Thermal Cycler (Bio-Rad, Hercules, CA) with PrimeSTAR HS polymerase (Takara Bio Inc., Shiga, Japan). The nucleotide sequences of all primers used in this study are listed in Supplementary Table S1. For easy construction of secretory expression systems, multiple cloning site (MCS) of pCES208 (Park et al., 2008) was slightly modified by adding two different *Sfi*I restriction enzyme sites between *Xba*I and *Not*I restriction enzyme sites. The new MCS was amplified by PCR with two primers, MCS-F and MCS-R. After digestion with *Kpn*I and *Not*I, the PCR product was cloned into the same restriction enzyme sites as pCES208 to yield pCES-NMCS. The *rrnB*T1T2 terminator sequence (350 bp) was amplified from pTrc99a by PCR with the primers, rrnBTer-F and rrnBTer-R, and after digestion with *Not*I, the PCR product was cloned into pCES-NMCS to yield pCES-PLPV.

The whole *cg1514* gene with its own promoter was amplified from *C. glutamicum* ATCC 13032 chromosome by PCR with two primers, Cg1514-F and Cg1514-F. After digestion with *Kpn*I and *Not*I, the PCR product was cloned into the pCES-PLPV to yield pCg1514. In this construct, a 6 × His tag (HHHHHH) was fused to the C-terminus of Cg1514 for purification and further analysis of the protein. For secretory production, four expression vectors, pCG-G, pCG-S, pCG-H36T, and pCG-H36A were constructed as depicted in Figure 1. To check the feasibility of whole Cg1514 protein for secretory production of recombinant proteins, the plasmid pCG-G was constructed. The whole *cg1514* gene with its own promoter (P_{cg1514}) was amplified from pCg1514 by PCR with two primers, Cg1514-F and Cg1514-R2. After digestion with *Kpn*I and *Xba*I the PCR product was cloned into the pCES-PLPV to yield pCG-G. The pCG-S vector was then constructed to use only the signal sequence of *cg1514* under its own promoter. From the pCg1514 template, the signal sequence of *cg1514* with its own promoter sequence was amplified by PCR with the primers, Cg1514-F and Cg1514ss-R. After digestion with *Kpn*I and *Xba*I, the PCR product was cloned into the pCES-PLPV to yield pCG-S. To introduce the H36 synthetic promoter (P_{H36}) which was previously isolated from the synthetic promoter library (Yim et al., 2013), P_{H36} sequence was extended in front of the *cg1514* signal sequence by PCR with primers: H36-F1, H36-cg1514-F2, and Cg1514ss-R from pCg1514. The PCR product was digested with *Kpn*I and *Xba*I and cloned into the pCES-PLPV to yield pCG-H36T. The original *cg1514* gene has the TTG codon as a start codon rather than the conventional ATG codon, so the plasmids pCg1514, pCG-G, pCG-S, and pCG-H36T also have TTG start codons. However, the TTG start codon is known to be non-optimal for leaderless translation of mRNA lacking 5′-UTR (untranslated region), which is the form of mRNA transcript from the synthetic H36 synthetic promoter (Moll et al., 2002; Yim et al., 2013). For pCG-H36A, the native TTG start codon of *cg1514* in pCG-H36T was replaced with the ATG codon for better combination of the *cg1514* signal sequence and the strong synthetic promoter P_{H36} by PCR using pCG-H36T as template and the primer pairs, H36-F1, H36-cg1514ATG-F2, and Cg1514ss-R. The PCR fragment was digested by *Kpn*I and *Xba*I and cloned into the pCES-PLPV to yield pCG-H36A.

To check the secretory pathway of Cg1514-based secretion, the GFP coding gene was amplified from pCES-H36-GFP (Yim et al., 2013) by PCR with the primers, aaGFP-F and GFP-R. After digestion

Table I. 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 lacIqZΔM15 Tn10 (Tet^r)]*	Stratagene[a]
C. glutamicum	Wild type	ATCC 13032
Plasmids		
pCES208	*E. coli - C. glutamicum* shuttle vector, Km^r	Park et al. [2008]
pET22b	5.5 kb, P_{T7}, β-lactamase gene (Ap^r)	Novagen[b]
pTrc99a	4.2 kb, P_{trc}, β-lactamase gene (Ap^r)	Pharmacia[c]
pCES-H36-GFP	6.7 kb, pCES208 derivative; P_{H36}, GFP	Yim et al. [2013]
pASJ104	8.1 kb, pCES208 derivative; P_{porB}, Signal sequence of *porB*, *xynA* from *S. coelicolor* A3(2)	An et al. [2013]
pCES-H36-XynA	7.9 kb, pCES208 derivative; P_{H36}, Signal sequence of *porB*, *xynA* from *S. coelicolor* A3(2)	Yim et al. [2013]
pUC57-cAbHuL22	3.1 kb, pUC57 derivative, cAbHuL22 VHH (15)	This study
pCES-NMCS	5.9 kb, pCES208 derivative	This study
pCES-PLPV	6.2 kb, pCES208 derivative	This study
pCg1514	7.2 kb, pCES208 derivative; P_{cg1514}, *cg1514*	This study
pCG-G	7.2 kb, pCES208 derivative; P_{cg1514}, *cg1514*	This study
pCG-S	6.5 kb, pCES208 derivative; P_{cg1514}, Signal sequence of *cg1514*	This study
pCG-H36T	6.3 kb, pCES208 derivative; P_{H36}, Signal sequence of *cg1514*	This study
pCG-H36A	6.3 kb, pCES208 derivative; P_{H36}, Signal sequence of *cg1514* (Start codon is changed to ATG)	This study
pCG-H36A-GFP	7.0 kb, pCG-H36A derivative; GFP	This study
pCG-G-XynA	8.5 kb, pCG-G derivative; *xynA* from *S. coelicolor* A3(2)	This study
pCG-S-XynA	7.8 kb, pCG-S derivative; *xynA* from *S. coelicolor* A3(2)	This study
pCG-H36T-XynA	7.6 kb, pCG-H36T derivative; *xynA* from *S. coelicolor* A3(2)	This study
pCG-H36A-XynA	7.6 kb, pCG-H36A derivative; *xynA* from *S. coelicolor* A3(2)	This study
pCG-S-AmyA	8.7 kb, pCG-S derivative; *amyA* from *S. bovis* KCTC 3959	This study
pCG-S-cAb	6.9 kb, pCG-S derivative; cAbHuL22 VHH	This study

[a]Stratagene (La Jolla, CA).
[b]Novagen (Darmstadt, Germany).
[c]Pharmacia Biotech (Uppsala, Sweden).

