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Development of a secretion system for the production of heterologous proteins in *Corynebacterium glutamicum* using the Porin B signal peptide

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ABSTRACT

Corynebacterium glutamicum is one of the useful hosts for the secretory production of heterologous proteins because of intrinsic attributes such as the presence of few endogenous proteins and proteases in culture medium. Here, we report the development of a new secretory system for the production of heterologous proteins by using the porin B (PorB) signal peptide in *C. glutamicum*. We examined two different endoxylanases and an antibody fragment (scFv) as model proteins for secretory production. In the flask cultivations, all the examined proteins were successfully produced as active forms into the culture medium with high efficiency. For the high-level production of endoxylanase, fed-batch cultivation was also performed in a lab-scale (5 L) bioreactor, and the endoxylanases were efficiently secreted in the culture medium at levels as high as 615 mg/L. From the culture supernatant, the secreted endoxylanases could be purified with high purity via one-step affinity column chromatography.

Introduction

Corynebacterium glutamicum is a gram-positive, non-sporulating, and non-pathogenic bacterium, which has traditionally been used for the industrial production of various L-amino acids such as glutamic acid and lysine, nucleic acids, organic acids, and others [1,2]. *C. glutamicum* is also considered an important host for the production of various heterologous proteins including industrial enzymes and pharmaceutical proteins [3]. Like other gram-positive bacteria, *C. glutamicum* has a single membrane and target proteins can be secreted into the extracellular medium simply by crossing this single membrane, which is a great advantage in protein production as compared with the periplasmic secretion system using *Escherichia coli* as the host. As a host for protein production, *C. glutamicum* has several important features that facilitate the secretion of proteins into the culture medium. Although the results of a recent proteome analysis indicated the presence of more than 140 proteins in the culture supernatant [4,5], the cultivation of *C. glutamicum* produces significantly fewer secreted endogenous proteins than other host systems and thus, the downstream process to purify target proteins can be remarkably simplified. In addition, the lack of detectable extracellular proteolytic enzyme activity [6] serves to improve the stability and productivity of heterologous proteins in *C. glutamicum*.

C. glutamicum possesses two major secretory pathways: (i) a general secretion pathway (Sec-dependent pathway) and (ii) Twin-Arginine translocation (TAT-dependent pathway) [7]. Generally, the Sec-dependent pathway is used for the secretion of unfolded proteins that are folded after secretion, whereas folded proteins are translocated via the TAT-dependent pathway. Different signal peptides are involved in each pathway, which enable the proteins to cross the cytoplasmic membrane. Previously, various signal peptides such as CspA [8], cgR_2070 [5], cgR_0949 [9,10] and *E. coli* TorA [11], and have been successfully used for the secretory production of heterologous proteins. In one successful example, Watanabe et al. [10] used the signal peptide of the CgR_0949 protein, which mediate the secretion via TAT-dependent pathway, and demonstrated a high yield (approximately 2.8 g/L) of green fluorescent protein (GFP)[1] secretion. However, in spite of several successful results, the yield and efficiency of secretory production in *C. glutamicum* need to be greatly improved and the previous studies were more focused on signal peptides for TAT-dependent pathway which are not suitable for the secretion of unfolded protein such as antibody. In addition, the approach is limited by the availability of few signal peptides that can be expressed in a *C. glutamicum* host. To expand the versatility of secretory production in *C. glutamicum*, isolation of new signal peptides that allow efficient secretion is necessary.

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[1] *Abbreviations used:* GFP, green fluorescent protein; AmyA, α-amylase; BHI, brain heart infusion; ELISA, enzyme-linked immunosorbent assay; LB, Luria–Bertani broth; PorB, porin B; PCR, polymerase chain reaction; PVDF, polyvinyl difluoride.

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To isolate new signal peptides for secretory production, we focused on the cell wall-associated proteins called porins [12]. Porin B (PorB), one of the four distinct porins in *C. glutamicum*, is known to be localized in the mycolic acid layer of cell wall as a pentameric form [13,14] and it plays a key role in the formation of the anion-selective cell wall channel [15]. Previously, due to its localization in cell wall, PorB was used as an anchoring motif for cell surface display in *C. glutamicum* [16]. Using fusion to PorB (99 amino acids), α-amylase (AmyA) from *Streptomyces bovis* 148 could be displayed on the surface of *C. glutamicum* with high efficiency. In cell surface display system, the efficiency of protein display is highly dependent on the signal peptide which is essential for translocation of protein to cell wall. In this aspect, the signal peptide of PorB which consists of 27-amino acids and mediate the SEC-dependent secretion [17], can be a good signal peptide for secretory production of heterologous proteins into culture medium.

