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Development of a Plasmid Vector for Overproduction of β-Galactosidase in *Escherichia coli* by Using Genetic Components of *groEx* from Symbiotic Bacteria in *Amoeba proteus*

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Abstract A plasmid vector, pXGPRMATG-*lac-Tgx*, was developed for overproduction of β-galactosidase in *Escherichia coli* using the genetic components of *groEx*, a heat-shock gene cloned from symbiotic X-bacteria in *Amoeba proteus*. The vector is composed of intragenic promoters P3 and P4 of *groEx*, the structural gene of *lac* operon, transcription terminator signals of *lac* and *groEx*, and *ColE1* and *amp'* of pBluescript SKII. The optimized host, *E. coli* DH5α, transformed with the vector constitutively produced 117,310-171,961 Miller units of β-galactosidase per mg protein in crude extract. The amount of enzyme in crude extract was 53% of total water-soluble proteins. About 43% of the enzyme could be purified to a specific activity of 322,249 Miller units/mg protein after two-fold purification, using two cycles of precipitation with ammonium sulfate and one step of gel filtration. Thus, the expression system developed in this study presents a low-cost and simple method for purifying overproduced β-galactosidase in *E. coli*.

Key words: *groE*, heat shock, promoter, transcription terminator, protein expression, purification

Vectors for expression of proteins must contain a promoter region recognized by RNA polymerase, a sequence for ribosome binding (SD), the start codon for translation, and the stop codon that signals termination of translation. For overproduction of specific proteins by recombinant technology, a strong promoter has to be produced first. For this purpose, promoters such as λ P_L [28], *trp* [4], *lacUV5*, *tac* [6], and *nar* [21] have been developed and utilized. For the efficient utilization of a plasmid for gene expression, it is useful to have a transcription-terminator sequence that controls the size of the mRNA produced [27]. However, maximal specific activities of β-galactosidase produced in *E. coli* using various vectors were less than 50,000 Miller units/mg protein in crude extract [21].

The *groEx* cloned from endosymbiotic X-bacteria in the xD strain of *Amoeba proteus* showed an unusually high expression in *E. coli* [1]. The gene contains two open reading frames analogous to *groES* and *groEL* of *E. coli* [2; GenBank Accession M86549] and has four promoters [23]. Among those promoters, two at the 5'-extended region of *groESx* are consensus promoters found in the *groE* family of heat shock genes. The other two, P3 and P4, located within the coding region of *groESx* are novel, potent, and σ^{70} factor-dependent promoters for this gene [23]. Thus, the *groELx* gene was strongly expressed in *E. coli* by simple culture at 30°C or above without any added inducer. The effectiveness of these promoters in the expression of cloned prokaryotic and eukaryotic genes was demonstrated by developing a GroELx-fusion vector, pUXGPRM, and an ATG-expression vector, pXGPRMATG [22].

In this gene, we found two high-GC stretches of 25 and 15 bases starting from nucleotide (nt) 1907 and 1975, respectively. The first stretch forms a hairpin loop with a 5 bp stem and the second, which is C-rich and G-poor, starts from the nucleotide immediately after the stop codon (nt 1971). There is an additional hairpin loop of an 11 bp stem (from nt 1996) behind the stop codon of the *groELx* gene. The two palindromic sequences flanking the stop codon are known to signal rho-independent termination [30]. On the other hand, the C-rich stretch of nucleotides has been reported to signal rho-dependent termination [3, 31]. Thus, the termination of *groEx* transcription may be controlled by multiple factors. These signal motifs appear to be additional genetic components for the strong expression of the *groEx* gene in X-bacteria and transformed *E. coli*.

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In this study, we developed an expression vector for β-galactosidase (pXGPRMATG-*lac-Tgx*) by integrating those intragenic promoters and transcription terminator signals of *groEx* (*Tgx*). The enzyme overproduced from transformed *E. coli* could be purified by simple procedures.

MATERIALS AND METHODS

Enzymes and Chemicals

Restriction enzymes, T4 DNA ligase, Klenow enzyme, and agarose were purchased from United States Biochemicals (Cleveland, OH, U.S.A.), and the QIAEXII gel extraction kit was from Qiagen (Chatsworth, CA, U.S.A.). Sephadex G-200 was obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ, U.S.A.). Acrylamide, *N,N'*-bisacrylamide, ammonium persulfate, TEMED, ammonium sulfate, and Tris were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). For comparing enzyme activities of β-galactosidase produced in this study with those of other enzymes, we purchased β-galactosidase of *E. coli* (Cat. No. G-5635) and *Aspergillus oryzae* (Cat. No. G-7138) from Sigma Chemical Co. The enzyme of *Kluyveromyces lactis* (Maxilact XL 50000; Gist-Brocades, Netherlands) was a gift from Pasteur Dairy in Korea.