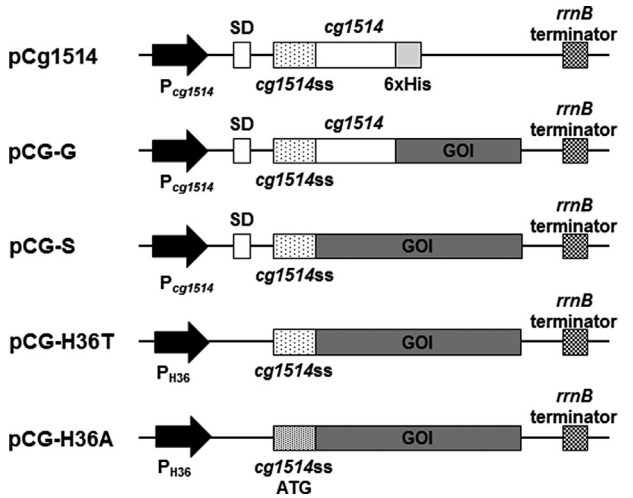

Figure 1. Schematic diagram of plasmids constructed for secretory expression system based on Cg1514. P_{cg1514} and P_{H36} indicate *cg1514* own promoter and H36 synthetic promoter, respectively. The *cg1514*ss indicates signal sequence of *cg1514*, and the *cg1514*ss ATG indicate *cg1514* signal sequence of which start codon (TTG) was changed to ATG. GOI is the abbreviation for gene of interest. In pCG-H36T and pCG-H36A, H36 promoter produces leaderless mRNA transcript, and so SD (Shine-Dalgarno) sequences were not included.

with *Sfi*I, the PCR product was cloned into pCG-H36A to yield pCG-H36A-GFP. For secretory production of endoxylanase (XynA) from *Streptomyces coelicolor* A3(2), the *xynA* gene was amplified from pCES-H36-XynA (Yim et al., 2013) by PCR with two primers, aaXynA-F and XynA-R. After digestion with *Sfi*I, the PCR product was cloned into the pCG-G, pCG-S, pCG-H36T, and pCG-H36A to yield pCG-G-XynA, pCG-S-XynA, pCG-H36T-XynA, and pCG-H36A-XynA, respectively. For the secretory production of α-amylase, the *amyA* gene was amplified from chromosomal DNA of *Streptococcus bovis* (KCTC 3659) by PCR with two primers: aaAmyA-F and aaAmyA-R. After digestion with *SfiI*, the PCR product was cloned into the pCG-S to yield pCG-S-AmyA. For secretory production of a cAbHuL22 single domain fragment (VHH) of a camelid heavy-chain antibody against human lysozyme (Chan et al., 2008), the coding gene was synthesized by GenScript Co. (Piscataway, NJ). The VHH gene was amplified from the synthesized gene by PCR with two primers: aacAbHuL22-F and cAbHuL22-R. After digestion with *Xba*I and *Not*I, the PCR product was cloned into the pCG-S to yield pCG-S-cAb.

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, 10 g/L) at 37°C. For production of recombinant proteins, *C. glutamicum* was cultivated in 100 mL flasks containing 20 mL of brain heart infusion (BHI; Difco Laboratories, Detroit, MI) medium at 30°C for 48 h at

200 rpm. In all cultivations, kanamycin (Km, 25 mg/L) was added to the culture medium as the sole antibiotic.

Fed-Batch Cultivation

As a seed culture, cells were inoculated into 200 mL of semi-defined medium containing 20 g/L of glucose in a 1 L baffled flask and cultivated at 30°C for 24 h with shaking at 200 rpm. The semi-defined medium consists of 3 g of K_2HPO_4, 1 g of KH_2PO_4, 2 g of urea, 10 g of $(NH_4)_2SO_4$, 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 $ZnSO_4$, 10 mg of $CaCl_2$, 2 g of yeast extract, and 7 g of casamino acid per liter with 25 mg/L of Km. The seed culture (200 mL) was inoculated into 2 L of fresh 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 when the pH dropped lower than the set point by 7. During the cultivation, glucose concentrations in the culture medium were monitored by a glucose analyzer (YSI 2700 SELECT™ Biochemistry Analyzer; YSI Life Science, Yellow Springs, OH). 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.

Protein Preparation and Activity Analysis

After cell cultivation in shaking 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 at 13,000 rpm for 30 min at 4°C, and the pellets were resuspended in phosphate-buffered saline (PBS, 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2PO_4, 1.4 mM KH_2PO_4, pH 7.2). Protein samples were stored at −20°C until further analysis. The activity of endoxylanase and α-amylase was assayed by the 3,5-dinitrosalicylic acid (DNS) method as described previously (Jeong et al., 1998). The activity of cAbHuL22 VHH was assayed by ELISA as described previously, but using 20 μM of its cognate antigen (human lysozyme) and 100 μL of culture supernatant as secreted VHH samples (Jeong and Rani, 2011). The signals were quantified by measuring the absorbance at 450 nm with the TECAN infinite M200 Pro (Tecan Group Ltd, Männedorf, Switzerland). One unit of activity was defined as the amount of enzyme required to release 1 μmol of xylose from xylan or 1 μmol of glucose from starch, respectively.