In this study, we have developed a new secretion system for the efficient production of heterologous proteins in *C. glutamicum* by using the PorB signal peptide (27 amino acids). To demonstrate its potential for secretory production, we examined two types of endoxylanases, one (20.4 kDa) from a *Bacillus* sp. (termed here XynA-Ba) and the other (48 kDa) from *Streptomyces coelicolor* A3(2) (termed here XynA-St), and an antibody fragment (M18 scFv). In all systems, all examined proteins were successfully secreted into the culture media with high efficiencies and high activities. In addition, fed-batch cultivation was performed for the high-level production of endoxylanase (XynA-St) into culture medium, and finally endoxylanase could be easily purified from the culture medium with a high recovery yield and purity.

Materials and methods

Bacterial strains, plasmids, and flask cultivations

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue was used as a host for gene cloning and plasmid maintenance, and *C. glutamicum* (ATCC 13032) was used as the main host for the production of heterologous proteins. Polymerase chain reaction (PCR) was performed on the C1000™ Thermal Cycler (Bio-Rad, Hercules, CA) with the PrimeSTAR HS Polymerase (Takara Bio Inc. Shiga, Japan). The nucleotide sequences of all primers used in this study are listed in Table S1. The PorB promoter (200 bp) was amplified by PCR from the chromosome of *C. glutamicum* with primers F1 and R1. The amplified PorB promoter gene was digested with 2 restriction enzymes (*Kpn*I and *Bam*HI), and then cloned into the plasmid pCES208 to yield pCES-PporB. For the construction of the protein secretion system, the PorB signal peptide gene (81 bp) was amplified via PCR from the chromosome of *C. glutamicum* by using the primers F2 and R3. After digestion with *Bam*HI and *Pst*I, the DNA fragment was cloned into pCES-PporB to yield pASJ100. The resultant plasmid (pASJ100) was used for the secretory production of 3 types of recombinant proteins (2 endoxylanases and an antibody fragment). The XynA-Ba gene from the *Bacillus* sp. was amplified from pKJX4 by PCR with the primers F3 and R4, and the XynA-St gene was amplified from chromosomal DNA of *Streptomyces coelicolor* A3(2) (GenBank Accession No. AL939125.1) by PCR with primers F4 and R5. A M18 scFv against the anthrax toxin PA was amplified from pAPEx-M18 by PCR with the primers F5 and R6. All three PCR products were digested with *Pst*I and *Not*I, and then cloned into the plasmid pASJ100. The resultant plasmids were designated as pASJ103, pASJ104, and pASJ105 containing the XynA-Ba, XynA-St, and M18 scFv genes, respectively. The schematic diagrams of plasmids constructed in this work are shown in Fig 1. After construction of plasmids in *E. coli* host, each plasmid was transformed into *C. glutamicum* via electroporation by using a Gene Pulser (Bio-Rad).

E. coli cells were cultivated in Luria–Bertani (LB) broth (tryptone 10 g/L, yeast extract 5 g/L, and NaCl 5 g/L) at 37 °C. For protein production, *C. glutamicum* cells were cultivated in 100 mL flasks containing 20 mL of brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) at 30 °C for 48 h with shaking at 200 rpm. For all cultivations, kanamycin (km, 25 μg/mL) was added to the culture medium as the sole antibiotic.

Fed-batch cultivation

C. glutamicum harboring pASJ104 was inoculated into 200 mL of defined medium containing 20 g/L glucose in a 1 L baffled flask, and cultivated at 30 °C for 20 h with shaking at 200 rpm. The defined medium consisted of 3 g K_2HPO_4, 1 g KH_2PO_4, 2 g urea, 10 g $(NH_4)_2SO_4$, 2 g $MgSO_4$, 200 μg biotin, 5 mg thiamine, 10 mg CPN, 10 mg $FeSO_4$, 1 mg $MnSO_4$, 1 mg $ZnSO_4$, 200 μg $CuSO_4$, and 10 mg $CaCl_2$ per liter with km (25 mg/L). The seed culture (200 mL) was inoculated into 1.8 L of fresh defined media in a 5-liter jar bioreactor (BioCNS, Daejeon, Korea). Throughout the cultivation, the temperature was maintained at 30 °C. The pH and dissolved oxygen (DO) concentrations were controlled at the set points using on-line monitoring. The DO concentration was maintained at 30% (v/v) by automatically increasing the agitation speed up to 1200 rpm and then mixing pure oxygen through a gas mixer. The pH was maintained at 7.0 by supplementing with 5N ammonia solution. During the cultivation, glucose concentration was monitored as follows. An aliquot from the culture was centrifuged, and the glucose concentration in the supernatant was determined using a glucose analyzer (YSI 2700 SELECT™ Biochemistry Analyzer, YSI Life Science,

Table 1
Bacterial strains and plasmids.

