Improving Soluble Expression of β-Galactosidase in *Escherichia coli* by Fusion with Thioredoxin*

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ABSTRACT : Recombinant heterologous proteins can be produced as insoluble aggregates partially or perfectly inactive in *Escherichia coli*. One of the strateges to improve the solubility of recombinant proteins is fusion with a partner that is excellent in producing soluble fusion proteins. To improve the production of soluble β-galactosidase, the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112 β-gal) was fused with thioredoxin gene, and optimization of its expression in *E. coli* TOP10 was performed. KNOUC112 β-gal in pET-5b was isolated out, fused with thioredoxin gene in pThioHis C, and transformed to *E. coli* TOP10. The β-galactosidase fused with thioredoxin was produced in *E. coli* TOP10 as dimer and trimer. The productivity of fusion β-galactosidase expressed *via* pThioHis C at 37°C was about 5 times higher than that of unfused β-galactosidase expressed *via* pET-5b at 37°C. Inclusion body of β-galactosidase was formed highly, regardless of the induction by IPTG when KNOUC112 β-gal was expressed *via* pET-5b at 37°C. Fusion β-galactosidase expressed at 37°C *via* pThioHis C without the induction by IPTG was soluble, but the induction by IPTG promoted the formation of inclusion body. Lowering the incubation temperature for the expression of fusion gene under 25°C prevented the formation of inclusion body, optimally at 25°C. 0.07 mM of IPTG was sufficient for the soluble expression of fusion gene at 25°C. The soluble production of *Thermus thermophilus* KNOUC112 β-galactosidase could be increased about 10 times by fusion with thioredoxin, and optimization of incubation temperature and IPTG concentration for induction. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 12 : 1751-1757)***

Key Words : β-Galactosidase, Thioredoxin, Inclusion Body, IPTG

INTRODUCTION

In enzyme technology, the improvement of enzyme production is important. Development of DNA manipulation from transferring gene between organisms to the technology of efficient production of protein has contributed to increase enzyme production. Nowadays it is general to clone the gene of enzyme in *Escherichia coli* of high reproduction power and express the gene to high amount of protein in a vector of strong promoter that is triggered by an inducer. However the expression of heterologous recombinant proteins in bacteria sometimes results in accumulation of the protein intracellularly in inactive forms often associating to form insoluble protein aggregates called inclusion bodies (Freedman, 1992; Krueger et al., 1990; Schein, 1989). An efficient strategy avoiding expression of recombinant gene as inactive protein aggregates is to link the gene of interest to a gene that is expressed well to highly water soluble protein in *E. coli*. Staphylococcus protein A (Nilsson et al., 1985), Schistosoma glutathion-S-transferase (Smith and Johnson, 1988), *E. coli* maltose binding protein (Maina et al., 1988) and *E. coli* thioredoxin (Holmgren, 1985) are fusion partners successfully producing soluble fusion proteins. *E. coli* thioredoxin has several characteristics suitable for production of recombinant proteins overexpressed in *E. coli* as active and soluble ones. Thioredoxin can be overexpressed by plasmid and accumulated to 40% of cellular protein in cell as soluble fraction (Lunn et al., 1984), the fraction is located on the cytoplasmic face between inner and outer membrane of *E. coli* cell so that it is easily released to exterior of cell by simple treatment of freezing and thawing or osmotic shock (Lunn and Pigiet, 1982), and thioredoxin's small size of 11.675 Da lets the fusion protein preserve it's property (Katti et al., 1990). Because of those properties above, thioredoxin was generally chosen to produce soluble fusion proteins (LaVallie et al., 1993; Tanaka and Yada, 1996).

β-Galactosidase (EC 3.2.1.23) hydrolysed lactose in milk, which can solve lactose intolerance world-widely prevalent for almost people of the world except Caucasian, more than 70% of population in the world. Most of countries, where lactose intolerant people are living, are not advanced economically enough to consume lactose hydrolyzed milk of high price. So cutting down the price of lactose hydrolzed milk is important to feed it to the lactose intolerant people, and the increasing productivity of β-galactosidase is one of strategies for it.