Protein Purification

After fed-batch cultivation, culture supernatant was prepared by centrifugation at 13,000 rpm 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 10 mL of filtrated samples were dialyzed against an equilibration buffer (50 mM Na_3PO_4, 300 mM NaCl) and the dialyzed sample was poured into Poly-prep® chromatography columns (Bio-Rad) filled with Talon metal affinity resin (Clontech, Mountain View, CA). The resin was washed twice with 10 mL of washing buffer (50 mM Na_3PO_4, 300 mM NaCl, 15 mM imidazole) and the 6 × His tag fused target proteins were eluted using 2 mL of the elution buffer (50 mM Na_3PO_4, 300 mM NaCl, 150 mM imidazole).

Two Dimensional Gel Electrophoresis (2D-GE) and MALDI-TOF

2D-GE was performed with IPGphor IEF system (GE Healthcare Bio-Science AB, Uppsala, Sweden) and Protean II xi Cell (Bio-Rad) as previously described (Han et al., 2005). After gel electrophoresis, protein spots were visualized using a silver staining kit (GE Healthcare) and the stained gels were scanned using a UMAX PowerLook 2100XL Scanner (UMAX Technologies, Taipei, Taiwan). Among the developed protein spots, the largest spot was excised from the gel and subjected to in-gel trypsin digestion (Shevchenko et al., 2006). After desalting using ZipTip C18 column (Pierce, Rockford, IL), the sample was analyzed by MALDI-TOF for the peptide finger printing method to identify the protein in EMASS Co. (Seoul, South Korea).

N-Terminal Sequencing of Protein

For N-terminal amino acid sequencing, the protein samples were transferred from SDS–PAGE gel to PVDF membrane and the transferred protein band on the membrane was analyzed using the Procise protein sequencing system model 492cLC (Applied biosystems, Foster City, CA) in Korea Basic Science Institute (KBSI, Korea).

Results

Identification of the Major Secreted Proteins by *C. glutamicum* in Fed-Batch Conditions

Recently, we reported the secretory production of the antibody fragment M18 scFv against anthrax toxin PA in *C. glutamicum* (Yim et al., 2014). In that work, a porin B signal peptide of *C. glutamicum* was used and M18 scFv as high as 68 mg/L was successfully produced in culture medium during the fed-batch cultivation. Interestingly, when we performed SDS–PAGE for the analysis of protein secretion into the culture medium, a very thick protein band of approximately 27 kDa molecular weight was detected, but it was not the band of the target protein M18 scFv (Supplementary, Fig. S1). This indicated that an endogenous protein of *C. glutamicum* might be secreted into culture medium more frequently than M18 scFv during fed-batch cultivation. This endogenous protein was not expressed from the multi-copy plasmid but from single-copy chromosomal DNA, and the high content of that protein in culture medium strongly indicates that its promoter and signal peptide can be a useful platform for enhanced secretion

of proteins in *C. glutamicum* in high cell density cultivation. We therefore sought to identify that major protein and use its promoter and signal peptide for development of an efficient secretory production system in high cell density cultivation conditions. The sample of extracellular proteins from the previous high cell density cultivation for M18 scFv production (Yim et al., 2014) was analyzed by two dimensional gel electrophoresis (2D-GE) for clear separation of the major protein from other proteins (Fig. 2A). For isoelectric focusing, the IPG stripe with a pH range of 3 to 10 was chosen so that no major proteins would be missed, although it was reported that extracellular proteins mostly possess a more acidic pI (Hansmeier et al., 2006). The major protein was then excised from the gel, digested with trypsin and analyzed by MALDI-TOF for peptide finger printing. These results were analyzed against the whole genome database of *C. glutamicum* ATCC 13032, and the overexpressed protein was found to be a hypothetical protein encoded by *cg1514*.

Characterization of the Cg1514 Signal Peptide

The Cg1514 protein consists of 250 amino acids (27 kDa) and its signal peptide was predicted by the Signal P server (Petersen et al., 2011). The first 32 amino acids were identified as a signal peptide, in which the last three amino acids (Ala-Gln-Ser) were well matched with the consensus motif (Ala-X-Ala or Ser) for signal peptide cleavage sites (Tuteja, 2005) (Fig. 2B). To confirm the production of Cg1514 and its secretion into culture medium, the whole gene encoding Cg1514 with its own promoter (P_{cg1514}) was cloned into pCES208 vector. After flask cultivation of *C. glutamicum* harboring pCg1514 (Fig. 1), the proteins in culture medium were analyzed by SDS–PAGE. As shown in Fig. 2C, one major protein was detected in the culture supernatant that highly matched the molecular weight of Cg1514 with a 6 × His tag (∼28 kDa). The protein was successfully purified (Fig. 2C) and subjected to N-terminal amino acid sequencing in order to identify the cleavage site of the signal peptide. The first five amino acids of the purified Cg1514 was verified to be LEQIT, which was identical to the first 5 amino acid sequences of mature Cg1514 protein predicted by the Signal P server (Fig. 2B). We therefore conclude that the first 32 aa-length peptide is the signal peptide of Cg1514.

