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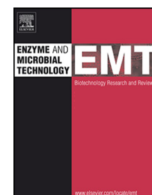

Application of repeated aspartate tags to improving extracellular production of *Escherichia coli* L-asparaginase isozyme II

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ABSTRACT

Asparaginase isozyme II from *Escherichia coli* is a popular enzyme that has been used as a therapeutic agent against acute lymphoblastic leukemia. Here, fusion tag systems consisting of the pelB signal sequence and various lengths of repeated aspartate tags were devised to highly express and to release active asparaginase isozyme II extracellularly in *E. coli*. Among several constructs, recombinant asparaginase isozyme II fused with the pelB signal sequence and five aspartate tag was secreted efficiently into culture medium at 34.6 U/mg cell of specific activity. By batch fermentation, recombinant *E. coli* produced 40.8 U/ml asparaginase isozyme II in the medium. In addition, deletion of the *gsp*DE gene reduced extracellular production of asparaginase isozyme II, indicating that secretion of recombinant asparaginase isozyme II was partially ascribed to the recognition by the general secretion machinery. This tag system composed of the pelB signal peptide, and repeated aspartates can be applied to extracellular production of other recombinant proteins.

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1. Introduction

Asparaginase isozyme II (AnsB) from *Escherichia coli* (*E. coli*) is a prokaryotic protein studied over 50 years as a therapeutic agent against acute lymphoblastic leukemia (ALL) [1]. In combination with other agents, AnsB is also used in the treatment of Hodgkin's disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, reticulosarcoma and melanosarcoma [2]. Studies have shown that only AnsB from *E. coli* is very effective in inhibiting tumors, while other bacterial asparaginases were either less active or completely inactive [3,4].

The *E. coli* AnsB enzyme naturally forms a homo-tetramer structure composed of two dimers of intimate dimers containing all the structural elements and functional groups, and the active enzyme is always a tetramer [5]. Several research efforts have been made to express AnsB actively in *E. coli*. Medium optimization was undertaken to improve production of AnsB from wild type *E. coli* ATCC 11303, but this approach is still limited by an inherent low expression yield [6]. Among the *trc*, *tac* and T7 promoter systems, the highest specific activity and expression level of recombinant AnsBs were obtained when the strong *T7* promoter was adopted [7–9]. A previous study showed that medium composition and induction strategy significantly influenced extracellular production of recombinant AnsB, and induction of the late log phase cells in TB medium was optimal for secretion of recombinant AnsB [9]. Among various signal sequences including native AnsB signal sequence, Bacillus endoxylanase leader sequence and the pelB leader sequence, expression of recombinant AnsB fused to pelB leader resulted in maximum production in culture medium. Interestingly, recombinant AnsB was produced extracellularly up to 75% of total AnsB proteins by unknown mechanism when it was combined with the pelB signal sequence [7]. While we also adopted pelB leader sequence to maximize AnsB secretion according to the previous studies. We sought to construct an efficient AnsB expression system in minimal medium because minimal medium is more beneficial than complex medium (TB medium) in terms of cost and tight control [10,11].

In most cases, extracellular production of target proteins in culture medium has several advantages over intracellular production: simplified downstream processing, enhanced biological activity, and higher protein stability and solubility [12,13]. For the extracellular expression of target proteins in *E. coli*, two prerequisites are required: maintenance of their soluble and active forms, and their delivery to the extracellular space by penetrating cytoplasmic and outer membranes. For soluble expression of tar-

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get proteins, many solubility-enhancing fusion partners have been devised: glutathione *S*-transferase (GST), maltose binding protein (MBP), thioredoxin (Trx), ubiquitin (Ub), *N*-utilization substance (NusA) and small ubiquitin-modifier (SUMO) [14,15]. To increase the active protein expression and secretion efficiency, combination of a signal sequence with the solubility-enhancing fusion partner is worthy to be considered. However, the fusion partners consisting of 76 (Ub) to 495 (NusA) amino acids are too big to go through the membrane structure and play their roles in the cytoplasmic space. Thus, other fusion partners should be developed to possess a small size and act in both cytoplasmic and periplasmic spaces.