Production of β-galactosidase of a thermophilic bacteria, *Thermus thermophilus* KNOUC112, could be increased about 250 times more by expressing it's gene in *E. coli* via pET-5b without using inducer for the promoter of pET-5b

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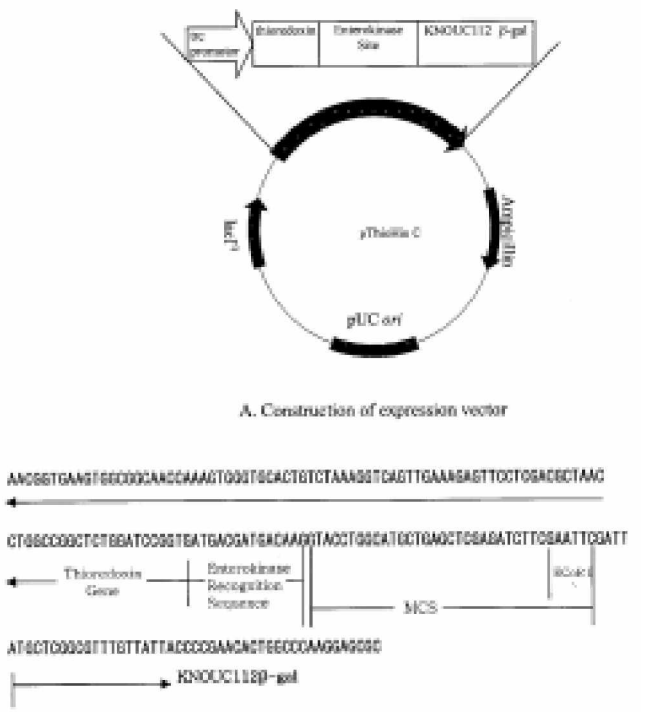

A. Construction of expression vector

B. DNA sequence at the connection region of thioredoxin gene and KNOUC112β-gal

Figure 1. Fusion of *Thermus thermophilus* KNOUC112 β-galactosidase gene (KNOUC112 β-gal) and thioredoxin gene.

(Nam et al., 2004). The productivity is expected to be improved more by fusing the gene of β-galactosidase with that of thioredoxin, and expressing it in *E. coli* via strong promoter in the presense of an appropriate inducer. In this research, improving the productivity of *Thermus thermophilus* KNOUC112 β-galactosidase was performed by fusing the gene of thioredoxin to the gene of *Thermus thermophilus* KNOUC112 β-galactosidase.

MATERIALS AND METHODS

Bacterial strains, vectors and culture condition

E. coli JM109 (DE3) and pET-5b (Promega), and *E. coli* TOP10 and pThioHis C (Invitrogen) were used for molecular cloning and expression. *E. coli* was cultivated in Luria-Bertani (LB) medium at 37°C or other temperatures suggested, if necessary, ampicillin was added to the medium at the concentration of 100 μg/ml. Cultivation of *E. coli* in liquid LB medium was performed aerobically by shaking at 220 rpm.

Fusion of β-galactosidase gene and thioredoxin gene

The β-galactosidase gene of *Thermus thermophilus* KNOUC112 (KNOUC112 β-gal) (Nam et al., 2004) in pET-5b was separated by EcoR I digestion, and purified by agarose gel electrophoresis and Gene clean method (Bio 101). The isolated KNOUC112 β-gal was ligated to the C-terminal of thioredoxin gene in pThioHis C allowing in frame fusion of *Thermus thermophilus* KNOUC112 β-galactosidase to thioredoxin. There is a enterokinase cleavage site between thioredoxin gene and KNOUC112 β-gal. Fusion of two genes was confirmed by plasmid size in agarose gel electrophoresis at 1% concentration of agarose, and by DNA sequencing at the border of thioredoxin gene and KNOUC112 β-gal. DNA sequencing was analyzed using the ABI 3730 DNA analyzer (Applied Biosystems, USA).

Expression of recombinant β-galactosidase gene

KNOUC112 β-gal in pET-5b was expressed in *E. coli* JM109 (DE3), and the fusion gene of KNOUC112 β-gal and thioredoxin gene in pThioHis C was transformed to and expressed in *E. coli* TOP10. Expression of KNOUC112 β-gal and the fusion gene of KNOUC112 β-gal and thioredoxin gene in *E. coli* on LB solid medium was confirmed by the colony's hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and that in *E. coli* cultured in liquid LB medium was done by activity of ONPG hydrolysis and X-gal hydrolysis by the gel of native polyacrylamide gel electrophoresis (PAGE) as described by Nam et al. (2004). SDS-PAGE and native PAGE were used to ascertain the expression of fusion β-galactosidase gene by a new protein band at the site of deduced molecular weight. Polyacrylamide electrophoresis (10% of acrylamide) was performed by the method of Laemmli (1970). To induce the expression driven by lacUV5 promoter in pET-5b, and by trc promoter in pThioHis C, isopropyl-β-D-thiogalactopyranoside (IPTG) was added when the liquid LB culture of *E. coli* harboring KNOUC112 β-gal in pET-5b or in pThioHis C arrived at 0.5 of A550 by incubation at 37°C, then induction was done by incubation more at the temperature suggested. It took about 3 h for the culture to arrive 0.5 of A550 after inoculation of *E. coli* preculture (0.5% of LB liquid medium containing ampicillin) at 37°C.