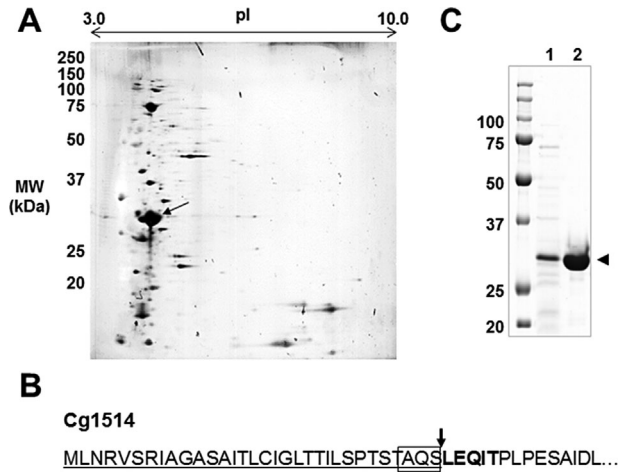

Figure 2. Identification of Cg1514 and verification of its signal peptide sequence. (**A**) 2-D map of the extracellular proteome (secretome) of *C. glutamicum* cultivated in the high cell density condition. Proteins were separated in a pH gradient from 3 to 10 and in the 12% SDS–PAGE that were silver-stained. The circle indicates the major spot (Cg1514) which was excised for MALDI-TOF analysis. (**B**) N-terminal amino acid sequences of Cg1514. Bold sequences (LEQIT) are N-terminal amino acid sequences determined by protein sequencer. Underlined 32 amino acid sequences were determined as signal peptide sequence. Arrow indicates a cleaved position. (**C**) SDS–PAGE analysis for expression and purification of Cg1514. Lane 1, extracellular proteins from *C. glutamicum* harboring pCg1514; Lane 2, purified Cg1514 protein. Arrow head indicates Cg1514.

C. glutamicum has two major secretory pathways as follows: (i) The Sec-dependent pathway used for secretion of unfolded proteins and (ii) The TAT (Twin-Arginine-Translocation)-dependent pathway used for secretion of folded proteins. GFP, which can be easily folded in the cytoplasm, is secreted via the TAT-dependent pathway not the Sec-dependent pathway therefore it has been widely used as a protein marker for the TAT-dependent pathway (Meissner et al., 2007). To determine the secretory pathway of the isolated Cg1514 signal peptide, the GFP coding gene was cloned into the pCG-H36A system, and the secretory production of GFP was examined. After flask cultivation of *C. glutamicum* harboring pCG-H36A-GFP, the protein fractions including cell lysates and culture supernatant were analyzed by SDS–PAGE (Fig. 3A). Most of the GFP was detected in the cell lysates fraction but was not detected in the culture supernatant. Fluorescence intensity analysis showed that fluorescence signal could only be observed in the cell fraction, not in culture supernatant (Fig. 3B). These results therefore indicate that the signal peptide of Cg1514 mediates the secretory production of target proteins via the Sec-dependent pathway. Taken together, we conclude that the Cg1514 protein is the major secretory protein of *C. glutamicum* in high cell density cultivation conditions and its 32 amino acid-length signal peptide can mediate the secretory production of recombinant proteins via the Sec-dependent pathway.

Secretory Production of Endoxylanase From *Streptomyces coelicolor* A3(2)

An *S. coelicolor* A3 endoxylanase (XynA), which plays a key role in xylan degradation was examined for secretory production in *C. glutamicum*. The XynA coding gene was cloned into a series of expression vectors (pCG-G, pCG-S, pCG-H36T, pCG-H36A) and the secretory production of XynA in each expression vector was examined in flask cultivation. In this experiment, two clones harboring pASJ104 (An et al., 2013) or pCES-H36-XynA (Yim et al., 2013) were used as controls, in which the signal peptide of *C. glutamicum*, Porin B, was used for secretory production of XynA under two different promoters, P_{porB} and P_{H36}, respectively. When the Cg1514 signal peptide was used (pCG-S-XynA and pCG-H36A-XynA), it was clearly observed that XynA (48 kDa) was a major secretory protein in culture supernatant and the production yields in both vectors were higher than those in the Porin B signal peptide (pASJ104 and pCES-H36-XynA) controls (Fig. 4A). It was also observed that the use of the *cg1514* promoter (pCG-S-XynA) allowed slightly higher production compared with the P_{H36}

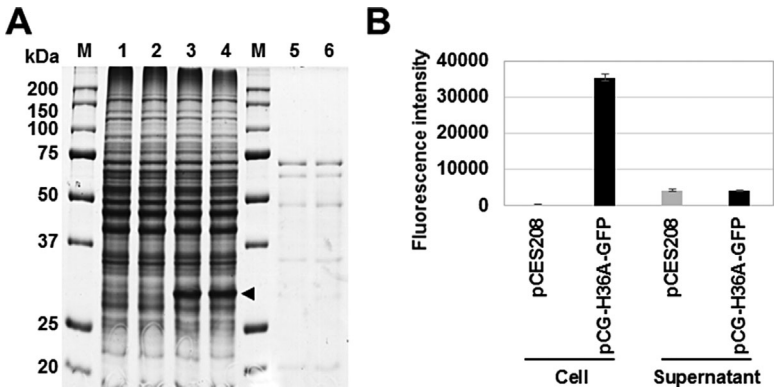

Figure 3. Determination of secretory pathway mediated by Cg1514 signal peptide. (**A**) SDS–PAGE analysis. Lanes 1 and 2, total lysate and soluble protein fraction of *C. glutamicum* harboring pCES208, respectively; Lanes 3 and 4, total lysate and soluble protein fraction of *C. glutamicum* harboring pCG-H36A-GFP, respectively; Lanes 5 and 6, 30 × concentrated culture supernatant of *C. glutamicum* harboring pCES208 or pCG-H36A-GFP, respectively. (**B**) Fluorescence intensity analysis of *C. glutamicum* harboring pCES208 or pCG-H36A-GFP.