Strain	Relevant characteristics	Reference or source
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}Z*ΔM15* Tn*10* (Tet^r)]	Stratagene[a]
C. glutamicum	Wild type	ATCC 13032
Plasmids	Relevant characteristics	Reference or source
pKJX4	*B. subtilis* endoxylanase expression system	[29]
pAPEx-M18	M18 scFv expression system in APEx	[21]
pCES208	*E. coli* – *C. glutamicum* shuttle vector, Km^r	[30]
pCES-PporB	pCES208 carrying PorB promoter (P_{porB})	This study
pASJ100	pCES208 carrying PorB promoter (P_{porB}) and PorB signal sequence	This study
pASJ103	pCES100 carrying *xynA-Ba* gene with His_6-tag	This study
pASJ104	pCES100 carrying *xynA-St* gene with His_6-tag	This study
pASJ105	pCES100 carrying the M18 scFv gene with FLAG-tag	This study

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

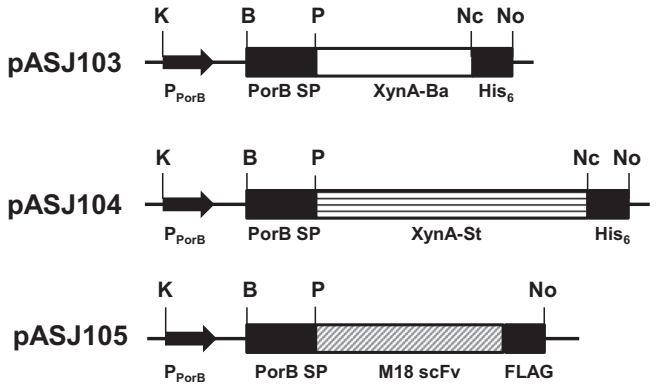

Fig. 1. Schematic diagram of plasmids used for secretory production of XynA-Ba (pASJ103), XynA-St (pASJ104) and M18 scFv (pASJ105) in *C. glutamicum*. P_{PorB} indicates the PorB promoter. Restriction enzymes: K, *Kpn*I; B, *Bam*HI; Nc, *Nco*I; No, *Not*I; P, *Pst*I.

OH). To prevent glucose starvation, a glucose solution (90 g in 150 mL) was added to the culture when the glucose was lower than 0.5% (w/v). Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Optizen POP, Mecasys, Daejeon, Korea).

Protein preparations

The extracellular proteins in the flask cultures were prepared using an acetone precipitation method [18]. The culture supernatant was mixed with two volumes of cold acetone and after incubation at −20 °C for 60 min, the proteins samples were collected by centrifugation (13,000 rpm, 30 min, 4 °C). The precipitated protein samples 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) and stored at −20 °C for further analysis.

SDS–PAGE and western blotting

All protein samples were analyzed by electrophoresis on a 12% (w/v) SDS–polyacrylamide gel. Gels were stained using Coomassie Brilliant Blue dye. Production yields and localization were further confirmed by western blotting. Following SDS–PAGE, all protein bands were transferred onto a polyvinyl difluoride (PVDF) membrane (Roche, Penzberg, Germany) for 60 min at 100 mA by using the Bio-Rad Trans-Blot® apparatus (Bio-Rad). The membrane was incubated with a blocking solution (5% (w/v) skim milk in 1× TBS-T buffered saline, 24.7 mM Tris, 137 mM NaCl, 2.7 mM KCl, and 0.5% Tween-20) for 1.5 h at room temperature. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated monoclonal ANTI-FLAG M2 antibody (Sigma–Aldrich Co., St Louis, MO) or a monoclonal Anti-poly Histidine-Peroxidase antibody (Sigma–Aldrich) for the immunodetection of FLAG-tagged or His_6-tagged protein, respectively. Each membrane was then washed 4 times with TBS-T for 5 min and an enhanced chemiluminescence kit (Amersham ECL Prime Western Blotting Detection Reagent; GE Healthcare) was used for the detection of proteins.

