융합 단백질을 이용한 내포체를 형성하는 탄수화물 합성 효소의 용해

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Solubilization of carbohydrate synthesis enzymes forming inclusion body by protein fusion

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서론

Renin binding protein(RhRnBp) is eucarytic protein with GlcNAc 2-epimerase activity. RhRnBp form homodimer. The folding and stability of RhRnBp is critically dependent on the presence of two inter disulfide bond within two monomer (1).

During protein biogenesis, cysteine oxidation occurs predominantly after a polypeptide chain has been exported from the highly reducing environment of the cytoplasm into a more oxidizing compartment, such as the mammalian endoplasmic reticulum or the bacterial periplasmic space. For this reason, secretion from the cytoplasm using a bacterial leader peptide is required for the expression of soluble eucaryotic protein in *Escherichia coli* (2). Studies on the expression of eucaryotic protein in the bacterial periplasm have indicated that the yield of active protein depends on the amino acid sequence. The low yields are thought to result primarily from poor translocation across the cytoplasmic membrane and/or aggregation in the periplasm. These problems can be alleviated to some degree by coexpression of periplasmic chaperones, by using gene fusion technology (3), or through protein engineering (4). Unfortunately, there are many antibody fragments for which none of these approaches is effective. Expression in the cytoplasm obviously circumvents cell toxicity issues associated with aberrant protein secretion. In addition, the presence of ATP-dependent chaperones in the cytoplasm could potentially assist impreventing off-pathway folding reactions leading to the formation of inclusion bodies. However, the cytoplasm is normally maintained at a low redox potential (approx. 2270 mV) via the action of thioredoxin and glutathione/glutaredoxin. As a result, oxidative protein folding does not normally occur in that compartment (5). Geneticstudies by Beckwith and co-workers first showed that null mutations in thioredoxin reductase (the *trxB* geneproduct) render the cytoplasm more oxidizing, allowing the formation of structural disulfide bonds in secreted proteins expressed without a leader peptide (6) . The expression of proteins with disulfide bonds, including antibody fragments in *trxB* strains, has

been investigated (7) . Proba *et al.* reported a low yield(2 mg/L) of a levan-binding(ABPC48) single-chain Fv antibody in shake flask cultures (8). Recently, Bessette *et al.* showed that an even higher yield of oxidized proteins can be obtained in the cytoplasm of *trxB gor* double mutants (*gor* is the gene encoding glutathione reductase). The *trxB gor* strain grows poorly in the absence of an exogenous reductantand accumulates suppressor mutations that restore rapid growth. In such a genetic background (strainFA113), a significant amount of enzymatically active alkaline phosphatase, containing two disulfide bonds, is formed. Proteins with a large number of cysteines are likely to form nonnative disulfide bonds during oxidative folding (9).

Currently, the most widely implemented carrier proteins for fusion protein expression in *E. coli* are ubiquitin(Han ef al, 1988), NusA (Gregory et al., 1999), *E. coli* maltose-binding protein (MBP) (di Guan et al., 1988), and *E. coli* thioredoxin (LaVallie et al. 1993)(10-13). MBP was chosen as fusion partners because of the combined advantage of high-level expression and affinity purification; on the other hand, ubiquitin, thioredoxin and NusA were chosen based on its high-level soluble protein expression.

In previous paper, It was reported that MBP seems to function as a molecular chaperone that promotes the solubility and stability of scFvs that are fused to it (14).

Herein, we report the construction of plasmid vectors for the expression of the RhRnBp in the *E.coli* cytoplasm. The yield of functional RhRnBp in a *trxB gor supp.* mutant strain was enhanced by the fusion of the soluble fusion partner. And fused proteins were cleaved by protease cleavage site (15). We measured the stability of MBP fusion protein.

본론

Genes of Fusion partners(Ubiquitin, MBP, thioredoxin, NusA) were fused to RhRnBp gene at amino teminal of fusion partners. Glycin and serin rich flexible linker was inserted between fusion partner and RhRnBp except ubiquitin fusion.

Expression of fusion proteins was achived in *E. coli* BL21 at 37C and 25C. BL21(DE3) is the host stain for pET system without OmpT. We expressed four fusion proeins(Ub-RhRnBp, Trx-RhRnBp, MBP-RhRnBp and NusA-RhRnBp) . Expressed fusion proteins and were divided two fractions-soluble protein and insoluble protein(inclusion body). The ratio of two fractions was analyzed by SDS-PAGE. When RhRnBp was expressed in 37C at O.D. 0.6, Whole RhRnBp was expressed into insoluble fraction. When RhRnBp was expressed in 37C at O.D. 3.0, 5% of whole RhRnBp was expressed into soluble fraction. This effect is due to the fact that low protein expression reduce the inclusion body formation. MBP and NusA showed high level soluble expression but Ub and TrxA didnt in BL21(DE3). Absolute value of soluble protein was highest in MBP fusion. The fusion partner having high molecular weight had a high soluble expression level. Fusion proteins and RhRnBp were also expressed in

origami(DE3) .
GlcNAc 2-epirmerase activities of expressed RhRnBp and four fusion proteins were measured. Initial rate of GlcNAc 2-epimerase reaction was measured and activity of epimerase could be determined. When RhRnBp was expressed in 37C at O.D. 0.6, RhRnBp did not have any activities.

