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

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text:

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.


KEYWORDS: Corynebacterium glutamicum; 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 processes 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
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 secreteme 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 (Shojosadati et al., 2008). Therefore, an extensive secreteme analysis in C. glutamicum under high cell density cultivation (higher than 100 of OD_{600nm} 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 secreteme analysis of C. glutamicum during fed-batch cultivation in order to develop an efficient secretory production system in C. glutamicum. From secreteme 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: endoxylnase (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 C1000TM 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 restriction enzyme sites between Xbal and NorI restriction enzyme sites. The new MCS was amplified by PCR with two primers, MCS-F and MCS-R. After digestion with KpnI and NorI, the PCR product was cloned into the same restriction enzyme sites as pCES208 to yield pCES-NMCS. The rrnBT1T2 terminator sequence (350 bp) was amplified from pTc95a by PCR with the primers, rrnBTer-F and rrnBTer-R, and after digestion with NorI, 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 KpnI and NorI, the PCR product was cloned into the pCES-PLPV to yield pCg1514. In this construct, a 6 × His tag (HHHHHHH) 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 KpnI and Xbal, 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 Cg1514-4S-R. After digestion with KpnI and Xbal, 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-F2, and Cg1514-F2 from pCg1514. The original signal sequence and the strong promoter sequence (P_{H36}) was 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 Cg1514-4S-R. The PCR product was digested by KpnI and Xbal 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 aaGFP-R. After digestion
with SfiI, 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 SfiI, the PCR product was cloned into 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 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 XbaI and NotI, the PCR product was cloned into 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

### Table 1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>XL1-Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (TeR)]</td>
<td>Stratagene&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>C. glutamicum</em></td>
<td>Wild type</td>
<td>ATCC 13032</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCES208</td>
<td><em>E. coli</em> - <em>C. glutamicum</em> shuttle vector, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Park et al. [2008]</td>
</tr>
<tr>
<td>pET22b</td>
<td>5.5 kb, P&lt;sub&gt;TE&lt;/sub&gt;, B-lactamase gene (Ap&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Novagen&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>pTrc99a</td>
<td>4.2 kb, P&lt;sub&gt;TR&lt;/sub&gt;, B-lactamase gene (Ap&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Pharmacia&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>pCES-H36-GFP</td>
<td>6.7 kb, pCES208 derivative; P&lt;sub&gt;H36&lt;/sub&gt;, GFP</td>
<td>Yim et al. [2013]</td>
</tr>
<tr>
<td>pASJ104</td>
<td>8.1 kb, pCES208 derivative; P&lt;sub&gt;proB&lt;/sub&gt;, Signal sequence of porB, xynA from <em>S. coelicolor</em> A3(2)</td>
<td>An et al. [2013]</td>
</tr>
<tr>
<td>pCES-H36-XynA</td>
<td>7.9 kb, pCES208 derivative; P&lt;sub&gt;H36&lt;/sub&gt;, Signal sequence of porB, xynA from <em>S. coelicolor</em> A3(2)</td>
<td>Yim et al. [2013]</td>
</tr>
<tr>
<td>pUC57-cAbHuL22</td>
<td>3.1 kb, pUC57 derivative, cAbHuL22 VHH (15)</td>
<td>This study</td>
</tr>
<tr>
<td>pCES-NMCS</td>
<td>5.9 kb, pCES208 derivative</td>
<td>This study</td>
</tr>
<tr>
<td>pCES-PLPV</td>
<td>6.2 kb, pCES208 derivative</td>
<td>This study</td>
</tr>
<tr>
<td>pCg1514</td>
<td>7.2 kb, pCES208 derivative; P&lt;sub&gt;cg1514&lt;/sub&gt; cG1514</td>
<td>This study</td>
</tr>
<tr>
<td>pCG-G</td>
<td>7.2 kb, pCES208 derivative; P&lt;sub&gt;cg1514&lt;/sub&gt; cG1514</td>
<td>This study</td>
</tr>
<tr>
<td>pCG-S</td>
<td>6.5 kb, pCES208 derivative; P&lt;sub&gt;cg1514&lt;/sub&gt; Signal sequence of cg1514</td>
<td>This study</td>
</tr>
<tr>
<td>pCG-H36T</td>
<td>6.3 kb, pCES208 derivative; P&lt;sub&gt;H36&lt;/sub&gt;, Signal sequence of cg1514</td>
<td>This study</td>
</tr>
<tr>
<td>pCG-H36A</td>
<td>6.3 kb, pCES208 derivative; P&lt;sub&gt;H36&lt;/sub&gt;, Signal sequence of cg1514 (Start codon is changed to ATG)</td>
<td>This study</td>
</tr>
<tr>
<td>pCG-H36A-GFP</td>
<td>7.0 kb, pCG-H36A derivative; GFP</td>
<td>This study</td>
</tr>
<tr>
<td>pCG-G-XynA</td>
<td>8.5 kb, pCG-G derivative; xynA from <em>S. coelicolor</em> A3(2)</td>
<td>This study</td>
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<td>pCG-S-XynA</td>
<td>7.8 kb, pCG-S derivative; xynA from <em>S. coelicolor</em> A3(2)</td>
<td>This study</td>
</tr>
<tr>
<td>pCG-H36T-XynA</td>
<td>7.6 kb, pCG-H36T derivative; xynA from <em>S. coelicolor</em> A3(2)</td>
<td>This study</td>
</tr>
<tr>
<td>pCG-H36A-XynA</td>
<td>7.6 kb, pCG-H36A derivative; xynA from <em>S. coelicolor</em> A3(2)</td>
<td>This study</td>
</tr>
<tr>
<td>pCG-S-AmyA</td>
<td>8.7 kb, pCG-S derivative; amyA from <em>S. bovis</em> KCTC 3959</td>
<td>This study</td>
</tr>
<tr>
<td>pCG-S-cAb</td>
<td>6.9 kb, pCG-S derivative; cAbHuL22 VHH</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup>Stratagene (La Jolla, CA).  
<sup>b</sup>Novagen (Darmstadt, Germany).  
<sup>c</sup>Pharmacia Biotech (Uppsala, Sweden).
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 K₂HPO₄, 1 g of KH₂PO₄, 2 g of urea, 10 g of (NH₄)₂SO₄, 2 g of MgSO₄, 200 μg of biotin, 5 mg of thiamine, 10 mg of calcium pantothenate, 10 mg of FeSO₄, 1 mg of MnSO₄, 1 mg of ZnSO₄, 10 mg of CaCl₂, 2 g of yeast extract, and 7 g of casamino acid. After cell cultivation in shaking for 30 min, the cell culture supernatant was vigorously mixed with two volumes of cold acetone and incubated at −20°C. 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. The residual insoluble matters in the culture supernatant were filtered using a 0.45 μm syringe filter (Sartorius Stedim Biotech, Gottingen, Germany). Approximately 10 mL of filtrated samples was dialyzed against an equilibration buffer (50 mM Na₃PO₄, 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 sample was washed with 10 mL of washing buffer (50 mM Na₃PO₄, 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 Na₃PO₄, 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, Uppsalä, 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 492LC (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 secretin
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 pl (Hansmeier et al., 2006). The major protein was then excised from the gel, digested with trypsin and analyzed by MALDI-TOF for peptide fingerprinting. 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 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 (Pcg1514) 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 64 × 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 LEQTL, 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.