Assay of endoxylanase activity

To determine endoxylanase secretion from *C. glutamicum*, cells were dotted on a BHI agar plate containing 0.5% (w/v) beechwood xylan (Sigma–Aldrich, Co.) and km (25 μg/L) using autoclaved toothpicks. After incubation at 30 °C for 48 h, the colonies that appeared on the agar plate were analyzed for the presence of a clear halo around them. The activity of endoxylanase was also measured using the 3,5-dinitrosalicylic acid (DNS) method [19]. To assay the activity of endoxylanase in the culture media, 0.5 mL of culture supernatant was collected by centrifugation and then mixed with 2 mL of the same 2.5% (w/v) beechwood xylan solution. After incubation of the reaction mixture at 30 °C for various durations (up to 24 h), the remaining xylan and cells were removed by centrifugation for 10 min at 12,000×*g* at room temperature. The reaction supernatants were mixed with 3 volumes of DNS solution containing (per liter) 7.5 g DNS, 14.0 g NaOH, 216.1 g Rochelle salt, 5.4 mL phenol, and 5.9 g $Na_2S_2O_5$. After boiling for 5 min, supernatants were cooled to room temperature for 5 min, and the absorbance of the reactant at 550 nm was detected using a spectrophotometer.

Enzyme-linked immunosorbent assay (ELISA)

The binding activity of the antibody fragment (M18 scFv) that was secreted into the culture medium was assayed using ELISA. Briefly, 50 μL of 5 μg/mL antigen (domain 4 of anthrax toxin PA) [20] dissolved in carbonate-bicarbonate buffer was coated on 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) 4 times, the coated antigens were blocked with 200 μL/well of 5% (w/v) bovine serum albumin (BSA; Invitrogen) solution in PBS for 1 h at room temperature. Serially diluted protein samples from the cultured media were added into the coated wells and allowed to bind for 1 h at room temperature. After washing, 50 μL of HRP-conjugated goat anti-FLAG IgG antibody dissolved in 5% (w/v) BSA solution in PBS (dilution 1:5000) was added into each well and allowed to react for 1 h. Then 50 μL of TMB peroxidase substrate system was added into each well for detection, with 50 μL of 2 M H_2SO_4 added as a stopping agent. The signals were quantified by measuring the absorbance at 450 nm using the TECAN Infinite M200 Pro (Tecan Group Ltd). As a negative control, BSA instead of the antigen was coated in the plate and all other steps were performed as described above.

Purification of endoxylanase

After the fed-batch cultivation, cells were removed by centrifugation at 5000×*g* at 4 °C for 30 min. To remove the residual insoluble matter, a 0.45 μm syringe filter (Sartorius Stedim Biotech, Goettingen, Germany) was used. Five milliliter of supernatant containing the endoxylanase was diluted with binding buffer (50 mM KH_2PO_4, 300 mM NaCl, pH 8.0) up to 50 mL and endoxylanase was then purified using a TALON metal affinity resin (Clontech, Mountain view, CA). Next, 0.5 mL of TALON metal affinity resin was loaded to Poly-Prep® chromatography columns (Bio-Rad) and equilibrated with 10 mL of binding buffer (50 mM KH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.0). The filtered supernatant samples were loaded onto the resin and the column was washed with 10 mL of binding buffer. 2 mL of elution buffer (50 mM KH_2PO_4, 300 mM NaCl, 150 mM imidazole, pH 7.0) was added for elution of the target protein. After purification of endoxylanase, proteins were quantified using a Bio-Rad protein assay kit (Bio-Rad) with BSA as a standard. The molecular mass of the purified XynA-St was determined using matrix-assisted laser desorption/ionization mass spectrometry (MALDI/TOF/MS, AB Sciex Instruments, Foster, CA).

Results

Secretory production of endoxylanase

We first examined two different endoxylanases, which degrade xylan to xylooligomers, as model proteins for secretory production using the PorB signal peptide: (i) XynA-Ba (20.4 kDa) from *Bacillus*