Extracellular proteins commonly have highly negative charge in the first five amino acid residues [16], whereas, AnsB has only one at position +18. In our previous report, recombinant *Candida antarctica* lipase B (CalB) with the N-terminal 5-aspartate tag dramatically increased both expression and secretion of CalB with the aid of the pelB signal sequence in recombinant *E. coli* [17]. These facts suggest that an adequate alteration of the N-terminal end of AnsB by fusion of anion amino acids could improve soluble expression and transport efficiency across the membranes as done for recombinant CalB. In this study, extracellular expression of AnsB was designed by its connection with the pelB signal sequence and a series of aspartate tag, of which strategy was verified in a simple batch culture. Moreover, we modulated the general secretion pathway (GSP) of *E. coli* and found that extracellular production of AnsB was partially ascribed to the recognition by the GSP components.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli TOP10 and BL21 star (DE3) (Invitrogen, Grand Island, NY, USA) strains were used for genetic manipulation and protein production, respectively. The AnsB gene and its recombinant genes were cloned behind the T7 promoter in derivatives of plasmid pET-26b(+) (EMD Millipore, Darmstadt, Germany), and their transcription was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG). The chromosomal DNA of *E. coli* K-12 was used as the source of the AnsB gene. A *gsp*DE-deficient background strain (designated here as ΔgspDE) lacking a part of the fifteen *gsp* genes was previously constructed [17].

2.2. Genetic manipulation

The AnsB genes were amplified by the polymerase chain reaction (PCR) using the chromosomal DNA of *E. coli* K-12 as a template. PCR DNA primers used for AnsB amplification were designed to be specific for the 5′- or 3′-end of the AnsB gene, and contain recognition sites of DNA digestion enzymes as shown in Table 1. To amplify the AnsB genes, DNA primers were formulated with a forward oligomer and a reverse oligomer of AnsB[R]. For example, AnsB[F] or D5-AnsB[F] and AnsB[R] were used to express the gene coding for the authentic AnsB without any tags and native signal sequence or recombinant AnsB with the N-terminal 5-aspartate tag, respectively. To combine each recombinant AnsB gene with the gene coding for the N-terminal pelB leader sequence in plasmid pET-26B(+), the forward primers were designed to remove the transcriptional initiation codon (ATG) of the AnsB gene. The AnsB gene was then located behind the leader sequence in frame. After the gene amplification, all PCR products were cut with appropriate restriction enzymes (Table 1) and ligated with plasmid pET-26b(+) digested with the same enzymes. The nucleotide sequences of the recombinant AnsB genes were verified by DNA sequencing. Names of plasmids, recombinant AnsB gene products and schematic structures are shown in Fig. 1.

2.3. Culture condition

The recombinant *E. coli* BL21 star (DE3) cells harboring each AnsB expression vector were pre-cultured in LB medium (5 g/l yeast extract and 10 g/l bacto-trypton) at 37 °C and 230 rpm for 12 h. After harvesting the cells, the cell pellets were used for inoculation. A batch culture using a 500 ml-scale baffled flask (Nalgene, Rochester, NY, USA) was carried out at 37 °C and 230 rpm in 100 ml of a defined medium (pH 6.8) [18] containing (per liter) 20 g glucose, 4 g $(NH_4)_2HPO_4$, 13.5 g KH_2PO_4, 1.7 g citric acid, 1.4 g $MgSO_4·7H_2O$ and 10 ml trace metal solution. The trace metal solution was composed of per liter of 5 mol HCl, 10 g $FeSO_4·7H_2O$, 2.25 g $ZnSO_4·7H_2O$, 1 g $CuSO_4·5H_2O$, 0.5 g $MnSO_4·4H_2O$, 0.23 g $Na_2B_4O_7·10H_2O$, 2 g $CaCl_2$, 0.1 g $(NH_4)_6Mo_7O_{24}$. At the logarithmic phase of growth (optical density at 600 nm (OD_{600}) ≈ 0.8–1.2), protein expression was induced by adding IPTG at a final concentration of 0.2 mM. The induced cells were cultivated at 20 °C and 200 rpm for additional 24 h.