Determination of β-galactosidase activity

The activity of β-galactosidase produced by *E. coli* cultured in liquid LB medium was determined by hydrolysis of o-nitrophenol-β-D-galactopyranose (ONPG). Cell free extracts of *E. coli* prepared by harvesting, washing and sonification was used for the assay of β-galactosidase activity as described by Nam et al. (2004). Both of soluble fraction and insoluble fraction of cell free extract were tested for β-galactosidase activity. Cell free extract produced by sonification was centrifuged at 12,500 g for 10 min. at 4°C, and the supernatant was used as the sample of soluble fraction. The precipitates were washed two times

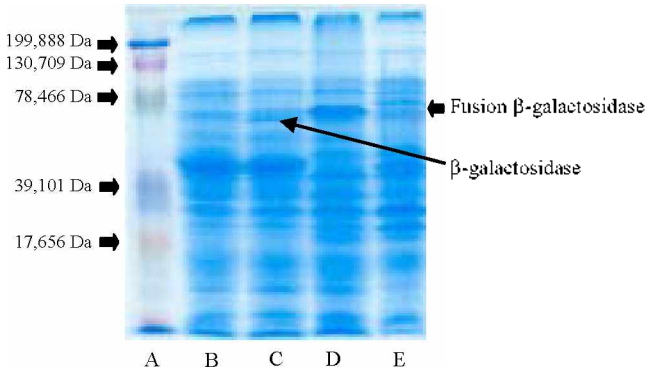

Figure 2. SDS-PAGE of cell free extracts from *E. coli* harboing the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112β-gal). A: molecular weight marker, B: *E. coli* JM109 (DE3) tranformed with pET-5b, C: *E. coli* JM109 (DE3) tranformed with pET-5b having KNOUC112 β-gal, D: *E. coli* TOP10 transformed with pThioHis C, E: *E. coli* TOP10 transformed with pThioHis C having KNOUC112 β-gal.

with Na-phosphate buffer (10 mM, pH 6.8), and used as the sample of insoluble fraction. One unit of β-galactosidase activity was defined as the amount of enzyme activity liberating 1 μmole of o-nitrophenol from ONPG per min at 70°C by the sample from 1 ml of culture.

Statistical analysis

Statistical analysis of data was carried out by comparing means according to LSD (least significant difference) test, using the GLM (general linear model) of the Procedure of SAS (2004) package program.

RESULTS AND DISCUSSIONS

Expression of *Thermus thermophilus* KNOUC112 β-galactosidase gene fused with thioredoxin gene in *Escherichia coli*

The gene of Thermus thermophilus KNOUC112 β-galactosidase was fused to the C-terminal site of enterokinase clevage site following thioredoxin gene in pThioHis C, as shown in Figure 1. The monomer of fusion gene expressed was a protein of 89 kDa bigger 16 kDa (sum of thioredoxin, enterokinase cleavage site and multiple