E. coli Strains and Plasmids

We used *E. coli* strains DH5α [17] from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.), XLI-Blue [8], BL21 [33], LE392 [7], and JM109 [34] from Stratagene (La Jolla, CA, U.S.A.), INVαF' [17] from Invitrogen (San Diego, CA, U.S.A.), MC4100 [10] from American Type Culture Collection (Waldorf, MD, U.S.A.), and KW251 from Promega (Madison, WI, U.S.A.) as hosts. Cells were grown in Luria-Bertani (LB) medium at 37°C in a rotary-shaker and transformed after Chung *et al.* [14]. Ampicillin (50 μg/ml) was added as a selective agent. Stock cultures of bacterial strains were stored at -70°C in LB broth containing 20% (v/v) glycerol. For the construction of vectors, we used plasmids pUXGPRM-*lac* [2], pXGPRMATG [22], pMC1403 [11], pMC1871 [32], and pBluescript SKII from Stratagene.

Vector Construction and DNA Procedures

Plasmid DNA was purified by the alkaline lysis method [24]. Parts of genetic components of *groEx* utilized in the construction of vectors are shown in Fig. 1. Digestion of DNA with restriction enzymes was performed according to the manufacturer's recommendation. DNA fragments were separated by electrophoresis on agarose gel using TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and purified by using the QIAEXII gel extraction kit by following the manufacturer's instructions. Ligation of

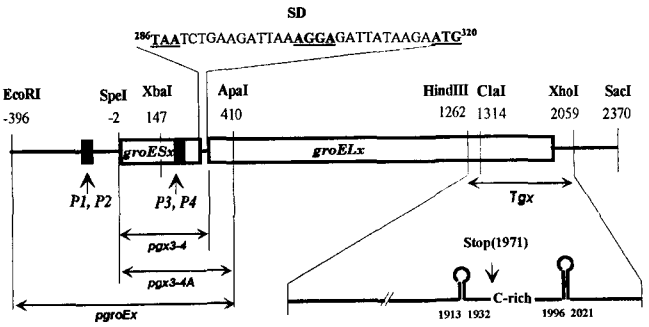

Fig. 1. The physical map of *groEx* to show parts of the gene used in the vector development.

Promoters of the *groEx* are shown with solid boxes; P1, P2: heat shock consensus promoters at nt -129~-65, and P3, P4: intragenic promoters unique to the *groEx* at nt 182~230 [2, 23]. The consensus ribosome-binding site (SD: AGGA) for *groELx* is located between the stop codon (TAA) for *groESx* and the start codon (ATG) for *groELx*. Transcription terminator of *groEx* (*Tgx*) contains two hairpin-loops (nt 1913-1932 and 1996-2021) and a C-rich fragment (nt 1975-1988). We used promoter-containing fragments, *pgx3-4*, for native protein production and *pgx3-4A* for fusion protein. Nucleotide sequences for *groEx* are available from GenBank Accession M86549 [2].

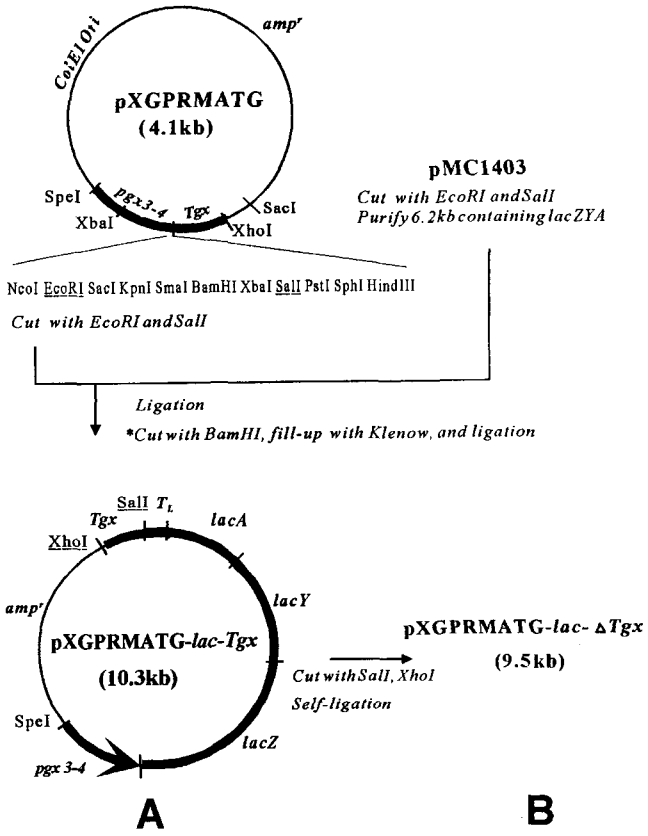

Fig. 2. The scheme for the construction of pXGPRMATG-*lac-Tgx* (A) and pXGPRMATG-*lac*-Δ*Tgx* (B).