2.4. Protein fractionation

To collect the AnsBs secreted from the recombinant cells, the culture broth was centrifuged at 15,000 rpm for 10 min, and its supernatant was named as the medium fraction. To obtain the intracellular protein fractions, the collected culture broth was concentrated or diluted to adjust its OD_{600} to be 10. This broth was centrifuged at 13,000 rpm and 4 °C for 10 min. The cell pellets were suspended in 50 mM Tris–Cl buffer (pH 9.0) and disrupted by an ultrasonic machine (Cole-Parmer, USA) on ice. The total, soluble and insoluble fractions of intracellular proteins were prepared as described in the previous report [19]. Briefly, after centrifugation of the cell lysate at 12,000 rpm and 4 °C for 30 min, the supernatant and pellets were collected individually and named as the soluble and insoluble protein fractions, respectively. The cell lysate containing both soluble and insoluble proteins before centrifugation was also regarded as the total protein fraction.

2.5. AnsB assays

To visualize recombinant AnsBs, the protein samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% acrylamide. Protein bands on SDS-PAGE gels were stained with Coomassie brilliant blue R-250 and quantitatively analyzed by a densitometer (TotalLab 1.01, Nonlinear Dynamics Ltd.).

To determine AnsB activity, the reaction mixture was composed of 140 μl of 0.1 M potassium phosphate buffer (pH 7.0), 50 μl of 40 mM L-asparagine and 10 μl of the crude enzyme solution, and preheated at 37 °C for 5 min. The reaction was terminated by addition of 50 μl of 15% (w/w) trichloroacetic acid, followed by centrifugation at 13,000 rpm for 2 min. 70 μl of the supernatant was transferred into a 96-well microplate and mixed with 130 μl of the Nessler's reagent for 10 min at room temperature. The absorbance change at 500 nm of wavelength was monitored by a 96-well microplate reader (Molecular Devices, Sunnyvale, CA, USA). Specific AnsB activity was calculated by the division of AnsB activity by dry cell weight.

2.6. Quantitative real-time PCR

Total cellular RNAs from the *E. coli* BL21 star (DE3) strains were isolated using the RNeasy Protect Bacteria Mini Kit in accordance with the manufacturer's protocol (Qiagen). Quantitative real-time PCRs were performed in a final volume of 20 μl of SYBR Premix Ex Taq (Takara) containing cDNA synthesized with the AccuPower RT PreMix system for reverse transcription-PCR (Bioneer). Real-time

Table 1
List of DNA oligomers used in this study. The italicized sequences indicate the recognition sites of the corresponding restriction enzymes. [F] and [R] mean the forward and reverse primers, respectively.

Name	Sequence (5′ → 3′)	Restriction enzyme	Amplified gene
AnsB[F]	GGAATTC*CATATG*TTACCCAATATCACCATTTTAGCAACC	*Nde*I	AnsB
P-AnsB[F]	CATGC*CATGG*CCTTACCCAATATCACCATTTTAGCAACC	*Nco*I	P-AnsB
P-D3-AnsB[F]	CATGC*CATGG*CCGATGATGATTTACCCAATATCACCATTTTAGCAACC	*Nco*I	P-D3-AnsB
P-D4-AnsB[F]	CATGC*CATGG*CCGATGATGATGATTTACCCAATATCACCATTTTAGCAACC	*Nco*I	P-D4-AnsB
D5-AnsB[F]	GGAATTC*CATATG*GATGATGATGATGATTTACCCAATATCACCATTTTAGCAACC	*Nde*I	D5-AnsB
P-D5-AnsB[F]	CATGC*CATGG*CCGATGATGATGATGATTTACCCAATATCACCATTTTAGCAACC	*Nco*I	P-D5-AnsB
P-D6-AnsB[F]	CATGC*CATGG*CCGATGATGATGATGATGATTTACCCAATATCACCATTTTAGCAACC	*Nco*I	P-D6-AnsB
AnsB[R]	CCGC*TCGAG*ATTAGTACTGATTGAAGATCTGCTGGAT	*Xho*I	[a]
gspD_RTPCR[F]	ATGAAAGGACTCAATAAAATCACCTGCTGC	–	GspD
gspD_RTPCR[R]	TCACCGTGACGATGGCGCAG	–	
gapC_RTPCR[F]	ATGAGTAAAGTTGGTATTAACGGTTTTGGTCG	–	GapC
gapC _RTPCR[R	TCAGAGTTTAGCGAATTTTTCGAGGGTG	–	

[a] In all cases of AnsB variants, AnsB[R] was used as the reverse primer.

