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Induction of the T7 Promoter Using Lactose for Production of Recombinant Plasminogen Kringle 1-3 in *Escherichia coli*

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Abstract A plasminogen kringle domain 1 to 3, rK1-3, was expressed in *Escherichia coli* under the control of T7 promoter. For the cost-effective production of rK1-3, the induction process was analyzed and optimized. Induction characteristics with lactose were analyzed in terms of induction time and inducer concentration in various culture conditions including batch and high-cell-density fed-batch cultures. In the fed-batch culture, the induction around 6 h after initiation of the DO-stat fed-batch culture resulted in the highest expression level of rK1-3 among the induction points examined. The highest demand of oxygen at this point was crucial for the maximum expression level of rK1-3. As the lactose concentration increased, the expression level also increased, though the expression level showed a plateau above a concentration of 14 mM of lactose. Lactose acted less specifically than IPTG since most of it was hydrolyzed to glucose and galactose. However, using lactose, the cell growth and the maximum expression level of rK1-3 increased by 20% and 24%, respectively, compared with those using IPTG in the fed-batch culture. The lactose seemed to be hydrolyzed by intracellular and extracellular β-galactosidase liberated by cell lysis at the same time. Residual concentration of glucose was maintained to a limit of detection by high performance liquid chromatography, and galactose was not consumed by the host strain *Escherichia coli* BL21(DE3).

Key words: rK1-3, T7 promoter, induction, lactose, *Escherichia coli*

To obtain oxygen and nutrients, many solid tumors stimulate the formation of new blood vessels (angiogenesis) from the existing healthy blood vessels into the cancer cells. As an anticancer agents, numerous anti-angiogenic agents have been recently examined and are currently under clinical trials [1, 2]

Among them, plasminogen kringle 1-3 (rK1-3) has been known as an anti-angiogenenic agent for cancer therapy [8, 10]. However, a large amount of rK1-3 with consistent quality should be supplied for testing of the efficacy and toxicity on various solid cancer models, because the effective dosage against solid cancer is so high that it ranges up to several hundred mg/kg-body weight. Therefore, the successful clinical trial and the commercialization of anti-angiogenic agent have been known to depend on the development of a cost-effective mass production system.

In this research, the expression system with the T7 promoter of *E. coli* was used, because it has been reported that the nonglycosylated form of rK1-3 from *E. coli* does not change its anti-angiogenic activity [8]. Isopropyl-β-D-thiogalactoside (IPTG) as an inducer is expensive and toxic to human, so it is insuitable for the industrial production of therapeutic proteins, especially for human uses. To circumvent these limitations, lactose was used to replace IPTG as the inducer in several studies [5, 7, 14, 15, 16, 17, 20]. In this study, the induction characteristics of recombinant *E. coli* producing rK1-3 were investigated and utilization of lactose was analyzed during the development of the production process using lactose as a substitute for IPTG.

MATERIALS AND METHODS

Construction of a Vector Expressing Recombinant Human Plasminogen Kringle 1-3

The expression vector under the control of T7 promoter, pET-11a (Novagen), was restricted with *Nde*I and *Bam*HI at a multicloning site and then it was ligated to the polymerase chain reaction (PCR) product of kringle 1-3 (rK1-3) which was amplified from human plasminogen cDNA [10]. rK1-3 (amino acid residues S82 to S335 of human

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plasminogen) was amplified by PCR using the following primers on a template consisting of the cDNA of human plasminogen: 5' primer, 5'-CGGGATCCCATATGTCAG-AGTGCAAGACTGGGA-3'; 3' primer, 3'-CATTCT ATG-GCAGGACACTGAGGATCATTCCTAGGGC-5' [8]. The resulting construct was transformed into *E. coli* BL21(DE3).

Cultivations

Seed culture was performed in a 500-ml baffled flask containing 100 ml of fortified Luria-Bertani (FLB) medium (10 g/l of bacto-trypton (Difco), 20 g/l of yeast extract (Difco), 5 g/l of NaCl) at 37°C shaking incubator for 12 h. The main culture was carried out in a 2.5 l fermentor, BiofloIII (New Brunswick Sci. Co., U.S.A.) with an initial volume of 1.0 l. As a control experiment, a DO-stat fed-batch culture was performed with complex rich medium (at initial medium; 40 g/l of glycerol, 40 g/l of yeast extract, 10.22 g/l of Na_2HPO_4, and 2.31 g/l of KH_2PO_4, at feed medium; 390 g/l of glycerol, 292 g/l of yeast extract, and 5.76 g/l of $MgSO_4$). This composition of complex medium was based on terrific broth (TB) [19]. The pH was objusted at 7.0 using phosphoric acid (10%, v/v) and ammonia solution (10%, w/v). A dissolved oxygen (DO) level was sustained not to decrease below 20% and feed medium was introduced in DO-stat manner, as described previously [12]. The expression of rK1-3 was induced by adding IPTG or lactose.