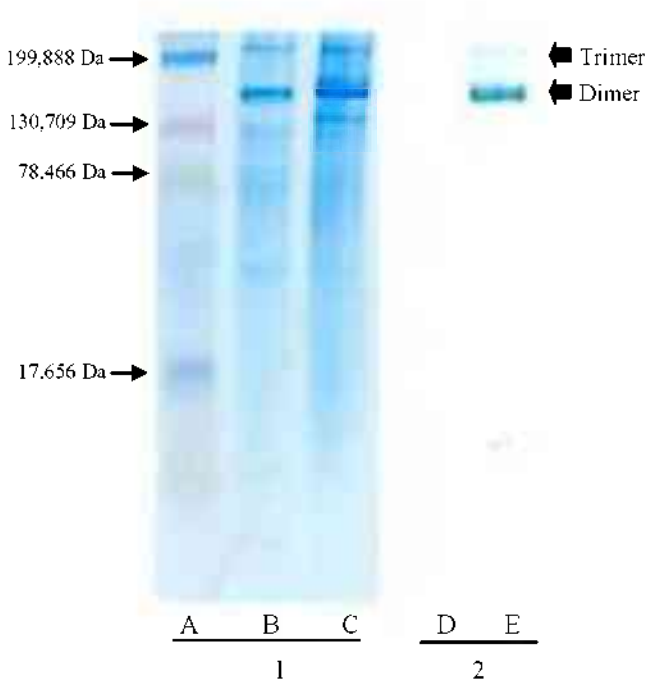

Figure 3. Native-PAGE of proteins in *E. coli* TOP10 harboring the gene *of thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112 β-gal) in pThioHis C. 1. Native PAGE, A: molecular marker, B: cell free extracts of *E. coli* TOP10 transformed with pThioHis C, C: cell free extracts of *E. coli* TOP10 transformed with pThioHis C having KNOUC β-gal. 2. X-gal hydrolysis by Native-PAGE gel at 70°C: D: cell free extracts of *E. coli* TOP10 transformed with pThioHis C, E: cell free extracts of *E. coli* TOP10 transformed with pThioHis C having KNOUC β-gal.

cloning site in pThioHis C) than the β-galactosidase of 73 kDa produced via pET-5b (Figure 2). It was expressed as two active forms that are shown at the sites of about 140 kD and about 210 kD in the result of native PAGE (Figure 3) meaning that the fusion β-galactosidase was expressed as active forms of dimer and trimer, coinciding with the result expressed via pET-5b (Nam et al., 2004).

The thioredoxin fused to KNOUC112 β-galactosidase did not interfere the activity of β-galactosidase, but increased it's productivity highly. The production of fusion β-galactosidase at 37°C (Table 2), indicated by the activity of ONPG hydrolysis, was about 5 times more than that of β-

Table 1. Growth of *E. coli* JM109 (DE3) harboring the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112 β-gal) in pET-5b and expression of KNOUC112 β-gal induced by different concentrations of IPTG at 37°C

	Incubation time post induction (h)	IPTG concentration			
		0 mM	0.07 mM	1 mM	SEM[1]
Growth (A_{550})	5	2.547[a]	1.307[b]	0.933[b]	0.253
	20	5.173[a]	2.913[b]	2.500[b]	0.423
	30	4.957[a]	3.723[b]	3.750[b]	0.208
Activity (Unit[2])	5	1.143[a]	1.053[a]	0.623[b]	0.082
	20	2.883[a]	0.667[b]	0.373[b]	0.399
	30	4.013[a]	0.863[b]	0.630[b]	0.564

[1] Standard error of mean. [2] Activity liberating 1 μmole of ONP from ONPG per minute by the sample from 1 ml of culture.
[a,b] Means different superscripts in the same row are significantly differ ($p<0.05$).

Table 2. Growth of *E. coli* Top10 harboring the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112β-gal) fused to the gene of thioredoxin in pthioHis C and expression of KNOUC112β-gal induced by different concentrations of IPTG at 37℃

	Incubation time post induction (h)	IPTG concentration			
		0 mM	0.07 mM	1 mM	SEM[1]
Growth	5	2.790[a]	2.320[b]	1.837[c]	0.151
(A_{550})	20	4.503[a]	3.660[b]	2.333[c]	0.324
	30	4.243[a]	3.567[b]	2.743[c]	0.221
Activity	5	7.393[a]	5.780[b]	1.750[c]	0.843
(Unit[2])	20	14.370[a]	8.417[b]	2.520[c]	1.767
	30	19.867[a]	13.717[b]	2.973[c]	2.485

[1] Standard error of mean. [2] Activity liberating 1 μmole of ONP from ONPG per minute by the sample from 1 ml of culture.
[a, b, c] Means different superscripts in the same row are significantly differ ($p<0.05$).