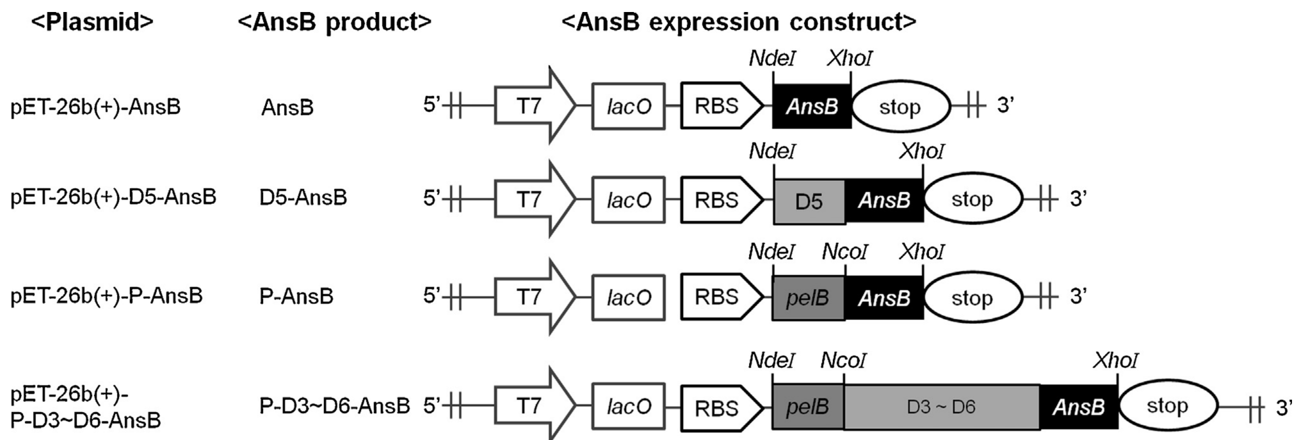

Fig. 1. Schematic diagram of the structures of recombinant AnsB expression cassettes in plasmid pET-26b(+) derivatives. The abbreviation and symbols are denoted as follows: T7, T7 promoter; *lacO*, *lac* operator; RBS, ribosomal binding site; *pelB*, signal sequence of pectate lyase B from *Erwinia carotovora*; *ansB*, *E. coli* L-asparaginase isozyme II; stop, translational stop codon; D5, 5 aspartates; D3–D6, 3–6 aspartates.

PCRs were performed in triplicate using the iCycler iQ real-time detection system (Bio-Rad) with two specific PCR primers (Table 1). The primers include a housekeeping gene, *gap*C, used for an internal reference for normalization.

3. Results

3.1. Design of AnsB expression systems with N-terminal repeated aspartates

Based on our hypothesis that polyanionic amino acids locating at the N-terminal end of AnsB may facilitate their extracellular expression, various protein expression systems were designed to possess different length of repeated aspartate molecules. To express and secrete recombinant AnsB in *E. coli*, the 5′-end of the AnsB gene without its own translational initiation codon and signal sequence was combined with the gene coding for the pelB signal sequence and 3–5 or 6 aspartates in a row. By addition of the repeated aspartates, isoeletric points of recombinant AnsBs decrease from pH 5.44 (no aspartate) to 4.79 (6 aspartates). As controls, the AnsB gene and AnsB with N-terminal 5 aspartates (D5-AnsB) without the pelB signal sequence were inserted into plasmid pET-26b(+). Schematic diagrams for the AnsB expression cassettes in pET-26b(+) plasmid derivatives and their names are displayed in Fig. 1.