Analytical methods

The expression level of rK1-3 was defined as a percent of rK1-3 of the total cellular protein. The percent expression level of rK1-3 in the total cell lysate of *E. coli* was determined by image analysis of SDS-PAGE gels of the total *E. coli* lysates, which was stained with Coomassie brilliant blue, as described previously [11].

Cell mass was observed using a spectrophotometer (LKB Biochrome Ltd., Ultrospec II, England) at a wavelength of 600 nm.

HPLC was performed to analyze residual concentration of lactose, glucose, and galactose. The HPLC system was composed of Shodex SUGAR SP0810 column with SP-G guard column (Shodex, SHOWA DENKO, Japan), Gilson 322 pump, Gilson Unipoint V1.9 software (Gilson, France), and Refractive Index (RI) detector (Shodex RI-71, Japan). Distilled water was used as a mobile phase. Flow rate was 0.5 ml/min and column temperature was 80°C.

RESULTS AND DISCUSSION

Induction of rK1-3 using IPTG

To optimize an induction strategy using IPTG, 5 l-scale fed-batch cultures were performed with various induction times. During the fed-batch culture, IPTG was introduced

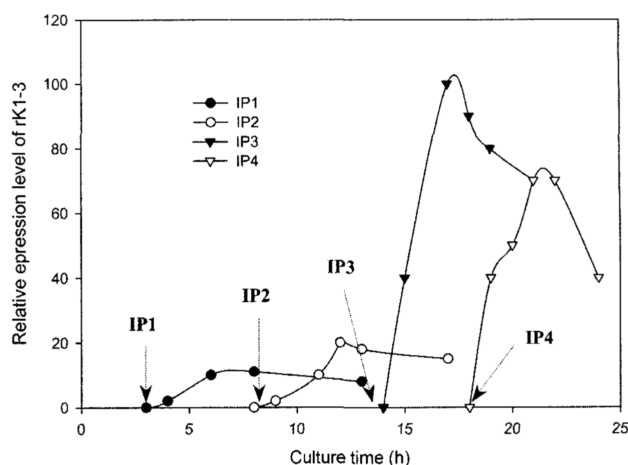

Fig. 1. Time profiles of the relative expression level of rK1-3 induced with IPTG at each induction time.
During the DO-stat fed-batch culture, IPTG was introduced with a concentration of 2 mM at IP1, IP2, IP3, and IP4. The relative expression level of rK1-3 was defined as a percent of rK1-3 of the total cellular protein with each induction point.

at several points to determine the optimal induction time. IPTG was introduced at the exponential growth phase (IP1), the start point of the DO-stat fed-batch culture (IP2), 6 h after initiation of feeding by a DO-stat manner (IP3), and 10 h after initiation of feeding by a DO-stat manner (IP4) in each fed-batch culture. As shown in Fig. 1, the optimal induction point was observed. In addition, the highest expression level of rK1-3 was observed at IP3. Moreover, at this point, the cell mass of 40 g/l was achieved and the cells were still metabolically active, which seems to be favorable for an induction of recombinant proteins. At induction point IP1, the cell mass was too low to achieve high-level expression. On the other hand, at IP4, the metabolic activity of the cells seemed to be low, although the cell mass was high enough.

As described in Materials and Methods, pure oxygen mixed with air was supplied to maintain DO level above 20%. If the DO decreased below 20% of saturation, the ratio of partial pressure of pure oxygen in inlet air-stream (O_2^{PR}) increased automatically by 0.5% of inlet air-stream volume and if the DO increased above 40% of saturation, O_2^{PR} decreased automatically by 0.5% using computer controlled gas mixer (Two-Gas mixer, New Brunswick Sci. Co., U.S.A.). As the cell density increased, the demand for oxygen increased and consequently O_2^{PR} increased automatically. As shown in Fig. 2, time profile of O_2^{PR} without induction reached its maximum value of about 40% at around IP3 point (6 h after initiation of feeding by a DO-stat manner) suggesting that cells were most vigorously growing at this point. Beyond this point, O_2^{PR} decreased gradually to about 30% at 15 h initiation of feeding by a DO-stat manner (Fig. 2). Several studies have reported the relationship between optimal induction time

