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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.