3.2. Effects of N-terminal repeated aspartates on AnsB expression

To investigate the effect of the aspartate tags on AnsB expression, recombinant *E. coli* BL21 star (DE3) strains overexpressing each recombinant AnsB gene were cultured batchwise and crude AnsB enzymes present in the intracellular and extracellular fractions were subjected to asparginase activity (Fig. 2) and SDS-PAGE (Fig. 3) analyses. The fusion of the pelB signal sequence improved both intracellular and extracellular asparaginase activities by more than 70%, relative to the control of the authentic AnsB. Among a series of repeated amino acids consisting of 3–6 aspartates, the four and five aspartates facilitated the secretion of AnsB and hence P-D5-AnsB showed a 1.9 times higher specific activity in the culture medium than P-AnsB. Even though the repeated aspartates did not affect intracellular accumulation of active asparaginase, they elevated a total expression level of AnsB by 51% and hence the best extracellular asparaginase activity (40.8 U/ml) was obtained by a simple batch fermentation of recombinant *E. coli* expressing P-D5-AnsB (Table 2). As well as the enzyme activity assay, SDS-PAGE analysis also showed efficient secretion of recombinant AnsB in the culture medium (Fig. 3). Protein band of the secreted AnsB for the case of P-D5-AnsB (Lane E) was much thicker than that for P-AnsB. It was located at a slightly higher position in the gel than the theoretical molecular weight (35 kDa) because the amino acid tag still remained at the N-terminus, and the pelB signal sequence was cut off by a signal peptidase, which was verified by the N-terminal

Table 2
Production of AnsBs in *E. coli*.

Strain	System	Activity[a] (U/ml)		Reference
		Intracellular	Extracellular	
E. coli ATCC11303		1.03		[5]
E. coli BL21 (DE3)	*T7* promoter	3.9		[7]
E. coli BLR (DE3)	PelB signal sequence	8.2	22.5	[6]
E. coli BL21 star (DE3)	PelB signal sequence + 5 aspartate tag	19.2	40.8	This study

[a] AnsB activity was determined by using L-asparagine as a substrate.

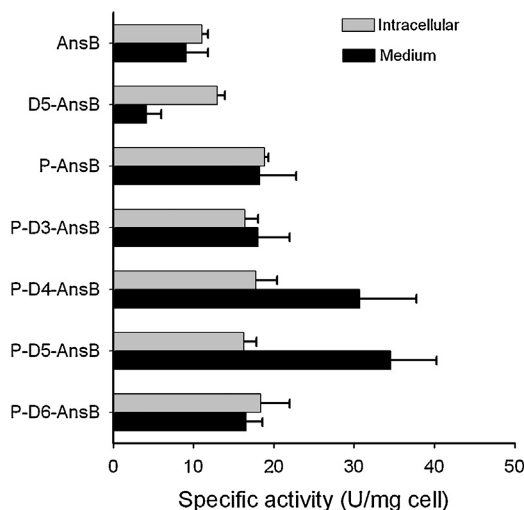

Fig. 2. Specific activities of recombinant AnsBs in the intracellular fraction and culture medium. After 24 h of IPTG induction, the recombinant *E. coli* cells were collected, harvested and disrupted. The intracellular fraction containing both cytoplasmic and periplasmic proteins, and culture medium were subjected to assay of asparaginase activity, which were measured in triplicate and normalized to dry cell mass.

amino acid sequencing of recombinant AnsBs present in the intracellular and extracellular spaces. Although the protein band of the secreted AnsB for the case of P-D3-AnsB was also thicker than that for P-AnsB, specific activities of P-AnsB and P-D3-AnsB in the culture medium were almost identical. We postulated that attachment of the tags to the N-terminal end of AnsB might alter the protein activity as previously reported [17,20], and hence, result in similar specific activities in medium.

3.3. Time-course expression of recombinant AnsB and D5-AnsB

Recombinant AnsBs were targeted to the periplasm via the SecB-dependant pathway, which is one of the type II secretion systems in *E. coli*. AnsB export via this pathway was initiated by the N-terminal pelB signal [21] and moreover the five-repeated aspartate tag played a role of protein transport through the outer membrane. To investigate the role of the five-repeated aspartate tag, batch cultures of the recombinant *E. coli* strain overexpressing P-AnsB or P-D5-AnsB were carried out and intracellular and extracellular asparaginase activities of recombinant AnsBs collected over time were analyzed. As shown in Fig. 4, the growth patterns of the two recombinant *E. coli* strains were almost identical. For asparaginase localization, the specific asparaginase activity of D5-AnsB (a processed form of P-D5-AnsB) in the culture medium (37.2 ± 2.7 U/mg cell) was about 2.0 times higher than 18.3 ± 2.7 U/mg cell of AnsB (a processed form of P-AnsB) in the broth whereas, the five-repeated aspartate tag did not affect intracellular accumulation of active asparaginase of which specific activity was around 24 U/mg cell. Although the relative amount of both extracellular AnsB and D5-AnsB increased over time, D5-AnsB had a much higher tendency of extracellular secretion than AnsB. These results indicated that the recombinant D5-AnsB in the periplasm has higher efficiency of transport across the outer membrane than the authentic AnsB.