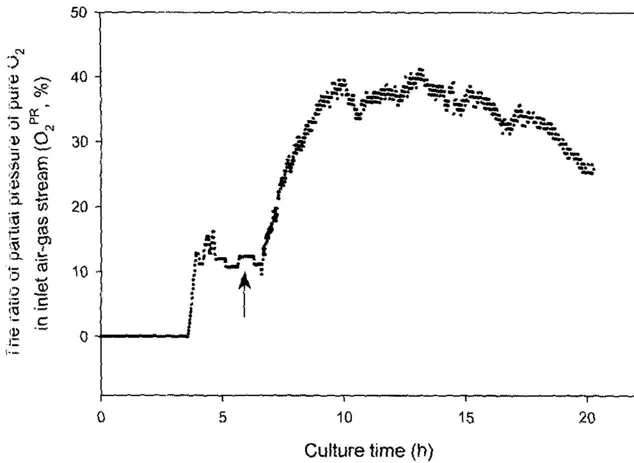

Fig. 2. Time-course profile of the ratio of partial pressure of pure oxygen in inlet air-stream (O_2^{PR}) during the DO-stat fed-batch culture without induction.

An arrow indicates the start point of the DO-stat fed-batch culture.

and oxygen uptake rate (OUR) or carbon dioxide evolution rate (CER) [4, 9]. According to these studies, the moment for the induction of recombinant protein expression was optimal when the oxygen uptake rate was maximized. Therefore, the demand for oxygen by the host cell, which was represented by O_2^{PR} in this study, could be used as an indicator for the metabolic state of the host cell during the fed-batch culture for optimizing induction time.

Induction of rK1-3 Using Lactose

IPTG, an inducer for T7 promoter, is inappropriate for mass production of therapeutic proteins for human uses

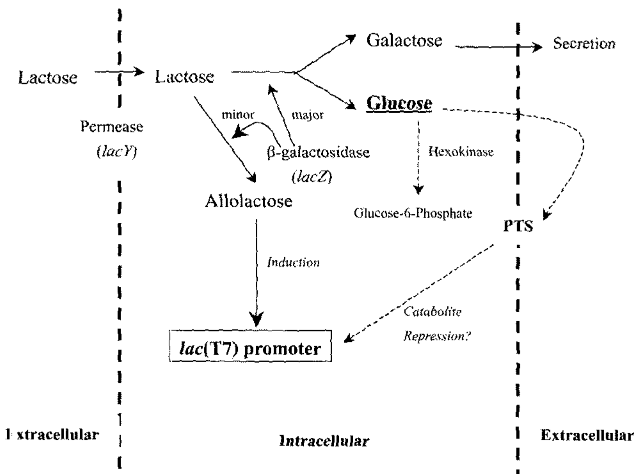

Fig. 3. Schematic diagram for possible actions and metabolic pathways of lactose as an inducer and a carbon source.

PTS; phospho*enol*pyruvate:sugar phosphotransferase system. Bold dotted lines indicate membrane of *E. coli*. Dotted arrows indicate the possible metabolism pathways of glucose liberated from lactose.

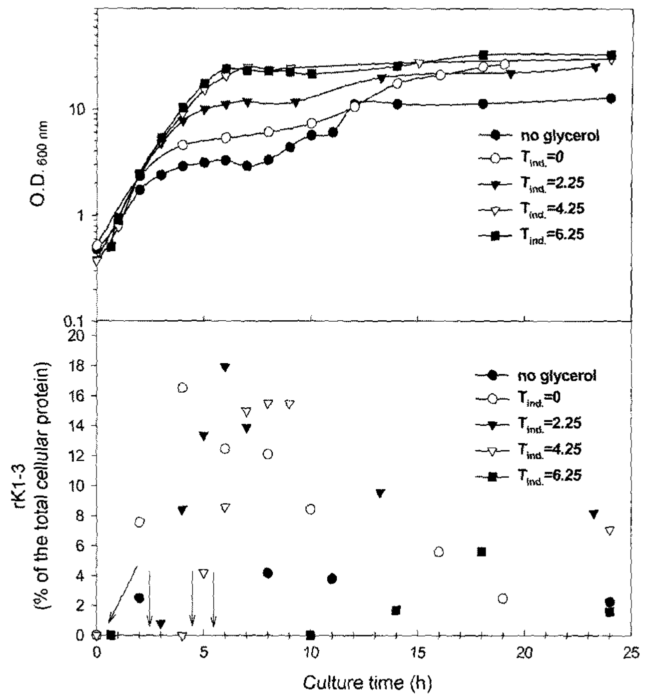

Fig. 4. Effect of induction time on the cell growth and expression level in the batch culture.