promoter (pCG-H36A-XynA). However, when the whole Cg1514 protein, including its signal peptide, was used as a fusion partner for endoxylanase secretion (pCG-G-XynA), there was extremely low-level secretion of the fused protein (75 kDa) into extracellular medium. As expected, when the original start codon (TTG) of the Cg1514 signal peptide was used under the P_{H36} promoter (pCG-H36T-XynA), protein bands could not be detected in SDS–PAGE analysis. On the other hand, using pCG-H36A-XynA, in which the original start codon (TTG) was replaced with the ATG codon, resulted in much higher production of XynA, indicating that the original TTG start codon is not preferable for the P_{H36} promoter. The activity of XynA in the culture supernatant from each expression vector was also evaluated by enzyme assay, and the results were similar to those observed in the SDS–PAGE analysis (Fig. 4B). To demonstrate the ability of Cg1514 signal peptide for secretion of protein via Sec-dependent pathway, we examined two other well-known signal peptides: CspB for Sec-dependent pathway and TorA for Tat-dependent pathway for the secretory production of endoxylanase. After flask cultivation, the protein fractions in culture medium were analyzed by SDS–PAGE, and it was clearly observed that the use of Cg1514 signal peptide resulted in the higher secretion of endoxylanase than other signal peptides (Supplementary, Fig. S2). As expected, the use of TorA signal peptide for TAT pathway failed to produce endoxylanase into the culture medium. The potential of *cg1514* promoter and signal peptide for secretory production, was further validated by comparing the production yields under two other well-known promoters (P_{Tuf} and P_{Sod}). The use of P_{cg1514} and Cg1514 signal peptide also exhibited the highest production yield of endoxylanase into culture medium (Supplementary, Fig. S3).

Next, to validate the usability of the Cg1514-based secretion system in large-scale production, we tested fed-batch cultivation with *C. glutamicum* harboring pCG-S-XynA, which exhibited the most efficient secretion of XynA among the examined expression vectors in flask cultivations. *C. glutamicum* harboring pCG-S-XynA continued to grow up to an OD_{600} of 136.4 for 28 h after which cell density slightly decreased (Fig. 5A). In this cultivation, cell specific growth rate in the exponential growth phase was 0.229 h^{-1}. During the cultivation, culture supernatant samples were periodically collected and secretory productions of XynA were analyzed by SDS–PAGE. The XynA band first appeared at 8 h then its concentration gradually increased (Fig. 5B). The maximum yield of XynA in culture medium (1.07 g/L) was obtained at the end of cultivation (32 h) and the final productivity was 33 mg × L^{-1} × h^{-1}. As shown in Fig. 5B, the XynA was produced as one major band in culture medium during the entire cultivation (>76% of extracellular proteins). The results of the fed-batch cultivations are summarized in Table II. Taken together, these results suggest that the pCG-S expression vector containing the signal peptide of Cg1514 with its own promoter (P_{cg1514}) can be a potential basis for the secretory production of recombinant proteins and its usability was further evaluated with other protein models.

Secretory Production of α-Amylase and Single-Domain Fragment (VHH)

To demonstrate the feasibility of the Cg1514-based system for the secretion of proteins with a broader range of molecular weights, we examined two more proteins: (i) α-amylase (AmyA) from *S. bovis* KCTC 3959 (78 kDa), which plays key roles in starch degradation (Tateno et al., 2007) and (ii) a single-domain fragment (VHH), cAbHuL22 (15 kDa), of a camelid heavy-chain antibody specific for the active site of human lysozyme (Chan et al., 2008). After cloning each gene into pCG-S, which showed the highest yield for XynA production among the four expression vectors, flask cultivations were carried out to check the secretory production of functional AmyA and cAbHuL22 into culture supernatant. In flask cultivations, both proteins were successfully produced and it was also clearly confirmed that both proteins have correct activities (Supplementary, Figs. S4 and S5).

Then, fed-batch cultivations were carried out and the secretory production yields were analyzed. In the case of AmyA, *C. glutamicum* harboring pCG-S-AmyA continued to grow up to an OD_{600} of 163.2 for 28 h after which cell density decreased (Fig. 6A). The cell specific growth rate in the

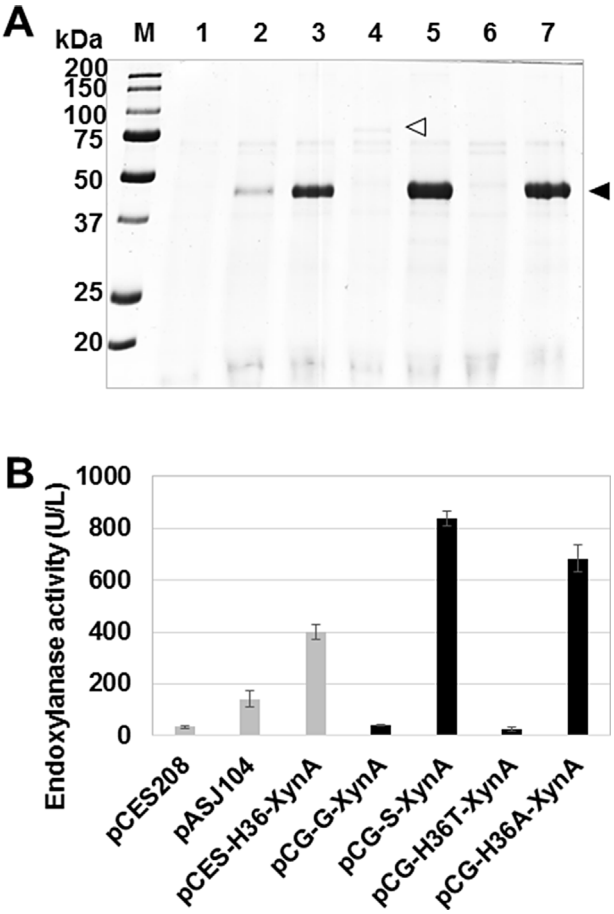

Figure 4. Secretory production of endoxylanase (XynA) in the flask cultivation. (**A**) SDS–PAGE analysis of culture supernatant. Lane 1, pCES208; Lane 2, pASJ104; Lane 3, pCES-H36-XynA; Lane 4, pCG-G-XynA; Lane 5, pCG-S-XynA; Lane 6, pCG-H36T-XynA; Lane 7, pCG-H36A-XynA. Same volume (10 μL) of five times concentrated culture supernatant was loaded on each lane. Open and closed arrowheads indicate whole Cg1514 protein-fused XynA and mature XynA, respectively. (**B**) Endoxylanase activity assay of the culture supernatant. *C. glutamicum* harboring pCES208 (●), pASJ104 (▲), pCES-H36-XynA (■), pCG-G-XynA (○), pCG-S-XynA (△), pCG-H36T-XynA (□), pCG-H36A-XynA (◇). One unit of activity was defined as the amount of enzyme required to release 1 μmol of xylose from xylan.

