The Soluble Expression of the Human Renin Binding Protein Using Fusion Partners: A Comparison of Ubiquitin, Thioredoxin, Maltose Binding Protein and NusA

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Abstract human renin binding protein (hRnBp), showing *N*-acetylglucosamine-2-epimerase activity, was over-expressed in *E. coli*, but was mainly present as an inclusion body. To improve its solubility and activity, ubiquitin (Ub), thioredoxin (Trx), maltose binding protein (MBP) and NusA, were used as fusion partners. The comparative solubilities of the fusion proteins were, from most to least soluble: NusA, MBP, Trx, Ub. Only the MBP fusion did not significantly reduce the activity of hRnBp, but enhanced the stability. The Origami (DE3), permitting a more oxidative environment for the cytoplasm in *E. coli*, helped to increase its functional activity.

Keywords: human renin binding protein, *N*-acetylglucosamine-2-epimerase, fusion protein, soluble expression

INTRODUCTION

The proper expression of eukaryotic proteins in bacterial cytoplasm and/or periplasmic space is often hampered by the incorrect folding and the inclusion body formation [1]. Incorrect folding often involves an incorrect disulfide bond formation, and together with the insolubility of the protein in the compartment [2], becomes a critical factor in the inclusion body formation. One of the major causes of the incorrect folding is the oxidation/reduction potential in the bacterial cytoplasm. A low redox potential in the cytoplasm is usually maintained by the action of thioredoxin and glutathion/glutathioredoxin [3], which guarantees proper disulfide bonds in the proteins expressed in the cytoplasm, indicating oxidative protein misfolding does not often occur there [3]. In the case of the protein expression in the periplasmic space, the disulfide bond formation predominantly occurs after a polypeptide chain has been exported from the cytoplasm of a highly reducing environment to the bacterial periplasmic space of a more oxidizing environment. A null mutation in the thioredoxin reductase, *TrxB*, in *E. coli* renders the cytoplasm a relatively oxidizing environment, allowing the formation of structural disulfide bonds in the secreted proteins to be expressed without a leader peptide [4]. The expressions of proteins with disulfide bonds, including antibody fragments in *trxB*$^-$ strains, have been successfully demonstrated [5]. Recently, a higher yield of alkaline phosphatase, containing two disulfide bonds, has been obtained in the cytoplasm of *trxB gor* double mutants, which additionally knocked out the glutathione reductase [6].

Protein solubility is another important issue in inclusion body formations. The over-expression of proteins in the cytoplasm, upon induction, transiently exceeds the solubility of the protein in the cytosol, due to the confined volume of the cell. As the degree of inclusion body formation usually depends on the rate of the recombinant protein expressions, the lower induction rate of the target protein, using a low concentration of inducer, such as isopropyl thiogalactopyranoside (IPTG), at a lower growth temperature, such as 20-25°C, often overcomes the transient excess of the target protein above its solubility. For proteins with genuine low solubilities in bacterial cytoplasm, the introduction of a fusion partner is partially successful. Fusion partners can confer different properties to the folded proteins, so that the solubility of the target protein can be drastically changed. Currently, the fusion partners most widely used in *E. coli* are ubiquitin [7], thioredoxin [8], NusA [9], maltose-binding protein (MBP) [10], and glutathione S-transferase (GST) [11].

The human renin binding protein (hRnBp) shows an *N*-acetylglucosamine 2-epimerase activity, which converts *N*-acetylglucosamine (GlcNAc) to *N*-acetylmannosamine (ManNAc), a precursor of sialic acid (NeuAc) synthesis [12]. This enzyme is of interest because the biosynthesis of ManNAc in bacteria remains to be elucidated, but is well known in eukaryotic cells, and has only