To construct pXGPRMATG-*lac-Tgx* (A), the structural gene of *lac* operon (6.2-kb) from pMC1403 [11] was inserted into pXGPRMATG containing *pgx3-4* and *Tgx*. Then, the reading frame of the inserted DNA was adjusted to the start codon (*).

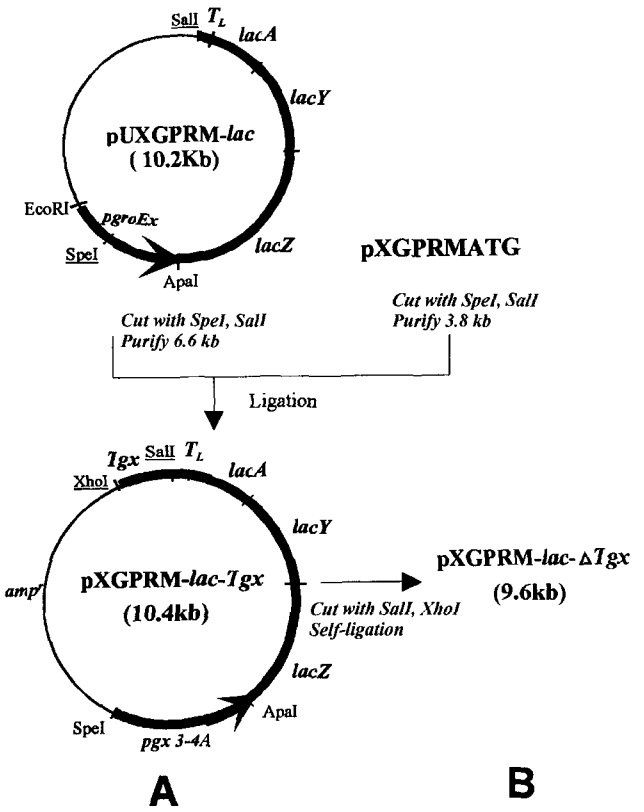

Fig. 3. The scheme for the construction of pXGPRM-*lac-Tgx* (A) and pXGPRM-*lac*-Δ*Tgx* (B).

To construct pXGPRM-*lac-Tgx* (A), the *pgx3-4* fragment of pXGPRMATG (Fig. 2) was replaced with a 6.6-kb fragment of pUXGPRM-*lac* containing *pgx3-4A* and *lac* structural gene.

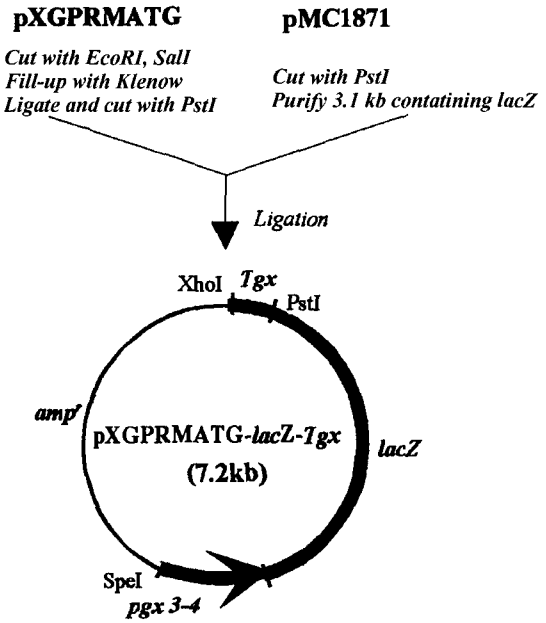

Fig. 4. The scheme for the construction of pXGPRMATG-*lacZ-Tgx*.

The structural gene of *lacZ* (3.1 kb) from pMC1871 [32] was inserted into pXGPRMATG after adjusting the reading frame to the start codon.

DNA fragments was also performed according to the manufacturer's recommendation. Schemes for the construction of plasmid vectors are summarized in Figs. 2-4.