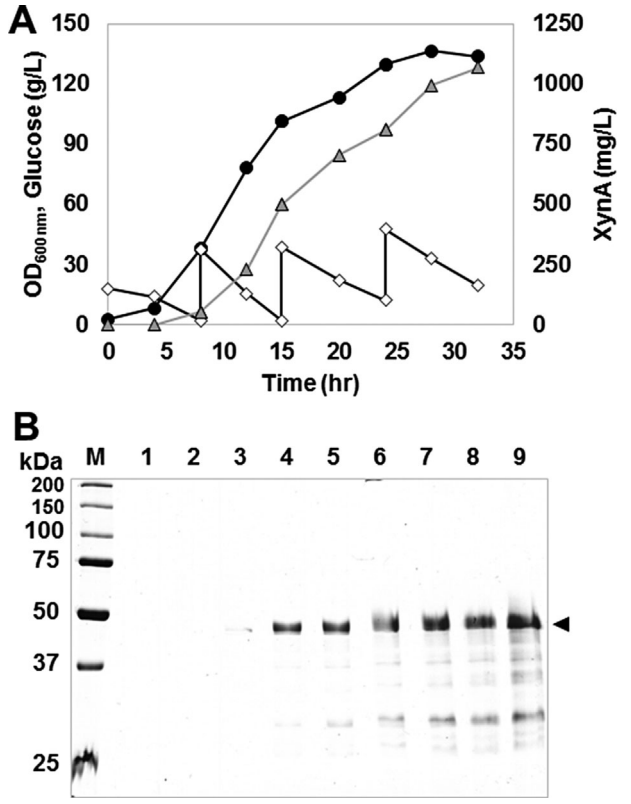

Figure 5. Fed-batch cultivation of *C. glutamicum* harboring pCG-S-XynA for the secretory production of endoxylanase (XynA). (**A**) Time profiles of cell growth (●), glucose concentration (◇), and XynA concentration (▲) in the culture supernatant. (**B**) SDS–PAGE analysis of the culture supernatant. Lanes 1 to 9, samples at 0, 4, 8, 12, 15, 20, 24, 28, and 32 h, respectively. At each time point, 8 μL of culture supernatant (without concentration) was loaded onto each lane. The arrowhead indicates XynA (~48 kDa).

exponential growth phase was 0.288 h^{-1}. The AmyA band first appeared at 8 h then its concentration gradually increased (Fig. 6B). The maximum yield of AmyA in culture medium (782.6 mg/L) was obtained at 21 h and the final productivity was 37 mg $\times$ L^{-1} $\times$ h^{-1} (Table II). Like as XynA, AmyA was also produced in the culture medium as one major protein with high purity (>90% of extracellular proteins) (Fig. 6B).

In the fed-batch cultivation of *C. glutamicum* producing cAbHuL22 (15 kDa), *C. glutamicum* harboring pCG-S-cAb grew up to an OD_{600} of 152 for 27 h with specific a growth rate of 0.194 h^{-1} in the exponential growth phase and then decreased (Fig. 7A). The cAbHuL22 VHH was first produced at 9 h then its concentration significantly increased up to 1.57 g/L at 30 h (Fig. 7B). The final productivity of cAbHuL22 VHH was 52 mg $\times$ L^{-1} $\times$ h^{-1} (Table II). As shown in Fig. 7B, cAbHuL22 was produced in the culture medium as a major protein with high purity (>82% of extracellular proteins).

Purification of the Secreted Proteins From Culture Supernatant

From all fed-batch cultivations, the proteins (XynA, AmyA, and, cAbHuL22 VHH) secreted into culture medium were purified as described in the materials and methods section. In the cases of XynA and AmyA, both proteins were successfully purified with high purity (>98%) and high recovery yields (>20%) by one-step column chromatography (Fig. 8). cAbHuL22 VHH was also

Table II. Summary of results from fed-batch fermentations and purification.

Protein	Specific cell growth rate (h^{-1})	Max. cell density (OD_{600})	Max. production yield (mg/L)	Productivity (mg $\times$ L^{-1} $\times$ h^{-1})	Purification yield (%)
Endoxylanase	0.229	136.4	1067	33	28.1
α-amylase	0.288	163.2	782.6	37	24.4
cAbHuL22 VHH	0.194	152	1,568.1	52	2.4