3.4. Effects of partial disruption of general secretion pathway on recombinant AnsB secretion

In most Gram-negative bacteria, the SecB-dependent pathway which allows proteins to cross the inner membrane is extended by terminal branches of the general secretion pathway (GSP) that permit extracellular secretion [22]. We have previously shown that the GSP machinery specifically exported the dimer form of D5-CalB [17]. However, a previous study reported that the *gsp* genes are transcriptionally silent in the *E. coli* K-12 strain [23]. To confirm the expression of the *gsp* genes, the transcriptional level of *gsp*D was analyzed in each chromosome of BL21 star (DE3) and a *gspDE*-deficient BL21 star (DE3) (ΔgspDE) strains using quantitative reverse transcription real-time PCR (Fig. 5A). The mRNA level of the *gspD* gene in the BL21 star (DE3) strain was 43.7-fold higher than that of the ΔgspDE, indicating that the *gspD* gene was expressed in BL21 star (DE3) under fermentation conditions performed in this

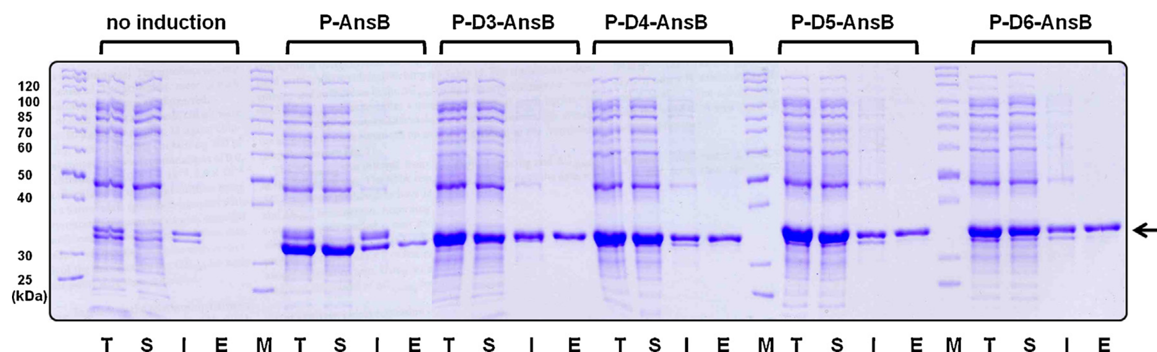

Fig. 3. SDS-PAGE analysis of recombinant AnsBs fused with the polyanionic tag consisting of 3–5 or 6 aspartates at the N-terminus of AnsB. After 24 h induction, the cells were harvested, disrupted and fractionated into total (T), soluble (S), insoluble (I) and extracellular (E) protein fractions. M indicates the protein size marker and the arrow points the protein band of recombinant AnsBs.