sp. and (ii) XynA-St (48 kDa) from *S. coelicolor* A3(2). Each gene was linked to PorB signal peptide coding gene and its expression was controlled under constitutive P_{PorB} promoter (Fig. 1). After cultivation of *C. glutamicum* harboring pASJ103 or pASJ104, secretory production of endoxylanases into the culture medium was analyzed by SDS–PAGE and western blotting. Of the two endoxylanases, XynA-St showed a very high yield in the culture medium. Using Coomassie Brilliant Blue dye-stained gels, the content of XynA-St in the culture supernatant was >90% of the total extracellular proteins (densitometric analysis) (Fig. 2A). Although XynA-Ba showed a much lower expression level than XynA-St, the results of western blotting clearly confirmed that the majority of XynA-Ba was secreted into the culture medium (Fig. 2B). The activities of the secreted endoxylanase were confirmed using 2 analytical methods. On BHI agar plates containing 0.5% xylan, *C. glutamicum* cells harboring either pASJ103 or pASJ104 showed clear halos around colonies but no clear halo was observed around the *C. glutamicum* control containing pCES208 (Fig. 2B). In particular, *C. glutamicum* (pASJ104) producing XynA-St showed larger and much clearer halo than *C. glutamicum* (pASJ103), indicating more secretory production of XynA-St into the culture medium than XynA-Ba, which correlated well with the results of SDS–PAGE and western blotting. The higher productivity of *C. glutamicum* (pASJ104) was also confirmed by DNS method. The endoxylanase activity achieved in the *C. glutamicum* (pASJ104) cultures was approximately 2 times that in *C. glutamicum* (pASJ103) cultures (Fig. 2C).

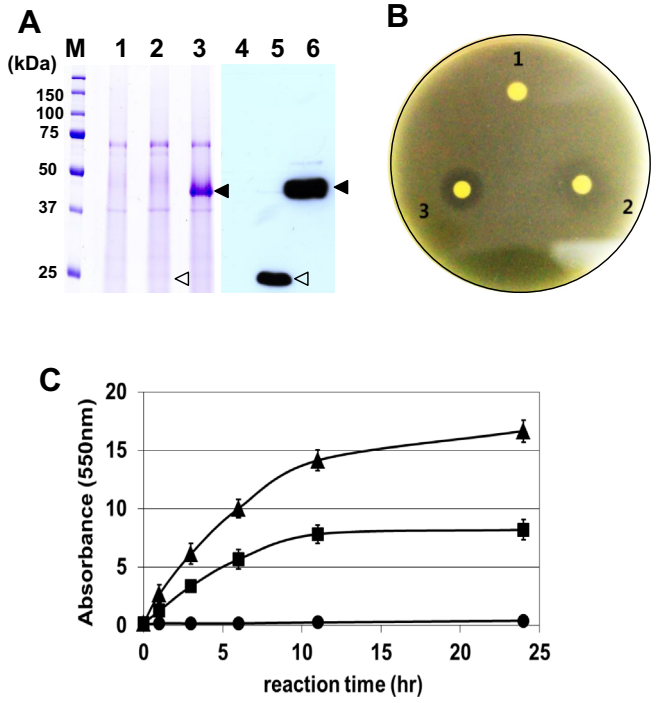

Fig. 2. Secretory production of endoxylanases in *C. glutamicum*. (A) Coomassie blue stained SDS–PAGE gel (lanes 1–3) and western blot analysis (lanes 4–6) of endoxylanases production. Lane M, molecular weight marker (kDa); Lanes 1 and 4, *C. glutamicum* harboring pCES208 as a control; Lanes 2 and 5, *C. glutamicum* harboring pASJ103; Lanes 3 and 6, *C. glutamicum* harboring pASJ104. Open and closed arrowheads indicate XynA-Ba (20.4 kDa) and XynA-St (48 kDa), respectively. (B) Activity assay of endoxylanase on BHI agar plates containing 0.5% (w/v) beechwood xylan. Numbers: 1, *C. glutamicum* harboring pCES208; 2, *C. glutamicum* harboring pASJ103; 3, *C. glutamicum* harboring pASJ104. (C) Activity assay of endoxylanase using DNS method. Symbols: ●, *C. glutamicum* harboring pCES208; ■, *C. glutamicum* harboring pASJ103; ▲, *C. glutamicum* harboring pASJ104.

Secretory production of antibody fragment

To demonstrate the general use of the PorB signal peptide for protein secretion, single chain variable fragment (scFv) was examined as an additional model protein. In this work, we used M18 scFv (30 kDa), which has a high affinity ($K_D \approx 35$ pM) to the anthrax PA toxin [21]. After flask cultivation of *C. glutamicum* harboring pASJ105, the secretory production of M18 scFv into the culture medium was analyzed by western blotting and ELISA. It was clearly confirmed by western blot that most of the M18 scFv was present in the culture supernatant, and not in the cytoplasm (Fig. 3A). An ELISA also confirmed that *C. glutamicum* (pASJ105) successfully secreted M18 scFv into the culture medium, and the high signal in the ELISA indicates that the secreted M18 scFv was highly active in the culture supernatant (Fig. 3B).