Cell Culture and Crude Extraction of Enzyme

A single colony of transformed *E. coli* was inoculated in 5 ml of LB medium and grown to an A_{600} of 1.5. Then, cells were diluted 100 times and inoculated in 250 ml of LB medium in a 1-liter flask and grown at 37°C in a 225 rpm rotary shaker to an A_{600} of 1.7. All enzyme preparations were carried out at 4°C unless otherwise stated. Cells harvested by spinning for 10 min at 4,000×g were suspended in BB buffer (200 mM Tris-HCl pH 7.6, 200 mM NaCl, 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, and 5% glycerol) and ruptured by passage through a French press at 1000 psi. The cell lysate was centrifuged for 30 min at 15,000×g and the supernatant was used as the crude enzyme extract.

Purification of β-Galactosidase

Three grams of wet cells from 500-ml culture were suspended in 30 ml of BB buffer and a crude enzyme extract was made by the same procedures as above. While stirring the crude enzyme extract in a beaker, saturated ammonium sulfate was slowly added to make 40% saturation of ammonium sulfate. After incubation for an hour at 4°C, the suspension was centrifuged for 30 min at 15,000×g and a protein pellet was collected. Then the pellet was dissolved in 30 ml of D buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 10 mM magnesium acetate, and 10 mM 2-mercaptoethanol) and dialyzed for 18 h in D buffer with two buffer changes. For the second precipitation, we added ammonium sulfate to 35% saturation and repeated the same centrifugation and dialysis as before. After the second dialysis, the solution was spun for 10 min at 15,000 × g and the supernatant was saved.

The enzyme was further purified by gel filtration column chromatography. Sephadex G-200 swollen for 5 h in A-buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM magnesium acetate, and 10 mM 2-mercaptoethanol) was loaded and equilibrated in a 1.5×100-cm gel filtration column (BioRad; Richmond, VA, U.S.A.). After loading 1.5-ml sample protein on top, the column was eluted at 10 ml/h using A-buffer.

Cell-free β-Galactosidase Assay

β-Galactosidase activity was assayed after Platt [29] using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. An aliquot of the 10~20 μl sample that had been diluted 1000 times in BB buffer was added to 1.0 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM

KCl, 1 mM $MgSO_4$, and 50 mM 2-mercaptoethanol, pH 7.0) and equilibrated for 3 min at 28°C in a water bath. The enzyme reaction was initiated by adding 0.2 ml of 4 mg/ml ONPG to the reaction mixture at 28°C and stopped by adding 0.5 ml of 1 M Na_2CO_3 when a yellow color developed due to hydrolysis of the substrate. As an enzyme blank, BB buffer was added. Then, the color density was read at 420 nm and the specific activity was calculated [29]. Here, one unit is defined as the amount of enzyme that hydrolyzes 10^{-9} mole ONPG per min per mg protein at 28°C.

Other Methods

Protein concentration was determined by the method of Lowry *et al.* [25]. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out essentially by the method of Laemmli [20] and stained with Brilliant Coomassie blue (BCB). The separating gel was 8% acrylamide and was run for 8 h at 20 mA/plate.

RESULTS

Description of Expression Vectors for β-Galactosidase Containing *groEx* Components

By combining DNA fragments of *groEx* shown in Fig. 1, we constructed several plasmid vectors (Figs. 2-4). The *pgx3-4* fragment contained the P3 and P4 promoters and the SD region of *groEx*, the *pgx3-4A* fragment contained *pgx3-4* and 94 nt for N-terminal portion of GroELx, and the *Tgx* fragment contained transcription terminator signal motifs of *groEx* (Fig. 1). The pXGPRMATG-*lac-Tgx* (10.3 kb, Fig. 2A) and pXGPRMATG-*lac-ΔTgx* (9.5 kb, Fig. 2B) were constructed by the scheme shown in Fig. 2 for the production of β-galactosidase in the native form. They contained *pgx3-4* and structural genes (*lacZYA*) and the transcription terminator (T_L) of *lac* in common. The difference is the *Tgx* connected at the downstream of T_L in pXGPRMATG-*lac-Tgx*. An ATG-expression vector with polycloning sites, pXGPRMATG, was cut with *Eco*RI/*Sal*I and replaced with *Eco*RI-*Sal*I fragment (6.2 kb) containing *lacZYA* and T_L from pMC1403 to make pXGPRMATG-*lac-Tgx*. The pXGPRMATG-*lac-ΔTgx* was made by removing the *Sal*I-*Xho*I fragment from pXGPRMATG-*lac-Tgx*.