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recently been cloned [12,13]. GlcNAc-2-epimerase was also recently cloned from a cyanobacterium *Synechocystis* sp. PCC6803, and used for the synthesis of NeuAc [14]. However, the properties of the eukaryotic enzyme, and its expression in prokaryotic systems, are still to be elucidated. As the hRnBp is a homodimeric protein, its folding and stability are very much dependent on the presence of two inter-disulfide bonds between the two monomers [12]. As this protein is always produced as an inclusion body in *E. coli*, we wanted to develop a means to produce it in a soluble form in the cell. To achieve this, we compared four fusion partners, *i.e.* ubiquitin, thioredoxin, maltose-binding protein and NusA, for the solubilization of hRnBp. Ubiquitins are multifunctional peptides involved in protein turnover and folding, but they also sometimes change the solubility of the fused proteins. Thioredoxin can catalyze disulfide bond formation, as mentioned above. NusA is well known for its improvement of the solubility of the fused proteins by increasing their hydrophilicity. MBP functions as a solubilizing agent and a molecular chaperone, and is famous for its ease of use in affinity purification. We also investigated the effect of cytoplasmic redox potentials on the production and stability of proteins.

MATERIALS AND METHODS

Materials

A pGME®-T easy vector system was purchased from the Promega Corporation (WI, USA). The enzymes used for the DNA manipulations were from Boehringer Mannheim GmbH (Mannheim, Germany). The primers were synthesized by Bioneer Co. (Chungwon, Korea). The amylose (amylose on agarose bead) affinity column and pMAL-c2x were from New England Biolabs (MA, USA). The pET23b, pET32.1a, pET43.1, *E. coli* BL21 (DE3) and *E. coli* Origami (DE3) were from Novagen (WI, USA). The pACYCUB harboring ubiquitin (Ub) gene was kindly donated by professor Jung, J. H. (Seoul National University).

Plasmid Construction

The DNA manipulations were performed according to the procedures described by Sambrook *et al.* [15], and the pGME®-T easy vector system was used for the cloning of PCR products. The PCR reaction was conducted in a 50 μL mixture, containing chromosomal DNA or plasmid, 50 pmoles of each primer, 2.5 mm dNTP, 1.5 mm $MgCl_2$ and 2.5 U of Taq polymerase (Takara, Japan). The conditions for the PCR cycling were: denaturation at 94°C for 30 sec, annealing at 40°C for 30 sec and extension at 72°C for 1 min. The plasmid amplification for the DNA manipulation was performed with *E. coli* DH5α.

The gene encoding recombinant human renin binding protein (RhRnBp) was amplified by PCR using the 5′ primer (TC*CATATG*GAGAAAGAGCGAGA) and the 3′ primer (CT*GAATTC*TTATTCCGCGCCTCG) from pUKHRB6, containing the recombinant human renin binding protein (RhRnBp) gene [12], and cloned into pET23b using NdeI and EcoRI resulting in pET-RhRnBp. The Ub gene was amplified by PCR using the 5′ primer (CT*CAT ATG*CAGATTTTCGTCAAGACT) and the 3′ primer (TC*G GATCC*ACCACCTCTTAGCCTCAGCAC) from pACYCUB, and cloned into the NdeI and BamHI sites of pET 43.1a to form pET-Ub. The RhRnBp gene was amplified by PCR using the 5′ primer (TC*GGATTC*ATGGAGAAA GAGCGAGA) and the 3′ primer (CT*AAGCTT*TTATTCC GCGCCTCG) from pUKHRB6, and separately cloned into pET43.1a, pET32.1a, pMalc2x and pET-Ub, using the BamHI and HindIII sites, to form pNusA-RhRnBp, pTrx-RhRnBp, pMBP-RhRnBp and pUb-RhRnBp, respectively.