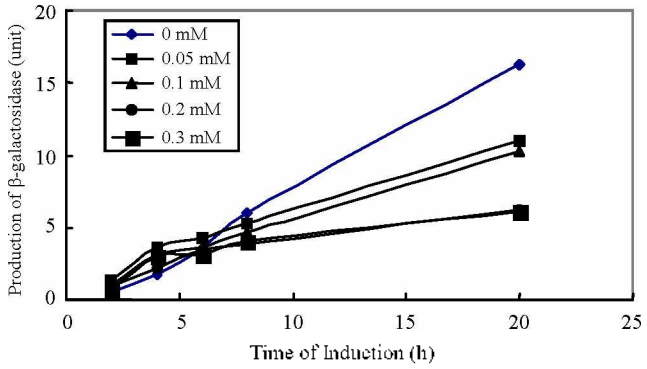

Figure 4. Effect of IPTG concentration on the expression of the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112 β-gal) fused with thioredoxin gene in *E. coli* TOP10 at 37°C.

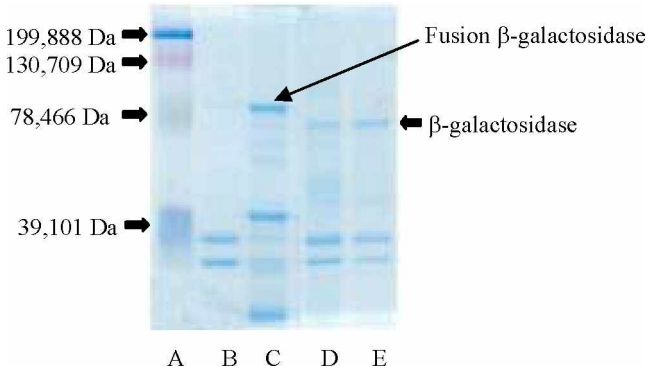

Figure 5. SDS-PAGE of insoluble protein in cell free extracts from *E. coli* harboring the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112 β-gal) in pET-5b or pThioHis C. A: molecular weight marker, B: insoluble PPT in cell free extracts from *E. coli* TOP10 harboring KNOUC112 β-gal in pThioHis C cultured at 37°C for 33 h without IPTG, C: Insoluble PPT in cell free extracts from *E. coli* TOP10 harboring KNOUC112 β-gal in pThioHis C induced by IPTG (0.07 mM) at 37°C for 30 h, D: insoluble PPT in cell free extracts from *E. coli* JM109 (DE3) harboring KNOUC112 β-gal in pET-5b cultured at 37°C for 33 h without IPTG, E: insoluble PPT in cell free extracts from *E. coli* JM109 (DE3) harboring KNOUC112 β-gal in pET-5b induced by IPTG (0.07 mM) at 37°C for 30 h.

galactosidase produced via pET-5b (Table 1), and the productivity per cell density showed similar trend of 5 to 6 times more.

Thioredoxin has high water solubility that increases the hydrophilicity of fusion protein (Lunn et al., 1984), the C-terminal of thioredoxin is accessible on the molecular surface (Katti et al., 1990) in a good position for fusion to other protein without disturbing the fused protein's shape and properties, and thioredoxin can increase thermostability of fusion proteins (LaVallie et al., 1993). Increasing solubility in water and improvement of stability by thioredoxin could be the reasons that improved the productivity of Thermus thermophilus KNOUC112 β-galactosidase fused with thioredoxin.

Overexpression by induction with IPTG and inclusion body formation

IPTG, the inducer of *lacUV5* promoter in pET-5b and trc promoter in pThioHis C, was added to induce the expression of KNOUC112 β-gal in pET-5b and KNOUC112 β-gal fused to thioredoxin gene in pThioHis C when cultures arrived at 0.5 of A550, and the cultures were incubated at 37°C. The β-galactosidase production for both of cultures decreased, and the growth was also inhibited (Tables 1 and 2). The decrease of β-galactosidase production and the inhibition of growth were more serious for the culture of *E. coli* harboring KNOUC112 β-gal in pET-5b than that harboring the fused gene in pThioHisC. The fused β-galactosidase production induced by low concentration of IPTG (0.05 mM to 0.3 mM) was higher than that of culture uninduced by IPTG in the first 4 h of incubation after addition of IPTG, but from 6 h of incubation after addition of IPTG it was reversed (Figure 4). In insoluble fractions of cell free extracts, there were no β-galactosidase activity, and found substances of same molecular weight with Thermus thermophilus KNOUC112 β-galactosidase and it's fusion one with thioredoxin as shown in the results of SDS-PAGE (Figure 5). There were similar amounts of inclusion body of β-galactosidase in the insoluble fractions from both of induced and uninduced cultures of *E. coli* JM109 (DE3) having KNOUC112 β-gal in pET-5b. But there was little inclusion body of fusion β-galactosidase in the insoluble fraction from the uninduced