For a comparison with the above nonfusion constructs, we made constructs to have 31 N-terminal amino acids of GroELx fused to LacZ. A 6.6-kb fragment containing *pgx3-4A* and *lac* structural gene was excised from pUXGPRM-*lac* and inserted into the *Spe*I/*Sal*I site of pXGPRMATG to make pXGPRM-*lac-Tgx* (10.4 kb, Fig. 3A). In order to test the effect of *Tgx* in this construct, we deleted *Tgx* by *Sal*I/*Xho*I digestion and made pXGPRM-*lac-ΔTgx* (9.6 kb, Fig. 3B).

We also constructed a plasmid pXGPRMATG-*lacZ-Tgx* (7.2 kb, Fig. 4) to have *lacZ* only to shorten the transcript size. The 3.1-kb fragment containing *lacZ* was excised from pMC1871 by cutting it with *Pst*I and inserted into the *Pst*I site of pXGPRMATG after adjusting the reading frame by removing the *Eco*RI-*Sal*I fragment and filling the overhang with Klenow enzyme.

All of these recombinant plasmids had *ColE1* origin and ampicillin resistance genes from pBluescript SKII. The genetic components of *groEx* and *lac* were inserted in reverse orientation to the *lacUV5* promoter of pBluescript SKII.

Comparisons of β-Galactosidase Production among Plasmid Vectors

The levels of specific activities of β-galactosidase in crude extracts of *E. coli* transformed with plasmids described above are shown in Fig. 5. In a comparison experiment, cells transformed with pBluescript SKII and induced with 1 mM IPTG produced 21 units/mg protein [2]. Thus, all clones containing P3 and P4 promoters of *groEx* overproduced the enzyme to the level of 1300~5800 fold of that induced with IPTG in pBluescript SKII plasmid. *E. coli* transformed with pXGPRMATG-*lac-Tgx* showed the highest specific activity of 122,703 units/mg protein. When the levels of enzymes among the plasmid clones with and without *Tgx* were compared, there was about a 1.8-fold and 1.2-fold increase, respectively, depending on nonfusion and *groELx*-fusion constructs (a and b, c and d in Fig. 5). On the other hand, fusion of 5'-*groELx* to *lacZ* reduced enzyme production

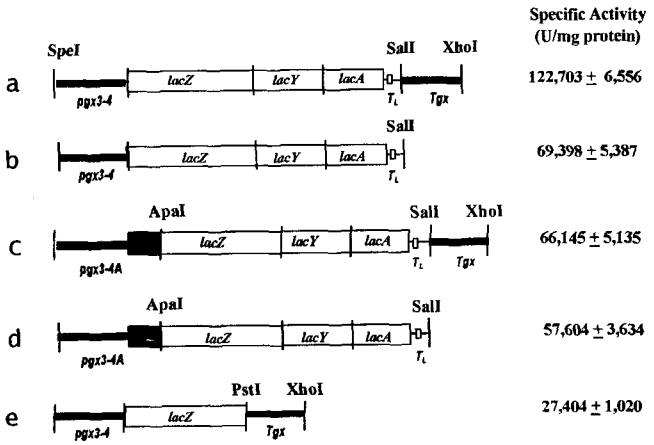

Fig. 5. Comparisons of levels of β-galactosidase produced from *lacZ* under the influence of *groEx* in various combinations.

The inserts a, in pXGPRMATG-*lac-Tgx*, b, in pXGPRMATG-*lac-ΔTgx*; c, in pXGPRM-*lac-Tgx*; d, in pXGPRM-*lac-ΔTgx*; and e, in pXGPRMATG-*lacZ-Tgx*. T_L represents the transcription terminator of *lac*. In c and d, β-galactosidase was produced as a fusion protein containing N-terminal 31-amino acids of GroELx [2]. Specific activity of the enzyme (in crude extract) is the average±standard deviation from three different cultures of *E. coli* DH5α transformed with respective plasmids.

by 43% and 17% depending on the presence or absence of *Tgx*, respectively (a and c, b and d in Fig. 5). This implies that *Tgx* had a strong positive effect on the production of the cloned enzyme. The effect was more pronounced when the enzyme was produced in its native form than as a fusion protein. Initially we constructed pXGPRMATG-*lacZ-Tgx* to overproduce the enzyme by removing unnecessary structural genes of the *lac* operon. However, deletion of *lacYA* and T_L greatly reduced the enzyme production to the level of 22% (Fig. 5a to 5e). It was apparent that *lacYA* and T_L were essential components for overproduction of the enzyme using this construct in *E. coli.*

Optimal Host for Overproduction of β-Galactosidase

In order to choose an optimal host for pXGPRMATG-*lac-Tgx*, we transformed eight genotypic strains of *E. coli* and compared the levels of β-galactosidase production by SDS PAGE (Fig. 6) and enzyme analysis. The specific activities of enzymes were proportional to the BCB-stained density of the gel (data not shown). DH5α strain of *E. coli* was found to be the best host for the expression of the gene, and the enzyme production was stable in successive cultivation.