High-level production of XynA-St by fed-batch cultivation

To achieve high-level secretory production of XynA-St, fed-batch cultivation of *C. glutamicum* (pASJ104) was carried out using a 5-L lab-scale bioreactor system. Based on the glucose level, new glucose solutions (90 g in each feeding) were fed and, cells were grown to an OD_{600} of 154.2 at 38 h at which point cell density decreased gradually (Fig. 4A). In the exponential growth phase, the specific growth rate (μ) was approx. 0.10 h^{-1}. During the cultivation, supernatant samples were taken periodically and analyzed by SDS–PAGE. At 13 h after inoculation, a band corresponding to the XynA-St band was seen first and the band density increased progressively (Fig. 4B). The XynA-St content in the culture supernatant were determined using a densitometer and the maximum content (approximately 43% of the total extracellular proteins) was obtained at 30 h and decreased gradually to 27% at the end of the cultivation. Based on the results of densitometric analysis and protein quantification, the maximum production yield was approximately 615 mg/L at 38 h (Fig. 4A) and the productivity of XynA-St was approximately 16.2 mg/L/h.

Purification of XynA-St from culture supernatant

The XynA-St was purified by single affinity column chromatography from the culture broth as described in Materials and methods. From only 5 mL of culture broth, approximately 1.1 mg of XynA-St could be purified with high purity (>95%) and recovery yield (ca. 33%) (Fig. 5A). Using the purified XynA-St, we determined the *N*-terminal sequence by Edman degradation method. The first 6 amino acids (S-R-A-E-S-T) were perfectly matched with the deduced amino acid sequence of XynA-St, and this result indicates

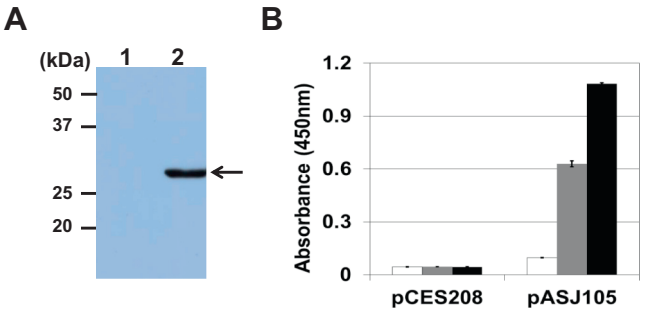

Fig. 3. Secretory production of M18 scFv in *C. glutamicum* (pASJ105). (A) Western blot analysis of M18 scFv production. Lane 1, cytoplasmic fraction; Lane 2, culture supernatant. (B) Analysis of M18 scFv binding activity by enzyme-linked immunosorbent assay (ELISA) using the culture supernatant of *C. glutamicum* harboring either pCES208 or pASJ105. White, gray and black bars represent antigen-coated well treated with 10 μL, 50 μL, and 100 μL, of supernatant, respectively.