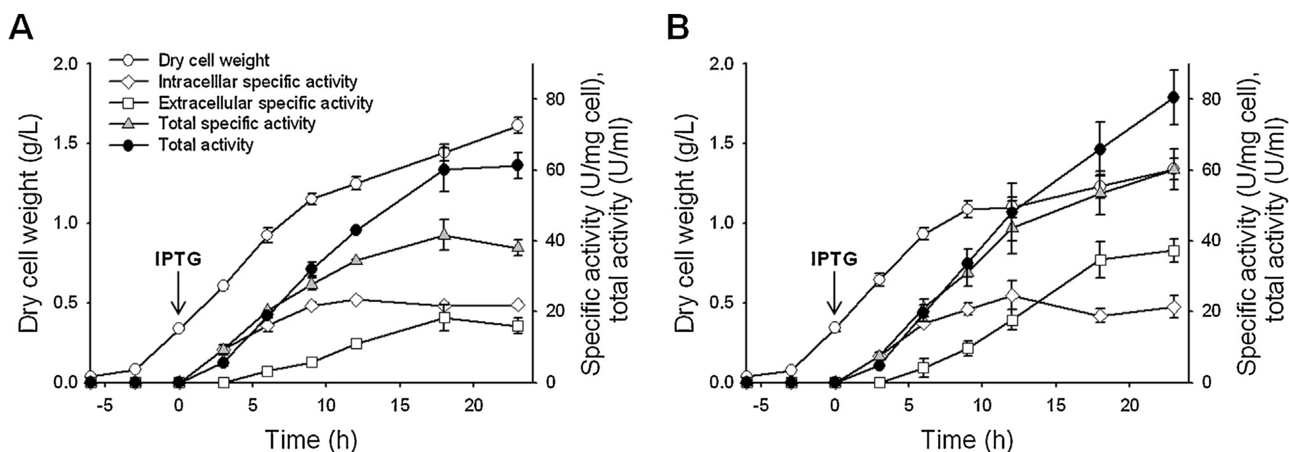

Fig. 4. Production of recombinant AnsBs in batch fermentation of recombinant *E. coli* strains. Batch production of P-AnsB (A) and P-D5-AnsB (B) were conducted in triplicate using 20 g/l glucose at 200 rpm and a culture temperature was shifted from 37 °C to 20 °C after IPTG induction.

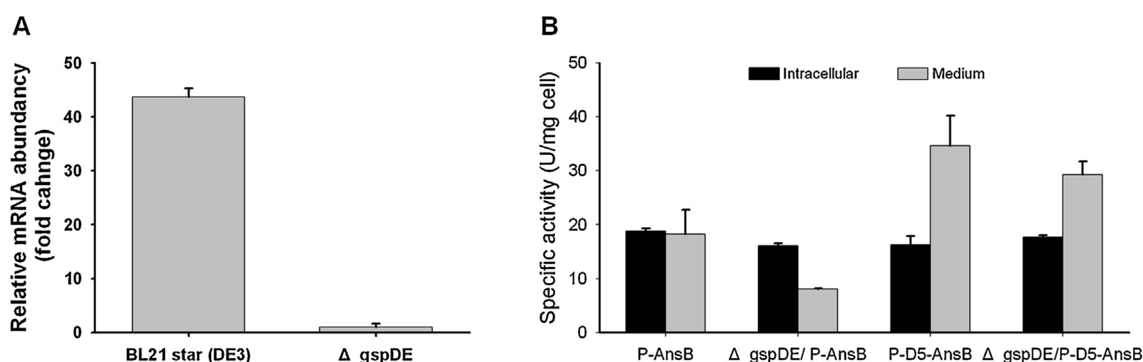

Fig. 5. Role of general secretion pathway in AnsB secretion. (A) The relative mRNA levels of *gspD* determined by quantitative real-time PCR analysis. Details for preparation of total cellular RNA and real-time PCR are given in materials and methods, and the mRNA levels of *gspD* were normalized to the *gapC* mRNA level. (B) Effects of the *gspDE* gene deletion on expression and localization of P-AnsB and P-D5-AnsB. Protein samples of AnsBs were prepared after 24 h of IPTG induction. *E. coli* BL21 star (DE3) strain and *gspDE*-deficient strain (△gspDE) were used as the host strains. The experiments were conducted in triplicate.

study. We hypothesized that the *gsp* genes encoding a putative secreton would be a factor specifically affecting transport efficiency of the D5-AnsB across the outer membrane as observed for D5-CalB. Based on this hypothesis, expression vectors of pET-26b(+)-P-AnsB and pET-26b(+)-P-D5-AnsB were transformed to ΔgspDE [17] lacking a part of the 15 *gsp* genes coding for all components of the GSP secreton. To assess the effects of destruction of a part of the GSP secreton on recombinant AnsBs expression, batch fermentations of the ΔgspDE/P-AnsB and ΔgspDE/P-D5-AnsB strains were performed, and the crude proteins were subjected to AnsB activity assay (Fig. 5B). Intracellular levels of AnsB and D5-AnsB were not influenced by the *gspDE* deletion, and the destruction of *gspDE* resulted in 56% and 15% reduced extracellular specific activities of AnsB and D5-AnsB, respectively. These results indicated that the transport efficiency enhancement of D5-AnsB was partially ascribed to the specific recognition by the GSP components, and a large fraction of D5-AnsB might be transported across the outer membrane by unknown mechanisms [24].