Expression and Extraction of Recombinant Fusion Protein

The overnight BL21 (DE3) cultures, with the fusion protein constructs, were diluted 50-fold in fresh Luria-Bertani (LB) media and grown in 300-mL baffled flasks (50 ml medium per flask) in a shaker at 250 rpm. The temperatures were controlled at either 37°C or 25°C until the cell density at OD_{600} was 0.6. Then, the cultures were induced with 1 mM IPTG for either 6 h (at 37°C) or 12 h (at 25°C). The cells were collected by centrifugation at 4,000 g at 4°C, and washed with ice-cold 50 mM TNE buffer (Tris/HCl, pH 7.5, 200 mM NaCl and 1 mM EDTA). The collected cell pellets were resuspended in 5 mL of 50 mM Tris/HCl buffer (pH7.5), containing 1 mM EDTA, and the lysis was performed by sonication for 10 min at 20 kHz, in an ice bath (Labcaire, XL2020 sonicator). The lysate was centrifuged for 20 min at 9,000 g and 4°C. The cell pellets, *i.e.* the insoluble fractions, were separated from the supernatant, *i.e.*, the soluble fractions. The insoluble fractions were washed twice with 15 mL of 50 mM Tris/HCl buffer (pH 7.5) containing 1 mM EDTA, and resuspended in 5 mL of a 1% SDS solution. Each fraction was either stored in ice water for immediate analysis, or frozen at -70°C until required

Purification of MBP-RhRnBP

To purify the expressed MBP-RhRnBP, a fraction of the soluble fusion protein was loaded onto the amylose affinity column (column size 1.5×3 cm, 4°C). The loaded column was washed thoroughly with 50 mM Tris/HCl buffer (pH 7.5) containing 200 mM NaCl, and eluted using the same buffer with an additional 10 mM maltose. The fractions containing the proteins were pooled and concentrated to 5 mg/mL using an ultrafiltration unit. The concentrated proteins were mixed with a 20% glycerol solution, in a 1:1 ratio, and the mixture stored at -20°C until required.

Assay of GlcNAc 2-epimerase Activity

The level of GlcNAc 2-epimerase activity was measured as follows: 50 mM Tris-HCl buffer (pH 7.0), containing 10 mM $MgCl_2$, 5 mM ATP and 100 mM GlcNAc,

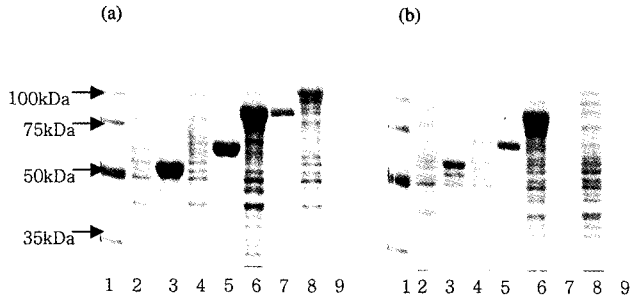

Fig. 1. 10% SDS-PAGE gel of fusion proteins expressed in BL21 (DE3) at two different temperatures (a) 37°C (b) 25°C. (lane 1: protein maker; lane 2 and 3: Ub fusion; lane 4 and 5 : Trx fusion; lane 6 and 7: MBP fusion; lane 8 and 9: NusA fusion; lane 2, 4, 6, 8: soluble fractions; lane 3, 5, 7, 9: insoluble fractions).

was mixed with a corresponding volume of the RhRnBp samples (a standard sample was at 10 μL), and the reaction mixtures were incubated for 2 h at 30°C. The substrate GlcNAc and the produced ManNAc were measured by HPLC (Waters 600) using an Aminex-87H column (Biorad) with UV detection at 210 nm. The column was eluted isocratically, with a 5 mm H_2SO_4 solution, at a flow rate of 0.7 mL/min at 40°C.

Analysis of SDS-PAGE Gel

SDS-PAGE was performed using 10% polyacrylamide gels. Stained PAGE gels were scanned with an HP scan jet, and the images obtained were analyzed using the 1-D Advance software (Amersham Biosciences), based on the measured optical densities.