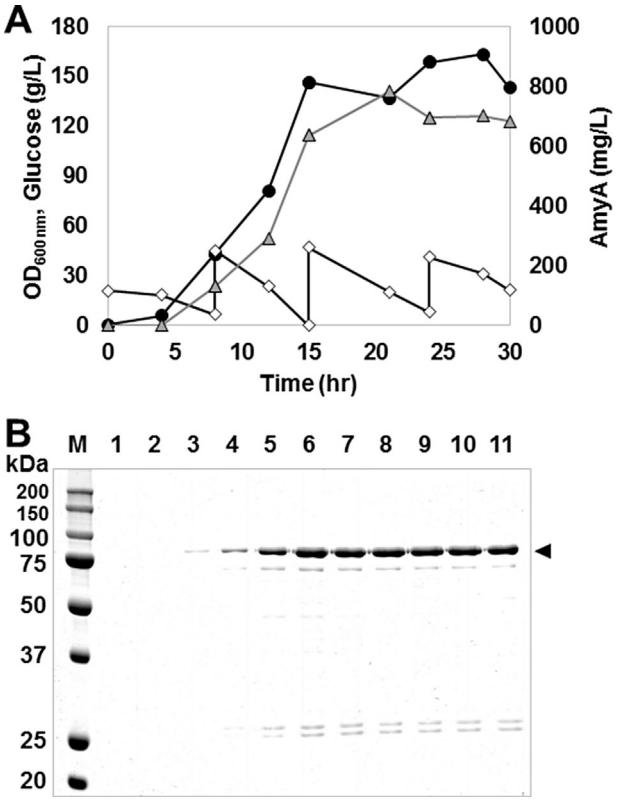

Figure 6. Fed-batch cultivation of *C. glutamicum* harboring pCG-S-AmyA for the secretory production of α-amylase (AmyA). (**A**) Time profiles of cell growth (●), glucose concentration (◇), and AmyA concentration (▲) in the culture supernatant. (**B**) SDS–PAGE analysis of culture supernatant during fed-batch cultivation. Lanes 1 to 11, samples at 0, 4, 8, 12, 15, 21, 24, 28, 30, 33, and 35 h, respectively. At each time point, 2 μL of culture supernatant (without concentration) was loaded onto each lane. The arrowhead indicates AmyA (~78 kDa).

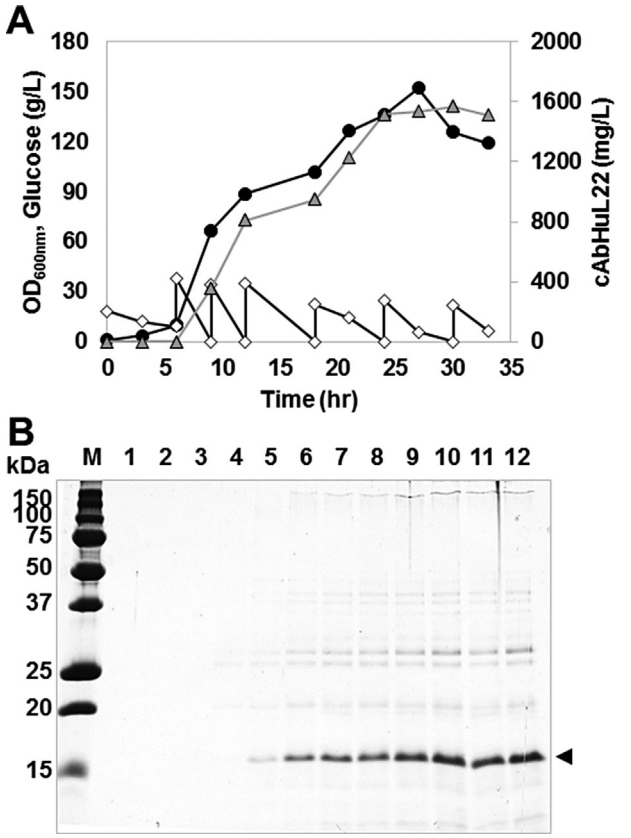

Figure 7. Fed-batch cultivation of *C. glutamicum* harboring pCG-S-cAb for the secretory production of cAbHuL22 VHH. (**A**) Time profiles of cell growth (●), glucose concentration (◇), and cAbHuL22 VHH concentration (▲) in the culture supernatant. (**B**) SDS–PAGE analysis of culture supernatant during fed-batch cultivation. Lanes 1 to 8, samples at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, and 33 h, respectively. At each time point, 0.8 μL of culture supernatant (without concentration) was loaded onto each lane. The arrowhead indicates cAbHuLL22 (~15 kDa).

successfully purified with high purity (Fig. 8) although its recovery yield was relatively low (2.4%).

Discussion

C. glutamicum has ideal features that facilitate efficient protein secretion and has been widely used for production of various recombinant proteins. Efforts to improve the protein secretion ability of *C. glutamicum* have previously focused on: over-expression of genes related to protein secretion channels (Kikuchi et al., 2009), isolation of factors for enhanced protein secretion (Watanabe et al., 2013), deletion of cell wall protein encoding genes (Matsuda et al., 2014), and optimization of culture conditions (Teramoto et al., 2011). However, considering the principal role of signal peptides in protein secretion, the isolation of a potential signal peptide can be a primary step towards building an efficient secretory expression system in *C. glutamicum*. For this purpose, a secretome analysis can be a useful method for discovering overexpressed proteins in extracellular medium and identifying the potential signal peptides of the secreted proteins. In the present work, we performed secretome analysis with culture supernatant from fed-batch cultivation and successfully isolated Cg1514 protein as a major protein. Previous studies have reported secretome analyses of *C. glutamicum* ATCC 13032 and *C. glutamicum* R, but those studies were performed with the samples of flask cultivations (Hansmeier et al., 2006; Suzuki et al., 2009). The secretome identified using fed-batch culture exhibited some similarity to the secretome identified using flask culture. A similar number of protein spots (approximately 90 spots) were found, with most of the proteins having an acidic pI in the 3.5–4.5 range, however a few proteins had acidic pIs in the 7–8 range (Fig. 2). In flask cultivation, Cg2052 was reported as a major protein (Hansmeier et al., 2006) and we also found that this protein was one of the major proteins (~24% of extracellular proteins) in the fed-batch cultivations (Supplementary, Fig. S1). Interestingly, Cg1514 was the most abundant protein (51% of extracellular proteins) in the fed-batch cultivation but was a minor protein detected as a small spot in the flask samples (Hansmeier et al., 2006). Previous studies indicated that Cg1514 is related to sulfur metabolism in *C. glutamicum* however the exact function of the protein is still

