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

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CMP-Neu5Ac synthetase is a key enzyme for the synthesis of CMP-Neu5Ac, which is an essential precursor of sialylated glycoconjugates. For the soluble expression of the CMP-Neu5Ac synthetase gene (neuA) from *Escherichia coli* K1, various heat shock proteins were co-expressed in *E. coli* BL21 (DE3) Star. In order to do this, a pG-KJE8 plasmid, encoding genes for GroEL-ES and DnaK-DnaJ-GrpE, was co-transformed with neuA and was expressed at 20°C by the addition of 0.01 mM IPTG and 0.005 mg/ml L-arabinose. The co-expression of a variety of heat shock proteins resulted in the remarkably improved production of soluble CMP-Neu5Ac synthetase in *E. coli*.

**Keywords:** CMP-Neu5Ac, heterologous protein, heat shock proteins, chaperone, recombinant *E. coli*

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.

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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-

teins [4, 10].

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'-CATGCCATGGGGATGAGAACAAAA-ATTATTGCG-3') and neuA-R (5'-CGCGGATCCTCATTT-AACAATCTCCGCTAT-3'). The *Nco*I *and Bam*HI sites introduced 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 Luria-Bertani (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 chlorampenicol (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-

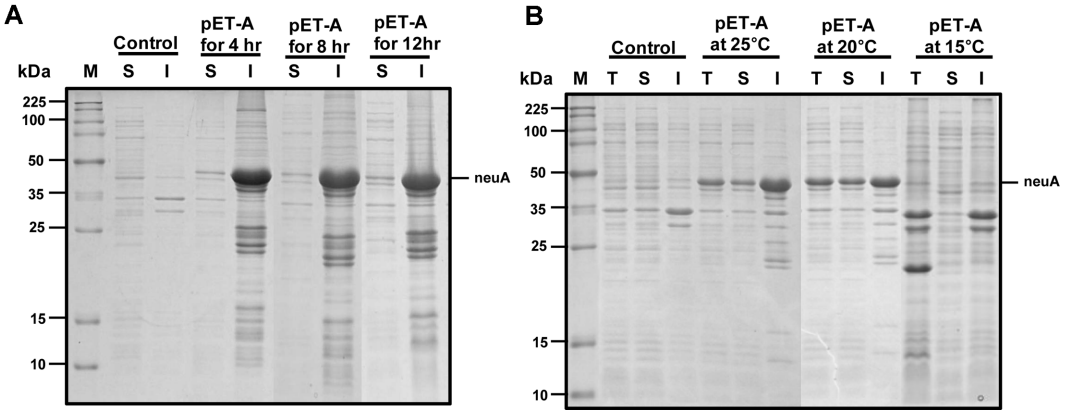


**Fig. 1. Optimization of cultivation period (A) and temperature (B) for neuA expression.** *Escherichia coli* BL21 (DE3) Star with the pET system harboring the neuA gene under the control of a T7 promoter was induced by adding 0.1 mM IPTG at 37°C. Control, negative control cells from *E. coli* harboring pETduet-1; pET-A, cells from *E. coli* harboring pET-A. Lane M, protein size markers (Promega); lane T, total protein; lane S, soluble protein fraction; lane I, insoluble protein fraction.

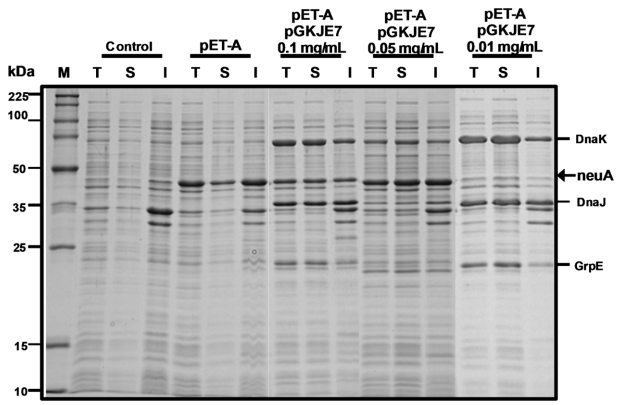


**Fig. 2. SDS–PAGE analysis of co-expressed pET-A and molecular chaperone vector, pGKJE7 induced with various concentrations of L-arabinose at 20°C.** Recombinant *Escherichia coli* were grown at 20°C for 12 h and induced with 0.01 mM IPTG for pET-A expression and 0.1 to 0.01 mg/ml L-arabinose for chaperone expression. Lane M, protein size markers (Promega); lane T, total protein; lane S, soluble protein fraction; lane I, insoluble protein fraction; control, negative control cells from *E. coli* harboring pETduet-1; pET-A, cells from *E. coli* harboring pET-A; pET-A and pGKJE7, cells from *E. coli* harboring pET-A and pKJE7.

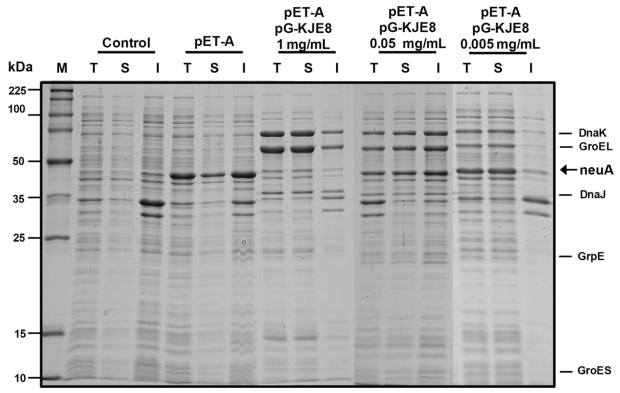


**Fig. 3. SDS-PAGE analysis of co-expressed pET-A and molecular chaperone vector pG-KJE8 induced with various concentrations of L-arabinose at 20°C.** Recombinant *Escherichia coli* was grown at 20°C for 12 h and induced with 0.01 mM IPTG for pET-A expression and 0.1 to 0.01 mg/ml L-arabinose for chaperone expression. Lane M, protein size marker (Promega); lane T, total protein; lane S, soluble protein fraction; lane I, insoluble protein fraction; control, negative control cells from *E. coli* harboring pETduet-1; pET-A, cells from *E. coli* harboring pET-A; pET-A and pG-KJE8, cells from *E. coli* harboring pET-A and pG-KJE8.