When RhRnBp was expressed in 37C at O.D. 3.0, RhRnBp had activity 1.5unit/mL. RhRnBp is highly insoluble enzyme in *E. coli.* RhRnBp was expressed into insoluble form in optimal growth condition. 10% of RhRnBp was expressed into soluble form in low expression level. When expressed in BL21, fusion proteins of Ub and TrxA did not show any activity but RhRnBp and fusion proteins of MalE and NusA had. Activity of MalE fusion was similar to activity of but that of NusA fusion have only 20% activity of RhRnBp. When expressed in origami(DE3), fusion proteins of Ub and NusA did not show any activity but fusion proteins of MBP and Trx had 0.9 unit/mL and 0.6 unit/mL.

MBP-RhRnBp had the highest solubility and activity in four fusion proteins. So we purifired MBP-RhRnBp using amylose column and MBP-RhRnBp was cut by factor Xa and divided two part-MBP and RhRnBp. Fig. 4 show the proteins divided by factor Xa. Activity of divided protein was measured. The activity of treated MBP-RhRnBp was 80% of untreated MBP-RhRnBp.

RhRnBp was originated from human. RhRnBp has epimerase activity. The protein form homodimer and 12 cyteine residues. Its active site residue is cysteine 380. Origami(DE3) is null mutant without trxB and gor. Thus Origami(DE3) has high oxidative cytoplasm. In oxidative condition, formation of disulfide bond is reinforced. So we expressed four fusion proteins and RhRnBp in Origami(DE3). The activities of proteins was showed in Table. 3. In this table, Activities in BL21 was higher than those in Origami except Trx fusion. Origami cell have lower growth rate than BL21, so when the cell was cultured in same condition, origami have low cell density and low protein quantity. When fusion proteins were expressed in origami, cell density had lower than BL21. Protein quantity of cell lysate was a half of BL21. It was thought that low protein quantity brought low activity. In the case of Trx fusion, Origami had activity higher than BL21. . It was thought that low level thioredoxin reductase reinforce the production of thioredoxin fusion protein in Origami.

RhRnBp has low thermal stability. So we measured thermal stabilities of RhRnBp, MBP-RhRnBp, Trx-RhRnBp, RhRnBp had no activities after 30min incuvation in 37C . But MBP and Trx have activity after 30 incuvation in 37C. MBP-RhRnBp and RnRnBp expressed in BL21 and MBP-RhRnBp expressed in Origami was incuvated in 37C. Incuvation for 2hr had no difference. But Incuvation for 4hr had difference. MBP-RhRnBp had 45% residual activity but RhRnBp had only 10% residual activity. MBP enhanced the stability of RhRnBp in fusion expression. This results indicate that MBP act as molecular chaperone in

MBP-RhRnBp fusion protein. MBP-RhRnBp expressed in Origami has higher stability than that expressed in BL21. This data exhibits that more oxidative atmosphere enhance the stability of MBP-RhRnBp.

.Maltose binding protein has high expression level, high molecular weight, high solubility and high stability in *E. coli*. It is deduced that High expression level of maltose binding protein enhance MBP-RhRnBp and high molecular weight and high solubility enhance soluble expression of fusion MBP-RhRnBp. Ubiquitin has high expression level, low molecular weight and high stability in *E. coli*. It is deduced that high expression level of ubiquitin enhance high expression of fusion protein and low molecular weight reduce the solubilization effect to fusion protein because the ubiquitin has the small potion in fusion protein. It is thought that ubiquitn fusion system is proper to expression of small in soluble protein. NusA has high molecular weight, high solubility and high expression. But the expression level of NusA is lower than that of Maltose binding protein and NusA has higher molecular weight than Maltose binding protein. It was thought that relatively low expression level of NuA reduce expression of NusA-RhRnBp and activity of whole cell extract. It is thought that NusA-RhRnBp has low activity because NuA domain interference RhRnBp domain in fusion protein. Thioredoxin has high solubility, moderate molecular weight and moderate high expression in *E. coli*. It is thought that moderate molecular weight is the cause of moderate solubilization effect to fusion protein because thioredoxin has moderate potion in fusion protein total weight

결론

MBP, NusA, thioredoxin and ubiquitin was tested for the fusion expression of RhRnBp. In this report, MBP fusion has highest solubility, highest expression level and highest enzymatic activity. NuA fusion exhibited high soluble expression and small enzymatic activity. Trx fusion exhibit exhibited small soluble expression and small enzymatic activity. MBP fusion enhance stability of RhRnBp.

Fusion protein system is important to expression of recombinant protein. But the study of fusion protein structure was not advanced. More extensive structural analysis is demanded. And another fusion partner will have to be searched.