Lactose was introduced at a concentration of 20 g/l.

since it is very expensive and also toxic. IPTG represented most of the cost of culture medium. Lactose can be a substitute for IPTG as it is cheap and not harmful to human body. But, its mechanisms as an inducer and a carbon source are rather complex because lactose uptake and allolactose induction may be influenced by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) through the hydrolysis of lactose and liberation of glucose [18]. Figure 3 depicts a schematic diagram for possible pathways of lactose uptake, action as an inducer, and metabolism of its hydrolyzates.

To optimize lactose induction processes in T7 promoter in recombinant *E. coli*, various lactose concentrations and induction times were tested in batch and fed-batch cultures. In batch culture, a late exponential phase, 4.25 h after induction, was found to be the optimal point (Fig. 4). In fed-batch culture, the induction at around 6 h after initiation of feeding by a DO-stat manner resulted in the highest expression of rK1-3 and the fastest cell growth among the induction points examined (Fig. 5). This result was consistent with the result obtained with IPTG in that maximum oxygen uptake rate was crucial for the determination of optimal induction time. The optimal induction time (around 6 h after initiation of feeding by a DO-stat manner) obtained in this study was reproducible and concomitant with the maximum value of O_2^{PR}. Plasmid stability was maintained above 90% throughout the culture at the optimal point of induction. As a results, using lactose,

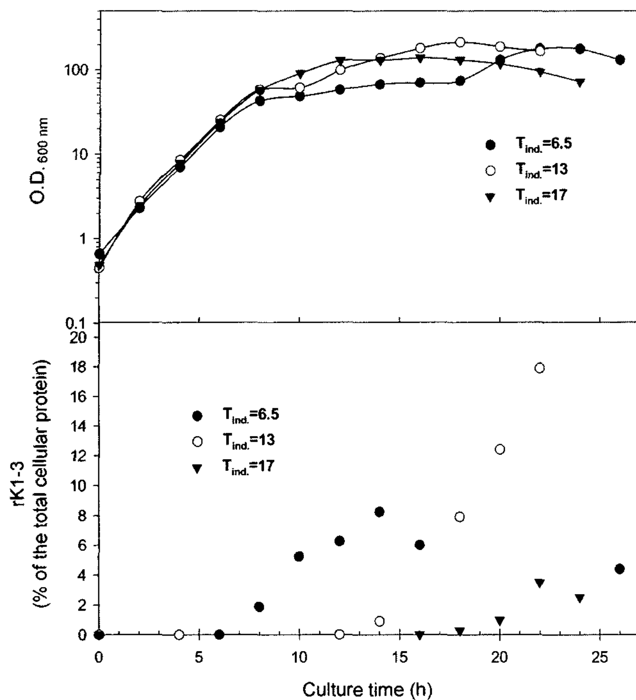

Fig. 5. Effect of induction time on the cell growth and expression level in the DO-stat fed-batch culture.
Lactose was introduced at a concentration of 20 g/l.

the cell growth and the maximum expression level of rK1-3 increased by 20% and 24%, respectively, compared with those using IPTG in the fed-batch culture.

Optimization of Lactose Concentration and Analysis of its Hydrolysis

As the inducer concentration increased, the maximum expression level also increased to 17.9% of the total cellular protein. However, an expression level showed a plateau above a concentration of 5 g/l lactose (Table 1). The concentration of 5 g/l corresponds to 14 mM lactose, which is a rather high concentration compared to 1 mM of IPTG used commonly in induction. It could be interpreted that lactose would act less specifically than IPTG since a small amount of lactose was transformed to allolactose by β-galactosidase, a real inducer of T7 promoter system, and the remainder was hydrolyzed to glucose and galactose. The activity of β-galactosidase increased just after the addition of lactose. Furthermore, maximum activity of β-galactosidase in the cells was proportional to the amount of lactose added at optimal induction time (data not shown).