ones were cotransformed and coexpressed in *E. coli.* When molecular chaperones were induced with various L-arabinose concentrations, all chaperone proteins were successfully expressed; however, the expression level of neuA was lowered (Figs. 2 and 3). This result could have been due to the fact that the induction strength of 0.1 mg/ml L-arabinose for chaperones was stronger than that of the 0.01 mM IPTG for neuA, thus resulting in higher expression of the chaperones than the neuA protein. Therefore, in the next experiment, we needed to weaken the induction strength of chaperones, and thus the L-arabinose concentrations were incrementally decreased. As shown in Fig. 2, when the chaperones of pGKJE7 were induced with 0.05 mg/ml L-arabinose, both chaperones and neuA had similar levels of expression; however, the insoluble neuA band still remained and significant solubilization of the neuA fraction was not observed. When the induction concentration of L-arabinose was lowered to 0.005 mg/ml, the soluble fraction of neuA was markedly increased by co-expression of pG-KJE8 (Fig. 3). Soluble neuA was most efficiently expressed when it was coexpressed with pG-KJE8 that encoded DnaK-DnaJ-GrpE and GroES-GroEL. However, the coexpression of a minichaperone, such as pGro7, did not increase the soluble expression of neuA (data not shown).

In conclusion, overexpression of DnaK-DnaJ-GrpE and GroELGroES increased the amount of neuA soluble protein, probably because interactions between DnaK-DnaJ-GrpE and nascent polypeptides are critical in an early step of neuA folding. Meanwhile, the total amount of neuA was decreased due to competitive consumption of limited amounts of metabolic energy and precursors in the cell, which are required to express DnaK-DnaJ-GrpE and/or GroEL-GroES as well as neuA. The recombinant *E. coli* system constructed in this study can be used as a microbial cell factory for the synthesis of CMP-Neu5Ac by intracellular expression of soluble CMP-Neu5Ac synthetase.

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## References

1. Boehm G, Stahl B. 2007. Oligosaccharides from milk. *J. Nutr.* **137**: 847-849.
2. Gilbert M, Cunningham AM, Watson DC, Martin A, Richards JC, Wakarchuk WW. 1997. Characterization of a recombinant *Neisseria meningitidis* alpha-2,3-sialyltransferase and its acceptor specificity. *Eur. J. Biochem.* **249**: 187-194.

3. Lee WH, Shin SY, Kim MD, Han NS, Seo JH. 2012. Modulation of guanosine nucleotides biosynthetic pathways enhanced GDP-L-fucose production in recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **93**: 2327-2334.
4. Mogk A, Tomoyasu T, Goloubinoff P, Rüdiger S, Röder D, Langen H, *et al*. 1999. Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. *Embo. J.* **18**: 6934-6949.
5. Nakano T, Sugawara M, Kawakami H. 2001. Sialic acid in human milk: composition and functions. *Acta Paediatr Taiwan.* **42**: 11-17.
6. Preston A, Mandrell RE, Gibson BW, Apicella MA. 1996. The lipooligosaccharides of pathogenic gram-negative bacteria. *Crit. Rev. Microbiol.* **22**: 139-180.
7. Ringenberg M, Lichtensteiger C, Vimr E. 2001. Redirection of sialic acid metabolism in genetically engineered *Escherichia coli*. *Glycobiology.* **11**: 533-539.
8. Schein CH. 1989. Production of Soluble Recombinant Proteins in Bacteria. *Nature.* **7**: 1141-1148.
9. Sørensen HP, Mortensen KK. 2005. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microb. Cell Fact.* **4**: 1.
10. Warrick JM, Chan HY, Gray-Board GL. 1999. Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70. *Nat. Genet.* **23**: 425-428.
11. Weickert MJ, Doherty DH, Best EA, Olins PO. 1996. Optimization of heterologous protein production in *Escherichia coli*. *Curr. Opin. Biotechnol.* **7**: 494-499.

## 국문초록

**샤페론 단백질 동시 발현기술을 이용한 수용성 CMP-Neu5Ac Synthetase 생산**

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CMP-Neu5Ac synthetase는 sialyated 된 glycoconjugates의 전구체로 사용되는 CMP-Neu5Ac를 합성하는데 관여하는 주요 효소이다. *Escherichia coli* K1에서 유래한 CMP-Neu5Ac synthetase 유전자 (neuA)는 평소 *E. coli* BL21(DE3)에서 비수용성으로 생성되는데, 이를 수용성 단백질로 생산하고자 여러 가지 샤페론 단백질 동시 발현기술을 이용하였다. 이를 위해, GroEL-ES와 DnaK-DnaJ-GrpE를 암호화하는 pG-KJE8 plasmid와 neuA를 동시 형질전환 시켰고 0.01 mM IPTG와 0.005 mg/ml의 L-arabinose로 유도하여 20°C에서 발현시켰다. 그 결과, *E. coli*에서의 수용성 CMP-Neu5Ac Synthetase 생산이 현저하게 증가하였다.