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Special Issue

Effect of hypoxia duration on the oxygen-dependent production of a recombinant protein, β-galactosidase, by an animal cell line, F6D2, with a hypoxia-inducible enhancer

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Abstract

Expression of specific genes is a strategy of animal cells for adaptation to oxygen deficiency and the mechanism underlying the hypoxic activation of gene expression may be useful for efficient production of recombinant proteins by animal cells, because oxygen is a limiting factor in animal cell cultures. We prepared an animal cell line harboring the plasmid in which expression of a reporter gene, β-galactosidase, is controlled by an enhancer responsible for the hypoxic activation of gene transcription. The purpose of this paper is to understand this hypoxic production of recombinant proteins quantitatively by a mathematical model originally developed based on the following hypotheses; 1 lacZ (the reporter gene) is transcribed after HIF-1 protein complex is bound to the hypoxic enhancer, 2. β-galactosidase synthesis rate is limited at the transcription of lacZ, 3. HIF-1 is an inactive form under a normal oxygen concentration, 4. Oxygen works as a repressor in the synthesis of HIF-1 protein, 5. Both β-galactosidase and HIF-1 are decomposed according to the first order reaction. The effects of hypoxic duration as well as oxygen concentration on the β-galactosidase production were successfully predicated by the model.

Abbreviations: EPO, erytrhopoitein; bp, abse pair; X-gal, 4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside; ONPG, o-nitrophenyl-β-D-galactopyranoside; ONP, o-nitrophenol; DO, dissolved oxygen

Introduction

Mammalian cells are used for production of recombinant proteins that require complicated folding and post-translational modification such as glycosylation for expression of high biological activities. Oxygen is a limiting nutrient in high cell density cultures because of its low solubility in the medium. To maintain appropriate oxygen levels continuously in animal cell cultures, a variety of physical methods for aeration with reduced detrimental effect on cells have been developed (Kelley *et al.*, 1993). Here we propose an alternate biological method to improve productivity of recombinant proteins under low oxygen concentrations by the use of an hypoxia-inducible enhancer element, which plays a key role in molecular adaptation of animals to hypoxia (Bunn and Poyton, 1996).

Since oxygen plays a dominant role in the metabolism and viability of cells, many organisms adapt to oxygen deficiency by activating expression of specific genes. In mammalian cells, a number of hypoxia-inducible genes have been demonstrated. These include genes encoding EPO, glycolytic enzymes, glucose transporters, vascular endothelial cell growth factor, nitric oxide synthase, and tyrosine hydroxylase, a key enzyme in catecholamine biosyn-

thesis (Bunn and Poyton, 1996). EPO gene has been studied most intensively. EPO is a major stimulator of erythropoiesis (Krantz, 1991; Jelkmann, 1992; Bunn and Poyton, 1996) and also functions as a neurotropic factor in the central nervous system (Masuda *et al.*, 1993, 1994, 1997; Nagao *et al.*, 1995; Moroshita *et al.*, 1996, 1997). The major production site of EPO in adults is the kidney and the kidney EPO circulates to reach erythropoietic tissues, bone marrow and spleen. EPO in fetus is produced by the liver, where erythropoiesis takes place. In the central nervous system, astrocytes produce brain EPO. Transcription of EPO gene in these sites is activated by hypoxia. Finding of the cis-acting element responsible for hypoxic induction of EPO gene has been made using a human hepatoma cell line, Hep3B, in which EPO gene transcription is activated by hypoxia. A short DNA stretch (∼40 bp) located ar 3' flanking region contains the enhancer element required for hypoxic activation of EPO gene transcription. This enhancer may be useful for production of recombinant proteins at high levels in hypoxic conditions.

In this paper we constructed the plasmid in which expression of β -galactosidase gene is under the control of EPO promoter and enhancer, and then a Hep3B cell line (F6D2) harboring this plasmid has been estimated to confirm that the β-galactosidase production is enhanced more than 20 times. In order to apply this production system to industry, it is necessary to find a optimum operation condition for producing a recommbinant protein using a hypoxia-inducible enhnacer. Using a mathematical model based on a hypoxic gene expression mechanism in cell, we here quantitatively examine the effect of hypoxia duration on the oxygen-dependent production of β-galactosidase by the F6D2 animal cell line with a hypoxia-inducible enhancer. This model system provides useful information for the development of an extended version with a high productivity.

Materials and methods

Construction of p4En0.2Z

Three fragments were ligated to construct p4En0.2Z. First fragment is a *Sac*I-*Eco*RI fragment which contains tandemly repeated four blunted *Apa*I-*Pvu*II fragments of human Epo 3' enhancer regio (Semenza *et al.*, 1991) flanked by *Sacl*-*Bam*HI linker pBluescript-tII(Stratagene) and blunted-*Hin*dIII.*Eco*RI fragment of pSVOALD5' (de Wet *et al.*, 1987). Second fragment is a *Hind*III-*Bam*HI fragment which was cut out from a plasmid containing blunted *Apal*-*Eco*521 fragment of human EPO promoter region(Tsuchiya *et al.*, 1993) ligated in blunted *Sac*I, *Pst*I sites of pUC18. Third fragment is a *Hin*dIII-*Bam*HI fragment from pCH110 (Pharmacia) which codes β -galactosidase.