XLI-Blue and INVαF' also showed appropriate production, but JM109, BL21, LE392, and KW251 produced only 10~25% of enzyme activity obtained from DH5α. During successive cultivation, DH5α, XLI-Blue, INVαF', and MC4100 showed stable expression. On the other hand, expression of the recombinant gene in BL21, LE392, KW251, and JM109 was unstable. JM109 that was similar in genotype with DH5α produced as much enzyme as DH5α in the early stage of transformation. However, the level of specific activity gradually decreased after successive cultivation on agar plates.

Growth and Enzyme Production of Transformed *E. coli*

Since the enzyme was produced in large amounts by transformed *E. coli*, we tested its effect on the cell growth. The growth of *E. coli* DH5α transformed with pXGPRMATG-*lac-Tgx* was compared with that of cells containing pBluescript SKII only as a control (Fig. 7). *E. coli* transformed with pXGPRMATG-*lac-Tgx* grew faster than control cells and arrived at saturation density in 10-h culture, which was 4 h earlier than the control cell culture. The cell density at the stationary phase (OD_{600} 1.7~1.8) was the same as that of cells transformed with pBluescript SKII. Thus, overproduction of α-galactosidase by harboring pXGPRMATG-*lac-Tgx* did not appear to affect cell growth.

In order to determine the optimal time for harvesting cells, we followed the change in specific activities of β-galactosidase with the cell growth (Fig. 7). The increase in enzyme activity was almost proportional to cell growth. Maximum activity was reached at the stationary phase of cell growth. Since the specific activity did not change while cells were in the stationary phase for 4 h,

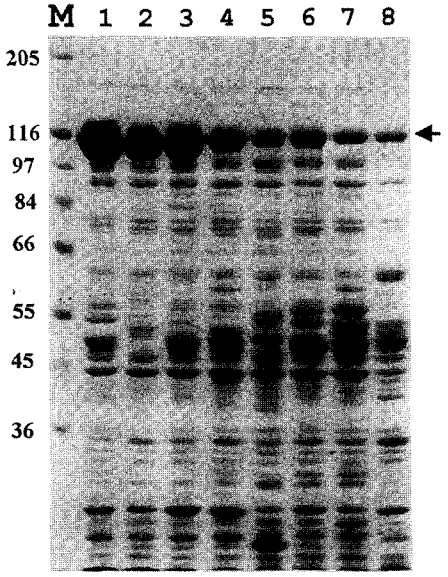

Fig. 6. Comparisons of expression level of β-galactosidase produced from various strains of *E. coli* transformed with pXGPRMATG-*lac-Tgx* shown in SDS polyacrylamide gel stained with Brilliant Coomassie blue.

Each lane was loaded with 100 μg protein of crude extract from *E. coli* strains: Lanes M; Molecular mass markers (kDa), 1; DH5α, 2; XLI-Blue, 3; INVαF', 4; MC4100, 5; BL21, 6; LE392, 7; KW251 and 8; JM109. The arrow indicates the band corresponding to β-galactosidase.

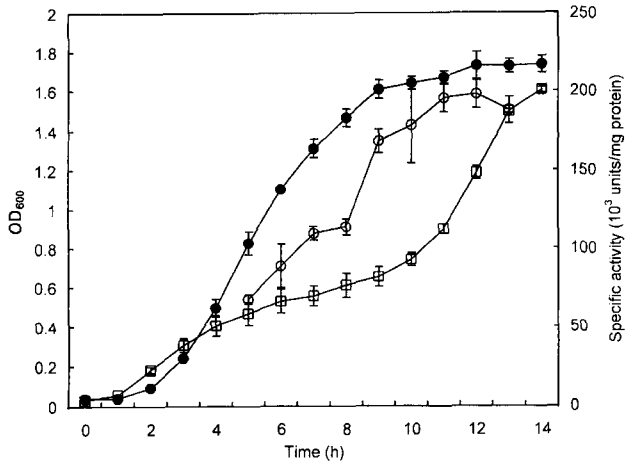

Fig. 7. Comparisons of the cell growth between *E. coli* DH5α transformed with pXGPRMATG-*lac-Tgx* (solid circles) and *E. coli* transformed with pBluescript SKII plasmid (open squares), and production of β-galactosidase (open circles) from *E. coli* DH5α transformed with pXGPRMATG-*lac-Tgx*.