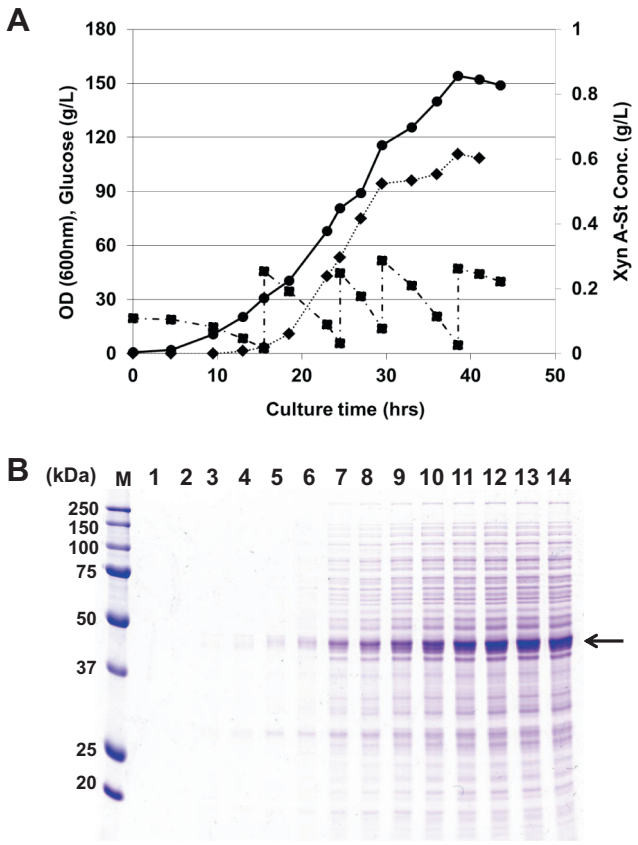

Fig. 4. Fed-batch cultivation of *C. glutamicum* (pASJ104). (A) Profiles of cell growth (●), glucose concentration (■), and concentration of XynA-St in the culture supernatant (♦) during fed-batch cultivation. (B) SDS–PAGE analysis of culture supernatant in fed-batch cultivation. At each time point, 5 μL of culture supernatant and an equal volume of sample buffer were applied to each slot and analyzed by SDS–PAGE. Lane M, molecular weight marker (kDa); Lanes 1–14, supernatant samples taken at 5 h, 9 h, 13 h, 16 h, 19 h, 23 h, 25 h, 28 h, 30 h, 34 h, 38 h, 40 h, 42 h, and 46 h after inoculation. Arrow indicates XynA-St (48 kDa).

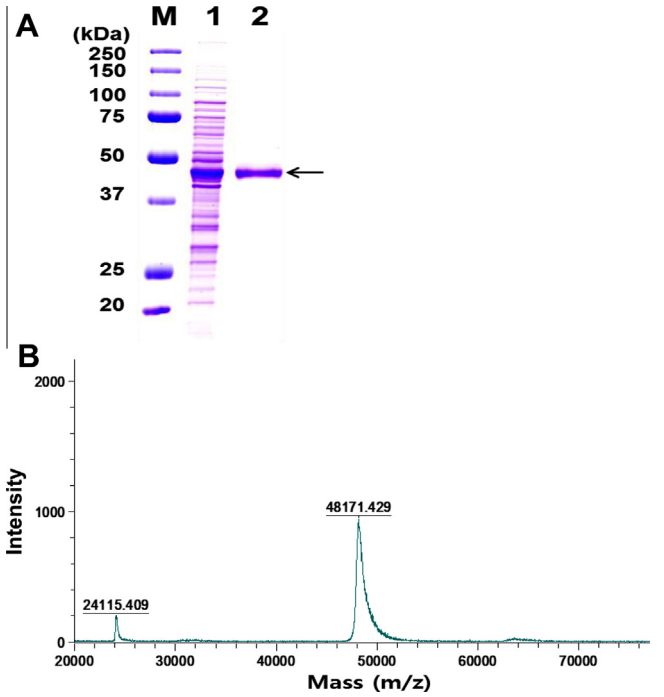

Fig. 5. Purification of XynA-St. (A) SDS–PAGE analysis. Lane M, molecular weight marker (kDa); Lane 1, culture supernatant (10 μL); Lane 2, eluate from Ni–NTA affinity column. Arrow indicates the XynA-St. (B) Matrix-assisted laser desorption/ionization (MALDI)-mass spectrum of purified XynA-St. The dominating peak (second peak) with a measured molecular mass of 48,171.4 represents XynA-St. The first peak (24,115.4) represents the doubly protonated form of the protein arising from the MALDI-mass spectrometric process.

that the PorB signal peptide was correctly removed during secretion into culture medium. In addition, the mass of purified XynA-St was measured by MALDI mass analysis and the measured mass (48,171.3 Da)coincided well with the estimated mass of mature XynA-St (48,090.7 Da)within error (Fig. 5B). Further, the dominant peak in the mass analysis demonstrated the high purity of the XynA-St in the purified sample.

Discussion

Many researchers have studied secretion pathways, developing various secretion systems in different hosts, because protein secretion has numerous advantages over intracellular production. In this study, we chose *C. glutamicum* as a production host and developed a secretion system using a new signal peptide of the PorB cell wall protein in *C. glutamicum*. To demonstrate the high efficiency and universality of the PorB signal peptide for secretory production, two types of endoxylanase as well as a single-chain antibody fragment (scFv) were examined as model proteins. Each model protein had diverse characteristics such as molecular weight (20–50 kDa) and folding (1–2 disulfide bonds), but all were successfully secreted in the culture medium with high efficiency. Particularly with XynA-St, production yields as high as 0.6 g/L could be achieved by fed-batch cultivation. Although this production yield is not the highest record achieved in *C. glutamicum* as a host [9,10], the efficiency of this system is quite competitive with other Sec-dependent secretion systems [7].