4. Discussion

An extracellular secretion strategy is beneficial for mass production of target proteins, reduction of metabolic burden on the host cells, prevention of intracellular proteolytic degradation, and easy separation and purification without a cell disruption process. Even though Gram positive bacteria and fungi are preferable for commercial production of various proteins, a work-horse of *E. coli* is also attractive as a host microorganism. Development of various signal peptides and modulation of periplasmic machineries have improved extracellular secretion of target proteins considerably, but of which titers are still lower than those expressed in other bacterial and fungal systems in many cases [21,24] because of its inherent problems. Meanwhile, the enhancement of target protein solubility for reduction of its inactive inclusion bodies has been somewhat achieved by connecting highly soluble and cytoplasmic fusion partners with coexpression of chaperones and foldases [14,15]. However, an application of current solubility-enhancing strategies is limited to cytoplasmic protein expression since the fusion partners are probably too big to penetrate the cytoplasmic membrane and may hinder the attachment of signal peptides onto the cytoplasmic membrane [25,26].

In the previous study, we developed novel N-terminal tag systems with only 1–9 repeated anionic amino acids to increase soluble expression or extracellular secretion of *C. antarctica* lipase B (CalB) by the pelB signal sequence [17]. Especially, the 5-aspartate tag substantially increased an extracellular expression level of recombinant CalB. AnsB is an *E. coli* periplasmic enzyme with a molecular weight of 35 kDa and forms a homo-tetrameric structure composed of closely packed dimmers [5] like CalB. Therefore, 3–6 aspartate tags were applied to increasing extracellular secretion of

AnsB. It was reported that recombinant AnsB fused with the pelB signal sequence was produced in the medium at about 5 g/l by an unknown mechanism [7]. Introduction of the pelB signal sequence increased extracellular expression of AnsB by a 2 fold, relative to the expression of AnsB only (Fig. 2). In addition, the pelB signal sequence triggered intracellular expression of recombinant AnsBs, that accords with the general concept of reduced metabolic burden by extracellular protein expression. When we insert five aspartates between the pelB signal sequence and AnsB, the total expression and extracellular secretion levels of AnsB further increased as the same as the case of CalB. Interestingly, expression patterns of recombinant AnsBs were quite different in each case. As shown in Fig. 4, intracellular expression profiles of both P-AnsB and P-D5-AnsB were similar. Consistent with the other studies, the extracellular expression level of P-D5-AnsB increased considerably after the intracellular expression reached a certain level [27,28]. Considering these phenomena, it was suggested that *E. coli* has a maximum intracellular space for protein accumulation and saturation of this space might be a prerequisite to efficiently secrete P-D5-AnsB into the medium. This hypothesis was confirmed by the observation that the destruction of *gspDE* resulted in decreases in extracellular activities of recombinant AnsBs without affecting their intracellular activities (Fig. 5B).

Although the *E. coli* K-12 strain contains the *gsp* genes that are homologous to those encoding other secretons, the *gsp* genes are transcriptionally silent under standard laboratory conditions [29]. However, we observed that endogenous expression levels of the *gsp* genes in the BL21 star (DE3) strain are sufficient to transfer AnsB into culture medium. Actually, more than half of AnsB (56%) were exported to the medium by the GSP machinery.

In the periplasmic space, proteins adopts tertiary and even quaternary structures in order to be recognized by the GSP secreton [30]. Attachment of the N-terminal five aspartate tag might induce a quaternary structure change of AnsB and affect the transport efficiency of AnsB across the outer membrane [17]. The five aspartates decreased the portion of extracellular AnsB exported by the GSP components from 56% to 15%. This result indicated that a large fraction of D5-AnsB might be transported across the outer membrane by other mechanisms. In conclusion, we clearly showed that simple fusion of aspartate could be applied to increasing total expression and extracellular secretion levels of *E. coli* AnsB up to 40.8 U/ml in fermentation medium. This pelB-anion amino acid tag-based expression system for AnsB can be extended to production of other proteins translocated to the periplasmic space by the pelB signal sequence and adopting a dimer-based oligomer as the same as recombinant AnsBs.

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