Soluble Production of CMP-Neu5Ac Synthetase by Co-expression of Chaperone Proteins in *Escherichia coli*

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Cytidine 5'-monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac) is an essential precursor for the synthesis of sialyloligosaccharides by sialyltransferase [3]. Free sialyloligosaccharides are found at high concentrations in human milk and are known to have both anti-infective and immunostimulating properties [1, 5]. Although most bacteria do not produce *N*-acetylneuraminic acid (Neu5Ac), several pathogenic bacteria, including species of *Neisseria* and *Campylobacter*, can synthesize it and display sialylated oligosaccharides on their cell surface to mimic mammalian cells and evade the host's immune system [6, 2]. The high cost of the essential substrate for sialyltransferase, CMP-Neu5Ac, limits the commercial production of sialyloligosaccharides [7]. For synthesis of CMP-Neu5Ac by conjugation of cytidine triphosphate (CTP) and Neu5Ac in recombinant *Escherichia coli*, the CMP-Neu5Ac synthetase gene (neuA) should be cloned and over-expressed in soluble form.

We have cloned the neuA gene from *E. coli* K1 and over-expressed it in *E. coli* K12; however, the proteins expressed accumulated in the form of biologically inactive inclusion bodies, which are aggregates and amorphous masses of expressed proteins (Fig. 1A). Various approaches to minimize inclusion body formation, such as expression at low temperature [8] and induction with low inducer concentration [11], were determined to be ineffective. Moreover, the denaturation-refolding process [9] is not applicable for enzymes in cells; soluble expression of heterologous proteins is a prerequisite for the operation of microbial cell factories.  

Here, we report a method that solves the above dilemma by recovering the capability of the quality control cell system in order to prevent the formation of misfolding-prone proteins by co-expression of 5 chaperone proteins (GroEL-ES and DnaK-DnaJ-GrpE). The molecular chaperones are a group of structurally diverse proteins highly conserved in all kingdoms of life, which form a complex network that assists with proper protein folding, prevents misfolded protein deposition, and dissolves deposits of misfolded pro-
For this, the neuA gene was amplified by PCR with Ex taq polymerase (Takara, Kyoto, Japan), using genomic DNA from *E. coli* K1 as a template and the following primers: neuA-F (5'-CATGCCATGGGGATGAGAACAAATTATTGC-3') and neuA-R (5'-CGCAGATCTCATTTAACATCTCGCTAT-3'). The *Nco* I and *Bam* HI sites are underlined.

*E. coli* K1 (KCTC 2441) was obtained from the Korean Collection for Type Culture. A 1,257-bp DNA fragment was cloned in pETduet-1 (Invitrogen, Carlsbad, CA, USA) and designated as pET-A. The vectors pG-KJE8 (GroEL, GroES, DnaK, DnaJ, and GrpE), pGKJE7 (DnaK, DnaJ, and GrpE), and pGro7 (GroEL and GroES) for the controlled expression of chaperones under the control of *L*-arabinose were obtained from Takara. The vectors were introduced into *E. coli* TOP 10 for cloning and *E. coli* BL21 star (DE3) cells for gene expression. The recombinant *E. coli* cells were cultured in 100 ml LB broth at various temperatures and cells were withdrawn after 0, 4, 8, and 12 h. When appropriate, antibiotics were added to the media at the following selective concentrations: ampicillin (100 µg/ml) for pETduet-1 and chloramphenicol (20 µg/ml) for other vectors. The cells were induced by various IPTG concentrations and collected by centrifugation at 8,000 × g. The pellets were disrupted by sonication and then aliquots of cell lysates were separated into total, soluble, and insoluble fractions. The insoluble fraction was concentrated 10-fold (v/v) more than the soluble fraction. The proteins expressed in each experiment were analyzed by SDS-PAGE and stained with Coomassie brilliant blue.

Fig. 1A shows that neuA protein was successfully expressed in the recombinant *E. coli* incubated at 37°C after 4, 8, and 12 h from induction with 0.1 mM IPTG. However, the enzyme was mainly detected in the insoluble fraction, indicating the formation of inclusion bodies under these conditions. In order to increase the soluble and active portion of the protein, the expression temperature was lowered to 25 and 20°C; however, this made the insoluble bands of the protein thicker. This result indicates that temperature control was not effective at solubilizing neuA in *E. coli* (Fig. 1B). Changes in IPTG concentrations (0.05 mM and 0.01 mM) were subsequently examined. The results showed that when neuA was induced with 0.05 mM IPTG, the inclusion body band was still thicker than the soluble protein band. However, when neuA was induced with 0.01 mM IPTG, the soluble protein band was of the same thickness as the insoluble protein band (data not shown). Therefore, we concluded that the optimal expression conditions for neuA protein are cultivation at 20°C and induction with 0.01 mM IPTG for 12 h.

The foregoing results clearly show that the translated neuA peptides are quickly aggregated in *E. coli*. To increase the soluble fraction of neuA, we optimized the expression conditions by lowering the culture temperature and IPTG concentration; however, the inclusion bodies were still observed as thick bands. Therefore, we employed a molecular chaperone that is known to be effective for increasing soluble expression of heterologous proteins. Plasmids containing the neuA gene and molecular chaper-