In this study, two different endoxylanases were examined and XynA-St showed a much higher production yield than did XynA-Ba. Among several factors that could have an effect on the production level, we considered that the differences in codon usage between the two endoxylanases gave the different production yields in the *C. glutamicum* host. Different hosts have different codon usages and codon usage is one of the important factors in protein production. It is well known that rare codons are strongly associated with low levels of protein expression, and thus codon usage should be considered in the construction of heterologous gene expression systems [22,23]. The expression level of target genes can be drastically improved through codon optimization, in which rare codons are changed to preferable codons. Jo et al. [24] optimized 12 *N*-terminal codons of the Poly(3-Hydroxybutyrate) (P[3HB]) synthetase gene (*phaC*) of *Ralstonia eutropha* towards preferable codons in a *C. glutamicum* host, and higher P(3HB) productivity could be achieved using codon-optimized genes. When we checked the codon usage of both endoxylanase genes, we found large differences in codon usage. In the case of XynA-Ba, the rare codon (below 5% frequency in the *C. glutamicum* host) content was 8.4% (18 codons of a total 214 codons) but the other endoxylanase (XynA-St) did not contain any rare codons in a total of 436 codons. This large difference in codon usage could affect translation and protein synthesis rate and consequently, may significantly affect the yield of secretory production. Another model protein M18 scFv also showed a relatively low-level production yield although secretion efficiency was high (Fig. 3). We also found that the M18 scFv gene has a relatively high content of rare codons (3.5%, 9 codons in a total of 257 codons) and this high content of rare codons may result in the low production yield. However, as

shown by other groups, we believe that translation efficiency and production yield of antibody fragments and XynA-Ba can be further increased through optimizing the codons toward *C. glutamicum*-preferable codons.

As shown in this study, secretory production using the PorB signal peptide worked quite well in large-scale fed-batch cultivations. By employing a constitutive PorB promoter, endoxylanase was constitutively secreted immediately after inoculation, and as cell density increased, quantities of endoxylanase in culture medium were also increased. Actually, high productivity (16.2 mg/L/h) was achieved and this means that total 1.54 g of XynA-St could be obtained from 2.5 L (final culture volume) culture. During the cultivation, we did not observe any significant reduction in cell growth rate. Cells continued to grow to an OD_{600} of 154 which was relatively high compared with other cultivation reports where the final cell densities were below an OD_{600} of 100 [9,25,26]. In the entire cultivation (46 h), proteolytic degradation of endoxylanase was not observed which was confirmed by western blotting with an anti-His_6 antibody (data not shown). As we mentioned in the Introduction section, this is one of the benefits of utilizing *C. glutamicum* as a production host, and productivity can be maximized without loss by proteolytic degradation in long-term processes. In fed-batch cultivation, the content of XynA-St in the extracellular fractions was relatively lower (approximately 40%) than that of the shake flask cultivation (greater than 90%) (Fig. 2A). As cell density increased rapidly during exponential growth phase, the portion of dead cells also increased, and thus proteins released from the dead cells may increasingly accumulate in the extracellular fraction. This indicates that the fed-batch cultivations need to be further optimized to minimize cell death and protein contamination in the extracellular fractions.

In conclusion, we have developed a Sec-dependent secretory production system using the PorB signal peptide in *C. glutamicum*. Using three model proteins, we successfully demonstrated that the PorB signal peptide facilitated the efficient secretion of the target proteins into the culture media in shake flask cultivation as well as in large scale fed-batch cultivation. Through the secretory production of endoxylanase (XynA-St) into the culture media, *C. glutamicum* can utilize hemicellulose (xylan), the second-most abundant bioresource on the planet, and it would be a useful tool in current white biotechnological and biorefinery processes. Additionally, the antibody fragment (M18 scFv) was successfully secreted into the culture medium, and to the best of our knowledge, this is the first report for the production of an antibody fragment in *C. glutamicum* host. In particular, due to the lack of endotoxins and the relatively low contamination of proteins and proteases in the culture medium, safe and cost-effective production of various pharmaceutical proteins can be achieved by using this host. Compared to eukaryotic hosts including *Pichia pastoris* which has been widely used for production of antibody and antibody fragments [27], *C. glutamicum* also has advantages such as relatively short cultivation time. In general, production of recombinant proteins in *P. pastoris* requires long cultivation (72 h or longer) [28], but as shown here, we could achieve good production yield in much shorter cultivation (40 h) which is very important for economic production of recombinant proteins in industrial scale cultivations. We believe that the combination of the efficient secretion system and the abovementioned intrinsic characteristics will make this organism a system of choice to produce proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2013.04.003.

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