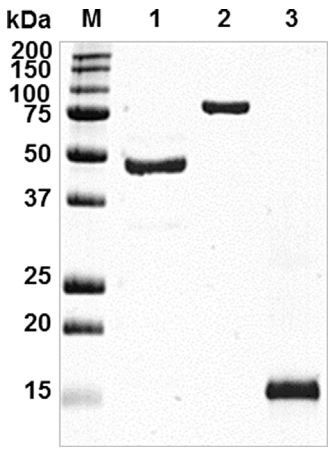

Figure 8. Purification of endoxylanase (XynA), α-amylase (AmyA), and cAbHuL22 VHH from fed-batch cultivation of *C. glutamicum* harboring pCG-S-XynA, pCG-S-AmyA, and pCG-S-cAb, respectively. Lane 1, purified XynA (48 kDa); Lane 2, purified AmyA (78 kDa); Lane 3, purified cAbHuL22 VHH (15 kDa).

unknown (Rückert et al., 2008). Expression of the *cg1514* gene has been shown to be up-regulated by CysR which is activated by O-acetyl-L-serine (OAS) or O-acetyl-L-homoserine (OAH) under sulfur limiting condition (Hoffmann et al., 2013; Rückert et al., 2008). In our work, however, there was no expectation for elevated cytosolic OAS or OAH concentrations during fed-batch cultivations because the medium used for cultivation, contained 10 g/L of ammonium sulfate and this concentration was high enough not to be limited during the cultivation. Also, in all fed-batch cultivations, protein production was highly associated with cell growth, indicating that the *cg1514* promoter mediated constitutive gene expression independent of CysR regulation. The reasons for the observed overproduction of Cg1514 in fed-batch cultivation is yet to be determined, and requires further investigation into the metabolic state of *C. glutamicum* in high cell density cultivation conditions.

The strong activity of the signal peptide and native promoter of the Cg1514 was verified by producing three recombinant proteins (endoxylanase, α-amylase, and camelid antibody fragment) into the extracellular medium in fed-batch cultivation. Each protein has different molecular weights from 15 kDa (VHH) to 78 kDa (α-amylase), yet all proteins were well secreted independent of their molecular weight. In addition, as shown in SDS–PAGE analysis, all examined proteins were produced as major proteins in culture medium with high purity (>80% of total proteins in culture medium), and the proteins could be easily purified by simple one-step purification (Fig. 8). To check the secretion efficiency, we also analyzed the cytoplasmic fractions, but we could not find any detectable amount of target proteins in cytoplasmic fractions (data not shown), which indicate the potential usability of Cg1514 signal peptides for the secretory production of recombinant proteins. Although the higher production yield was obtained with *cg1514* promoter than H36 promoter, it does not mean that *cg1514* promoter has higher strength in gene expression than P_{H36} promoter which was previously isolated as the strongest promoter (Yim et al., 2013). When we examined the cytoplasmic expression of GFP (without signal peptide) under P_{H36} and P_{cg1514} promoters, the use of H36 promoter resulted in much higher production of GFP in the cytoplasm of *C. glutamicum* (Supplementary, Fig. S6A and B). The quantitative analysis of mRNA transcript by qRT-PCR also clearly confirmed the higher transcription under the H36 synthetic promoter than *cg1514* promoter (Supplementary, Fig. S6C). These results indicate that H36 promoter is indeed strong promoter. We think that the incompatible results between the cytoplasmic and secretory production might be resulted from different translational efficiencies of mRNA transcripts synthesized under each promoter. The H36 synthetic promoter produces leaderless transcript which does not contain UTR and SD sequence, but mRNA transcript synthesized under *cg1514* promoter contains its own UTR and SD sequence which might be more preferable for translation of mRNA of Cg1514 signal peptide linked endoxylanase in *C. glutamicum*. In all fed-batch cultivations, cells showed quite good growth rate and high cell density could be achieved (Table II), which indicated that the secretory production of recombinant proteins did not give any deleterious effect on cell growth. Among the three protein models, the highest productivity (1.57 g/L) could be achieved with camelid antibody fragment (VHH). Previous studies using other signal peptides and different model proteins reported higher productivities than our results; however, those results were obtained with the assistance of additional factors. Teramoto et al. (2011) reported on the production of GFP using the Tat-type signal peptide CgR0949, demonstrating that GFP productivity was dramatically increased, as high as 1.36 g/L, upon addition of Ca^{2+} in the medium. More recently, the same group reported significantly improved GFP productivity (2.5 g/L) using SigB inactivation in the host (Watanabe et al., 2013). In the present work, there were no modifications to the cell or media and only the signal peptide was used for protein secretion, and to the best of our knowledge, this is the highest record for the secretory production of recombinant protein in *C. glutamicum* without further cellular engineering (Frenken et al., 2000; Tanha et al., 2002; Thomassen et al., 2002).

In conclusion, we successfully isolated a new signal peptide of Cg1514 based on secretome analysis of *C. glutamicum* in high cell density cultivation; using its own promoter and signal peptide, an efficient secretion system was developed. We confirmed that the Cg1514 signal peptide mediates secretory production via the Sec-dependent pathway, which could be another useful property, since many recombinant proteins derived from human and other higher organisms cannot be folded in the reduced cytoplasmic environment and therefore need Sec-type signal peptides for secretion in *C. glutamicum* (Marco, 2009; Yoon et al., 2010). The potential usefulness of our system in high cell density cultivations were also successfully demonstrated with three protein models with a broad range of molecular weights (15, 48, and 78 kDa). In large-scale cultivations, the purification of proteins is also important in the consideration of production cost. As shown here, all examined proteins were successfully produced in the culture medium with high purity, and all of them could be purified by

simple centrifugation and one-step column chromatography. We believe that this simple and efficient purification process can greatly contribute to decreasing production cost and Cg1514 secretion system can serve as a potential platform for protein production in bio-industry.

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Supporting Information

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