Table 3. Expression of the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112 β-gal) fused with thioredoxin gene in *E. coli* TOP10 at different temperatures (β-galactosidase activity, unit[2])

Incubation hour post induction	Concentration (mM)	Temperature (°C)				
		20	25	30	37	SEM[1]
20	0	3.193^{e}	3.693^{c}	8.897^{b}	14.340^{a}	1.404
	0.07	13.363^{b}	26.370^{a}	12.957^{b}	8.417^{b}	2.147
	1	13.590^{b}	31.360^{a}	7.433^{bc}	2.520^{c}	3.398
30	0	5.720^{c}	4.437^{c}	9.077^{b}	19.867^{a}	1.840
	0.07	25.087^{b}	43.510^{a}	16.197^{c}	13.717^{c}	3.585
	1	26.013^{b}	51.277^{a}	9.177^{e}	2.973^{d}	5.657

[1] Standard error of mean. [2] Activity liberating 1 μmole of ONP from ONPG per minute by the sample from 1 ml of culture.
^{a,b,c,d} Means different superscripts in the same row are significantly differ ($p<0.05$).

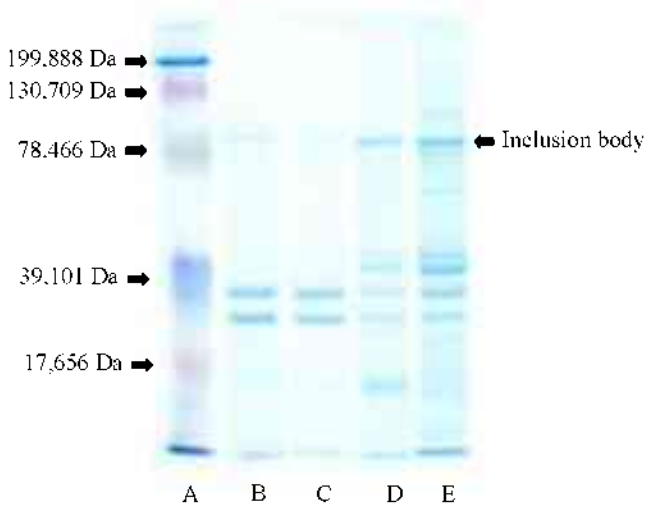

Figure 6. SDS-PAGE of insoluble fractions in cell free extract of *E. coli* TOP10 having p1hioHis C harboring the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112 β-gal) cultured at different temperatures in the presence of IPTG (0.07 mM). A: molecular weight marker, B: insoluble fraction from cell free extracts of *E. coli* TOP10 harboring KNOUC112 β-gal in pThioHis C induced by IPTG (0.07 mM) at 20°C for 30 h, C: insoluble fraction from cell free extracts of *E. coli* TOP10 harboring KNOUC112 β-gal in p1hioHis C induced by IPTG (0.07 mM) at 25°C for 30 h, D: insoluble fraction from cell free extracts of *E. coli* TOP10 harboring KNOUC112 β-gal in p1hioHis C induced by IPTG (0.07 mM) at 30°C for 30 h, E: insoluble fraction from cell free extracts of *E. coli* TOP10 harboring KNOUC112 β-gal in p1hioHis C induced by IPTG (0.07 mM) at 37°C for 30 h.

culture of *E. coli* TOP10 having KNOUC112 β-gal in pThioHis C meaning that fusion of thioredoxin to KNOUC112 β-galactosidase improved the soluble expression of KNOUC112 β-gal.

The folding process of protein in *E. coli* can be divided into several steps of polypeptide synthesis, moving to target organ and place, and folding (Grisshammer and Tate, 1995; de Gier and Luirink, 2001). All steps of the process have to be balanced for correct folding to the active structure. Many recombinant proteins tend to be produced as inclusion bodies upon production in *E. coli* especially when the recombinant protein is produced at high level exceeding the capacity of the host cell to perform the folding steps properly (Davis et al., 1999). The inclusion bodies and low β-galactosidase activity of cell free extract from *E. coli* JM109 DE3 harboring KNOUC112 β-gal in pET-5b and *E. coli* TOP10 harboring KNOUC112 β-gal in pThioHis C induced by IPTG may be due to the overexpression of those genes that are too much for host cells to handle properly.