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 PAS104 (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, PporB and PH36, 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 (PAS104 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 PH36.
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 PH36 promoter. The potential of Cg1514 signal peptide was further evaluated with other protein models. To demonstrate the feasibility of the Cg1514-based system 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 slightly decreased (Fig. 6A). The 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 secreto production of recombinant proteins and its usability was further evaluated with other protein models.
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 L\(^{-1}\) 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 a specific growth rate of 0.194 h\(^{-1}\) in the exponential growth phase and then decreased (Fig. 7A). The cAbHuL22 VHH was first produced at 15 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 L\(^{-1}\) 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).

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 purified with high purity (>90%) and high recovery yields (>20%) by one-step column chromatography (Table II).

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

<table>
<thead>
<tr>
<th>Protein</th>
<th>Specific cell growth rate (h(^{-1}))</th>
<th>Max. cell density (OD(_{600}))</th>
<th>Max. production yield (mg/L)</th>
<th>Productivity (mg/L h(^{-1}))</th>
<th>Purification yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoxylanase</td>
<td>0.229</td>
<td>136.4</td>
<td>1867</td>
<td>33</td>
<td>28.1</td>
</tr>
<tr>
<td>(\alpha)-amylase</td>
<td>0.288</td>
<td>163.2</td>
<td>782.6</td>
<td>37</td>
<td>24.4</td>
</tr>
<tr>
<td>cAbHuL22 VHH</td>
<td>0.194</td>
<td>152</td>
<td>1,568.1</td>
<td>52</td>
<td>2.4</td>
</tr>
</tbody>
</table>

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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: overexpression 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
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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References

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