RESULTS AND DISCUSSION

Effect of Fusion Partner on the Expression of RhRnBp

To investigate the effect of fusion partners on the solubility of the RhRnBp, each fusion protein was expressed in *E. coli* BL21 (DE3) at 37°C and 25°C. The cell extracts were divided into the soluble and the insoluble fractions containing the inclusion body, and analyzed by SDS-PAGE. Fig. 1 shows that the MBP-RhRnBp and NusA-RhRnBp were mainly expressed in the soluble forms, both at 37°C and 25°C. The fusion of Ub and Trx resulted in the expression of insoluble proteins. The total expression level at 37°C was higher than that at 25°C, but the ratios of the expressed fusion proteins were not significantly different at either temperature. The soluble protein contents were larger at 37°C. Table 1 shows the ratio of soluble fusion protein to total fusion protein at 37°C, the relative amount of soluble protein, and the GlcNAc-2-epimerase activity of the soluble fraction. The MBP and NusA fusions both exhibited high levels of soluble contents and GlcNAc-2-pimerase activity, whereas the Ub and Trx showed no

Table 1. Expression ratios of the soluble fusion protein and GlcNAc-2-epimerase activities (expressed in BL21(DE3))

Fusion partner	Soluble form (%)[a]	Relative soluble RhRnBp[b]	Relative activity[d]
No fusion	0	0	nd[c]
Ub	0	0	nd
Trx	0	0	1.3
MBP	65	100	100
NusA	98	15	20

[a] Soluble form (%) means the percentage of soluble fusion protein per total fusion protein. [b] Relative soluble amount was determined by setting the amount of MBP-RhRnBp as 100. [c] nd: not detected. [d] Relative activities were determined by setting the activity of MBP-RhRnBp as 100.

Table 2. GlcNAc-2-epimerase activities of fusion proteins expressed in the origami (DE3)

Fusion partner	Relative activity
No fusion	100
Ub	nd
Trx	600
MBP	900
NusA	nd

Relative activities were determined by setting the activity for the intact enzyme activity as 100.

improvement in the solubility of the fused proteins. The highest proportion of soluble protein was obtained with the MBP fusion, because the amount of the total protein in the MBP fusion was higher than that from the NusA fusion.

Expression of the Fusion Protein in Origami (DE3)

When the cell O.D. at the induction point was 3.0, equivalent to the stationary phase, *ca.*, 5% of the total RhRnBp was expressed in the soluble form (data not shown). This indicates that the environment of the inner part of the cells, corresponding to the cell growth conditions, affects the degree of soluble protein expression. *E. coli* Origami (DE3), trxB and the gor deletion mutant, allows for a relatively high oxidative environment in the cytoplasm. Thus, we would expect more functional RhRnBp to be produced in the Origami (DE3) compared to the corresponding BL21 (DE3). The GlcNAc-2-epimerase activities of the soluble RhRnBp, using pET23b-RhRnBp and each fusion protein, were compared using the Origami (DE3) as an expression host. No activity was observed with the fusion proteins of Ub and NusA, but relatively high activities were observed for those of the MBP and Trx (Table 2). The Trx and NusA fusions exhibited different expression trends, unlike in Table 1, and even the expression of the intact RhRnBp showed GlcNAc-2-epimerase activity in the Origami (DE3). These results suggest that a change in the cytoplasmic condition affected the expression of the RhRnBp.

Table 3. Activity of the MBP-RhRnBp and the cleaved MBP-RhRnBp

Condition	Relative activity
Purified MBP-RhRnBp	100
MBP-RhRnBp incubated at 25°C for 2 hr	87
MBP-RhRnBp cleaved by factor Xa at 25°C for 2 hr	73

Relative activities were determined by setting the activity for the purified enzyme activity at 0 hr as 100.