Soluble expression of KNOUC112 β-gal fused with the gene of thioredoxin harbored in pThioHis induced by IPTG at low temperature

Fast expression is one for reasons of inclusion body formation (Davis et al., 1999). Therefore to slow down the expression of fusion gene of *Thermus thermophilus* KNOUC112 β-galactosidase so that host cells could have enough time to fold expressed proteins properly, the gene was expressed at low temperature by incubating culture at lower than 37°C after addition of IPTG. As in Table 3, in the absence of IPTG, incubation at 37°C showed the highest β-galactosidase activity. But in the presence of IPTG, incubation at lower temperatures than 37°C showed higher β-galactosidase activity than at 37°C. Induction by IPTG at 25°C was the best for active β-galactosidase production. the incubation temperature of 30°C was too high to produce active β-galactosidase properly, and the incubation at 20°C showed a good amount of β-galactosidase activity higher than those at 30°C and 37°C. There are high amount of inclusion bodies in the insoluble fractions of cell free extracts cultured at 30°C and 37°C in the presence of IPTG, but little inclusion bodies were found in those cultured at 20°C and 25°C (Figure 6).

Generally the solubility of the recombinant protein in *E. coli* increased when the incubation temperature of culture decreased (Schein and Noteborn, 1988). The optimum incubation temperatures for soluble expression of human β-defensin-2 (Peng et al., 2004), bovine procarboxypeptidase A (Seddi et al., 2003), human IL-6 and human BMP-2 (LaVallie et al., 1993) were 28°C, 15°C and 25°C,

Table 4. Soluble β-galactosidase production in *E. coli* TOP10 harboring the gene of *Thermus thermophilus* KNOUC112 β-galactosidase (KNOUC112 β-gal) fused with thioredoxin gene induced by different concentrations of IPTG at 25°C (β-galactosidase activity, unit[2])

Incubation hour post induction	Concentration (mM)							
	0	0.03	0.07	0.3	0.7	1	1.5	SEM[1]
0	0.887	0.887	0.887	0.887	0.887	0.887	0.887	0
3	0.897	1.440	1.710	2.937	3.067	3.053	2.600	0.319
5	1.290^{c}	2.387^{bc}	3.093^{ab}	4.143^{ab}	4.710^{a}	3.920^{ab}	3.137^{ab}	0.304
7	1.800^{b}	5.387^{a}	6.250^{a}	7.570^{a}	6.713^{a}	7.237^{a}	5.567^{a}	0.538
10	1.927^{b}	11.470^{a}	11.137^{a}	11.920^{a}	11.730^{a}	11.460^{a}	8.993^{a}	0.867
21	3.083^{b}	30.347^{a}	32.767^{a}	32.980^{a}	34.177^{a}	31.670^{a}	31.633^{a}	2.381

[1] Standard error of mean. [2] Activity liberating 1 μmole of ONP from ONPG per minute by the sample from 1 ml of culture.
[a, b, c, d] Means with different superscripts in the same row are significantly differ ($p<0.05$).

respectively, that are far from the optimum growth temperature of 37°C for their host cell, *E. coli*. Inclusion body formation of HIVgp41 fused with β-galactosidase could be prevented at 42°C (Hoffman et al., 2004). For *E. coli* TOP10, the incubation temperature of 30°C and 37°C were too high to prevent the formation of inclusion body, and that of 20°C might be too low for metabolism to produce soluble β-galactosidase optimally.

To find the optimal concentration for the induction of fusion gene of KNOUC112 β-gal and thioredoxin at 25°C, IPTG concentrations from 0.03 mM to 1.5 mM were tested. As shown in Table 4, in the beginning of induction until 5 h, at 0.3 mM to 1.0 mM of IPTG the production of active β-galactosidase was high, highest at 0.07 mM. But in the later incubation from 7 h of induction, there was no significant difference among 0.03 mM to 1.5 mM of IPTG, but induction by 0.07 mM to 0.7 mM of IPTG showed a little higher activity suggesting that the proper concentration of IPTG is 0.07 mM.

The production of active β-galactosidase while incubation for 23 to 24 h was increased from 2.88 unit via pET-5b to 14.37 unit by fusion with thioredoxin (Tables 1 and 2), and could be increased to 32.77 unit by inducing the fusion gene of KNOUC112 β-gal and thioredoxin gene with 0.07 mM of IPTG at 25°C (Table 4), finally about 11 times more.

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