The growth of cells in LB media and production of the enzyme are shown as OD_{600} and as specific activity in crude extracts, respectively. Bars represent standard deviations from three different experiments.

the enzyme appeared to be stable and was not significantly degraded during this period.

Purification of β-Galactosidase

Three grams of cells obtained from a 500-ml culture of *E. coli* DH5α transformed with pXGPRMATG-*lac-Tgx* were processed for enzyme purification. Enzymes in the crude extract were precipitated twice with ammonium sulfate and purified by one cycle of column chromatography on Sephadex G-200. The purity and activities of β-galactosidase in the fractions obtained from each step were analyzed by SDS-PAGE (Fig. 8) and cell-free β-galactosidase assay (Table 1). About 66% of the enzyme contained in the crude extract (171,961 units/mg protein) could be precipitated with 40% ammonium sulfate. The precipitate already had an enzyme activity of 257,911 units/mg protein that was higher than that of the same enzyme commercially available (Table 2). After the second precipitation in 35% ammonium sulfate, we obtained a specific activity of 295,694 units/mg protein that was equivalent to a pure grade [29]. By simple gel filtration, we further purified the enzyme to have a specific activity of 322,248 units/mg protein. This was a 1.9-fold purification. The final yield was 43% of the enzyme activity present in the crude extract. About 150 mg of the highly purified enzyme could be isolated from a liter of culture.

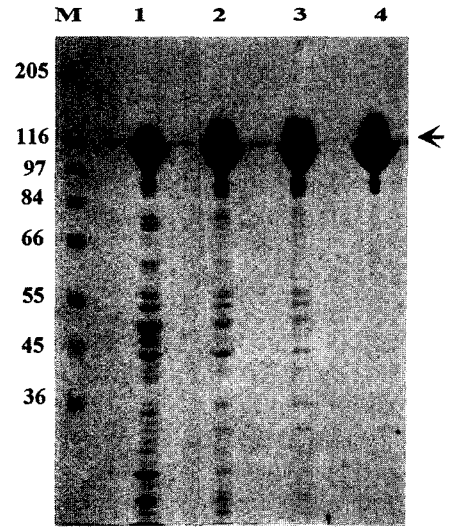

Fig. 8. Brilliant Coomassie blue-stained 10% SDS-polyacrylamide gel showing the purity of β-galactosidase obtained in purification procedures of the enzyme from *E. coli* DH5α transformed with pXGPRMATG-*lac-Tgx*.

Lanes M, molecular mass markers (kDa); 1, crude extract; 2, the first precipitate formed by 40% ammonium sulfate; 3, the second precipitate formed by 35% ammonium sulfate; and 4, proteins purified by gel filtration using the second precipitate as starting material. Each lane was loaded with 100-μg protein. The arrow indicates the band corresponding to β-galactosidase.

DISCUSSION

By developing an expression system using genetic components of the *groEx* gene, it has been possible to overproduce β-galactosidase in *E. coli* DH5α. The expression system developed in this study presents a low-cost and simple method for purifying overproduced β-galactosidase. In this system, β-galactosidase was constitutively expressed in soluble form in contrast to the formation of large amounts of inclusion bodies to attain high-level expression in bacterial hosts. This takes advantage of the transcriptional and translational apparatus of *groEx* containing unique intragenic promoters (P3, P4), a ribosome-binding site, and combined transcriptional termination sequences.

When the amount of enzymes produced by the vectors with or without *Tgx* was compared, *Tgx* appeared to have a

Table 1. Activities and yields of β-galactosidase in purification procedures from *E. coli* DH5α transformed with pXGPRMATG-*lac-Tgx*.

Purification Steps	Specific Activity (Units/mg protein)	Purification Factor	Total Activity* (Units)	Yield (%)
Crude extract	171,961	1	58,549,281	100
1st Ammonium sulfate ppt (40%)	257,911	1.5	38,697,773	66
2nd Ammonium sulfate ppt (35%)	295,694	1.7	32,034,305	55
Sephadex G200 gel filtration	322,248	1.9	24,895,591	43

*Total available activity of β-galactosidase in each step of purification. The starting material was 3.0 g of wet cells obtained from a 500-ml culture.

Table 2. Comparisons of specific activities of β-galactosidase obtained from *E. coli* DH5α transformed with pXGPRMATG-*lac-Tgx* with those from other sources.