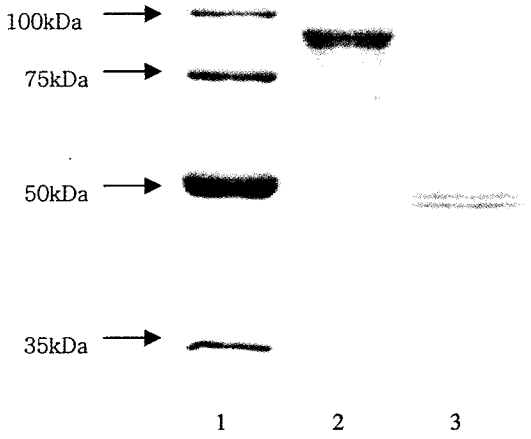

Fig. 2. 10% SDS-PAGE gel of purified MBP-RhRnBp and MBP-RhRnBp treated with factor Xa. (lane 1: protein maker; lane 2: purified MBP-RhRnBp; lane 3: cleaved MBP-RhRnBp by factor Xa).

Effect of MBP Fusion on the RhRnBp Activity and Stability

As mentioned in the Introduction, the RhRnBp is a GlcNAc-2-epimerase, which can be used as a biocatalyst for the production of sialic acid from GlcNAc. The activity and stability of the fusion proteins might be important factors for the efficient production of sialic acid. Among the four fusion proteins studied, only the MBP-RhRnBp was examined after over-expression as the soluble form (Fig. 1 and Table 1). Then, we compared the activity and stability of the MBP-RhRnBp with those of free RhRnBp.

The MBP-RhRnBp was purified through an amylose affinity column, and the purified fusion protein incubated with, or without, factor Xa. As the MBP-RhRnBp contains the cleavage site for factor Xa in the linkage of the two proteins, the addition of factor Xa can remove the free RhRnBp. Fig. 2 illustrates that the purified MBP-RhRnBp (M.W. about 90 kDa) was properly digested to the free RhRnBp (M.W 46.5 kDa) and MBP (MW. 43.3 kDa). The activity of the MBP-RhRnBp treated by factor Xa was observed to be approximately 80% that of the untreated MBP-RhRnBp, indicating that MBP fusion does not change the enzyme activity to any great extent (Table 3).

Table 4. Functional stability of the RhRnBp and MBP-RhRnBp

Form of RhRnBp and strain	Residual activity (%)		
	0 hr	2 hr	4 hr
RhRnBp in BL21	100	55	10
MBP-RhRnBp	100	55	45
MBP-RhRnBp (Origami)	100	65	60

To determine the thermal stability of the RhRnBp, the cell lysates producing RhRnBp and MBP-RhRnBp were divided into 50 μl aliquots and incubated at 37°C. Each incubated lysate was sampled at 0, 2, 4 hr, and the activity of GlcNAc 2-epimerase in each lysate measured. Residual activities were determined by setting the activity for each set of conditions at 0 hr as 100.

To investigate the effect of the MBP fusion on the stability of the RhRnBp, the extracts containing the MBP-RhRnBp or free RnRnBp expressed in BL21 (DE3) were incubated at 37°C, and their GlcNAc-2-epimerase activities measured. Table 4 shows that the MBP-RhRnBp retained 45% of its residual activity, but the intact RhRnBp retained only 10% of its residual activity after 4 h, suggesting that the MBP enhanced the stability of the RhRnBp in the fusion expression. This result also indicates that MBP can act as a molecular chaperone in the MBP-RhRnBp fusion protein [16]. The MBP-RhRnBp expressed in the Origami (DE3) showed greater stability than that expressed in the BL21, indicating that a more oxidative environment can additionally enhance the stability of the MBP-RhRnBp.

CONCLUSION

We have tested four proteins, ubiquitin (Ub), thioredoxin (Trx), maltose binding protein (MBP) and NusA, as a fusion partners for the recombinant human rennin binding protein (RhRnBP), to overcome its inclusion body formation. The fusion of MBP has improved the soluble portion of RhRnBP and a stability of the protein. The soluble production of RhRnBP, *i.e.* GlcNAc-2-epimerase, would be a ground work for the large-scale production of sialic acids from GlcNAc.

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