It is not exactly known by which pathways the cell consumes the glucose formed from lactose and allolactose. There are two possibilities. The first one is that the glucose is phosphorylated by hexokinase to glucose-6-phosphate inside the cell. Enterobacteria are known to contain hexokinase [3, 6]. Although its function in lactose metabolism has not been documented, it is possible that a primary function of hexokinase would be to phosphorylate glucose formed from the hydrolysis of lactose or other carbohydrates. The second possibility is that glucose resulting from lactose hydrolysis is secreted from the cell (actively or passively) and phosphorylated upon reentry into the cell through PTS. Out of these two models, only the second model can exert catabolite repression mediated by cAMP concentration (Fig. 3). Gombert and Kilikian [7] suggested that the residual lactose concentration played an important role in the production of heterologous proteins in the T7 promoter system. In the report by Neubauer and Hofmann [15], the VP1 (main anitigenic coat protein of the foot and mouth disease) gene was expressed only after lactose was depleted in the culture broth in a T7-RNA polymerase expression system.

On assumption that high residual concentration of lactose repressed the *lac*-derived promoter, there might be catabolite repression by glucose secreted from the cell. HPLC analysis of the culture broth in batch culture with 20 g/l lactose as a sole carbon source showed that the residual concentration of lactose decreased and the residual concentration of galactose increased with culture time. On the other hand, glucose was not detected in the culture broth. It was observed that the host strain *E. coli* BL21(DE3) was not able to consume galactose as a carbon source (Fig. 6) and seemed to release unusable galactose (Fig. 3). In a wild-type *E. coli*, the galactose was converted to glucose-1-phosphate by reactions sequentially mediated by galactose kinase, galactose-1-phosphate uridyl transferase, and UDP-galactose 4-epimerase [13]. Theoretically, all lactose should be translocated into the cell by lactose permease and hydrolyzed intracellulary by β-galactosidase, but in

Table 1. Effects of lactose concentration on cell growth, expression level, and plasmid stability in fed-batch culture.

Initial concentration of lactose (g/l)	Max. O.D. (600 nm)	% of rK1-3 to the total cellular protein	Plasmid stability (%)[a]
0.5	172	2.4	98
1	144	4.3	98
2.5	161	8.8	98
5	203	17.0	95
10	213	17.9	98

[a]Plasmid stabilities at the time showing maximal expression levels.

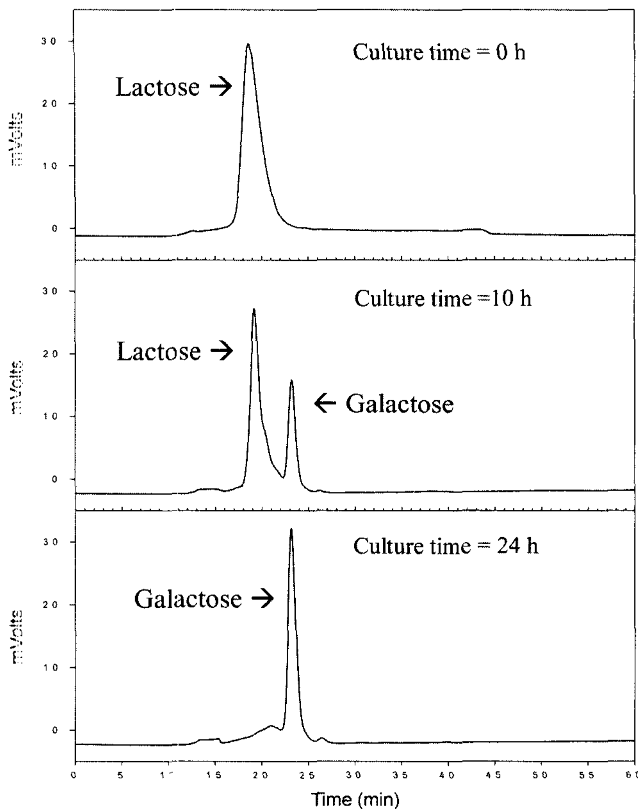

Fig. 6. HPLC analysis of residual lactose, glucose, and galactose in the batch culture.

practical, there seemed to be a partial extracellular hydrorysis of lactose by β-galactosidase liberated by cell lysis during high-cell density fed-batch culture. The residual concentration of glucose was maintained to the limit of detection by HPLC. Even if glucose diffused out of the cell, as suggested by the second model, glucose was assumed to be consumed immediately by cells after secretion.

In conclusion, there were the optimal conditions for the lactose induction, but it was difficult to explain the exact destiny of lactose in each induction condition because of the complexity of a high-cell density culture system of *E. coli*. For further study, the extent of external and internal hydrolysis of lactose should be quantitatively examined by studying the kinetics of consumption of lactose during the culture.

Acknowledgments

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