Enzyme sources	*E. coli*[1]	*Aspergillus oryzae*[2]	*Kluyveromyces lactis*[3]	*E. coli* DH5α (pXGPRMATG-*lac-Tgx*)
Units/mg protein	210,870	4,485 29,920*	161,000	322,248

[1,2]G-5635 and G-7138 were purchased from Sigma Chemical Co., respectively. [3]Maxilact XL 50000 from Gist-Brocades. *Reaction buffer was pH 4.5. All others were pH 7.0.

positive effect on the production of the cloned enzyme. By placing *Tgx* at the downstream of T_L, it was possible to enhance the enzyme production by 1.8 times. This could be due to a proper transcription termination and stabilization of the cloned gene, or to stabilization of mRNA by having an additional hairpin loop at its 3'-end (Fig. 1).

The transcription terminator motif of lac (T_L) is composed of three hairpin structures, the first hairpin at 13, the second at 81, and the third at 967 bases down from the stop codon of *lacA* [18]. In the expression of *lac* a prominent stop was made at the 3'-end of the second G+C rich hairpin structure that is 110 bases down from the stop codon. When the promoterless *lac* containing T_L was cloned under the control of *pgx3-4A*, *E. coli* transformed with the gene produced a large amount of fusion proteins (Fig. 5). However, the plasmid and expression of the gene controlled by the strong promoter alone were unstable during successive cultivation.

Plasmids exhibiting a strong promoter activity interfere with the maintenance of recombinants in *E. coli*. Vectors carrying efficient transcriptional terminators surrounding the insertion site have been found valuable for cloning DNA and stabilizing recombinants [13]. Strong promoters are known to be stable only in vectors in which efficient terminator signals protect control elements of the plasmid from excessive transcription [13, 16]. By inserting *Tgx* in this study, the recombinant became stable in successive cultivation of *E. coli*.

We approximated the transcription terminator signal region of *groEx* by Bal31 mutagenesis and determined the messenger endpoint of GroELx by S1-nuclease mapping (in preparation). The messenger endpoint (nt 1988) was 15 bp down from the stop codon for GroELx and was located in the middle of a C-rich motif which was followed by the second hairpin of 11-bp stem and 4-bp loop [2]. This implies that the C-rich motif and the hairpin present at the downstream of the *groELx* gene may have an essential role for the termination of gene transcription.

Other points noted in this study are the effects of *lacYA* and T_L on the production of the enzyme from the cloned gene. Contrary to our expectation, a reduction of the size of insert DNA reduced the production of the enzyme (Fig. 5e). In addition, presence of *Tgx* at the downstream of *lacZYA* enhanced the enzyme production by 1.2 to 1.8 times. This implies that a lengthening of the 3'-end containing additional hairpin-stem loops stabilizes the transcript [19, 15]. Further work is in progress to determine the effect of *Tgx* on the expression of foreign genes.

Another essential component for the overproduction of proteins in cloned cells is an appropriate ribosome-binding site (*SD*). An *SD* derived from gene *10* (*g10*) of bacteriophage T7 causes a pronounced stimulation of gene expression when placed upstream of various genes due to a stimulation of translation efficiency in *E. coli*. The *SD* of *g10* contains an A/U-rich 9-base sequence, which has the potential for forming a novel base-paired interaction with bases 458-466 of the 16 S rRNA of *E. coli* [9]. There was a 110-fold increase in the translation efficiency of the *lacZ* when the sequence was placed upstream of the gene [26]. The SD sequence for *groELx* located within an A/U-rich 11-base domain (Fig. 1) appears to have a comparable function to that of *g10*.

Overproduction of proteins encoded by a plasmid can be a principal metabolic burden to the host and may result in reduced cell growth and repression of overall protein synthesis by the host [5]. However, *E. coli* DH5α transformed with pXGPRMATG-*lac-Tgx* showed a normal growth and produced the enzyme proportional to the cell growth (Fig. 7). As we obtained extraordinarily high levels of β-galactosidase production by the construction of a vector containing a strong promoter and transcriptional terminator in an optimal host, purification of the enzyme was almost trivial. Homogeneity of the enzyme could be achieved using classical procedures, ammonium sulfate precipitation and gel filtration.

β-Galactosidase has been used mostly to hydrolyze lactose in dairy production and in the treatment of whey, which is a major by-product in cheese manufacturing and a significant waste. The β-galactosidase enzyme is still relatively expensive compared with other industrial enzymes such as α-amylases and proteases. Enzymes of *E. coli* have not been allowed in dairy production due to possible biohazards. However, the overproduced β-galactosidase in *E. coli*, as shown in this study, could be used in immobilizing procedures as an alternative approach to reduce the cost for enzyme treatment [12].

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