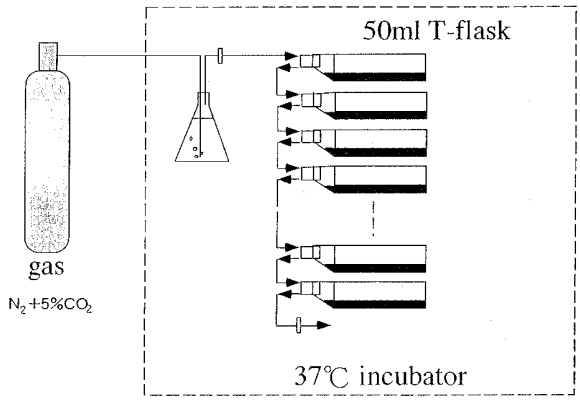

Figure 1. Experimental apparatus.

Cell

Hep3B, human hepatoma cells were co-transfected with p4En0.2Z and pKSV10neo using lipofectamine reagents (GIBCO) and G418 resistant clones were screened in the presence of 400 μg/ml of G418. After two round of cloning by limiting dilution. F6D2 cells which could indicate expression of β-galactosidase most strikingly by X-gal staining (Sanes *et al.*, 1986) after cultured only in hypoxic (1% O_2) conditions, not in normoxic were established among G418 resistant clones.

Experimental procedure

A Dulbecco's modified Eagle medium (DMEM) (Nissui Co. Ltd., Japan) with 10% fetal calf serum was used for maintaining cells and producing β-galactosidase. Cells were cultivated statistically in 50 ml culture flasks. The effect of hypoxic duration on the β-galactosidase production was examined using an experimental set-up shown in Figure 1. Culture flasks cultivating F6D2 cells were connected in series in a thermostatic chamber in which temperature was maintained at 37 °C. Cells were cultivated first under a normoxic condition with atmospheric air and 5% CO_2. When cell growth was in the middle of an exponential growth (5×10^5 cells/ml), air with 5% CO_2 supply-

ing to the culture flasks was switched to nitrogen gas with 5% CO_2 to give a hypoxic condition. At definite intervals the culture flasks was removed from the tail of the series for analyzing β-galactosidase activity and other components concentrations. The effect of oxygen concentration on β-galactosidase production was investigated by supplying 2%, 10% and 21% oxygen in nitrogen gas with 5% CO_2, respectively, for 24 hours instead of nitrogen gas with 5% CO_2. Every gas used was humidified before supplying to culture flasks to avoid any change in components concentrations in the culture medium due to vaporization.

Assay

The cell number was counted on a Burker-Turk hematometer after cells were removed from the culture flask. The cell viability was confirmed by a Trypan Blue dye exclusion method. A β-galactosidase activity was measured using an enzymatic reaction from o-nitrophenyl-β-D-galactopyranoside (ONPG) to o-nitrophenol (ONP) and galactose. One unit of β-galactosidase was defined as the amount of enzyme catalyzing to produce 1 nmol of ONP for 1 minute at pH 7.0 at 28 °C. Before analyzing β-galactosidase activity, cells were ruptured using a trace of 0.2% SDS solution and chloroform in a Z-buffer (Na_2HPO_4 $7H_2O$; 16.1g/l, NaH_2PO_4 H_2O; 5.5g/l, KCl; 0.75g/l, $MgSO_4$ $7H_2O$; 0.25g/l and 2-mercaptethanol; 2.7ml/l). Decomposition of β-galactosidase was confirmed by examining a change in β-galactosidase activity after any protein synthesis was inhibited by adding 100 μ mol cycloheximide. Concentrations of glucose, glutamine, lactate and ammonium in the culture medium were enzymatically analyzed using commercially available assay kits. An ATP concentration in cell was measured using enzymatic reactions including luciferase coupled with $NADP^+$ and NADPH conversion after cells were ruptured. The ATP concentration was estimated from a change in the absorbance of $NADP^+$ at 340 nm. The oxygen uptake rate of the F6D2 cells was determined by measuring a change in dissolved oxygen (DO) concentration in a culture flask with a full of fresh culture medium with no air space in the flask with a DO electrode. The bottom of the flask was occupied by the F6D2 cells adhering and the averaged cell concentration in the flask was 4.7×10^4 cells/ml.

Mathematical modeling

β-galactosidase synthesis

The following hypotheses were assumed for the β-galactosidase synthesis:

1. lacZ is transcribed after HIF-1 protein is bound to the hypoxic enhancer.
1. β-galactosidase synthesis rate is limited at the transcription of lacZ.
3. HIF-1 becomes an inactive form under a normal oxygen concentration.
4. Oxygen works as a repressor in the synthesis of HIF-1 protein.
5. Both β-galactosidase and HIF-1 are decomposed according to the first order reaction.

The rate limiting step is shown below;

$$\text{DNA} + \text{HIF-1} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{DNA}$$

$$\text{HIF-1} \xrightarrow{k_{+2}} \text{mRNA}$$

where DNA HIF-1 is a complex of DNA and HIF-1, k_{+1}, k_{-1} and K_{+2} are the rate constants. Since HIF-1 becomes an inactive form with increase in oxygen concentration,

$$\text{HIF-1} \underset{k_{-3}}{\overset{+O_2 k_{+3}}{\rightleftharpoons}} \text{DNA}_{\text{inactive}}$$

where HIF-1_{inactive} is inactive HIF-1 and k_{+3} and k_{-3} are the rate constants. It is generally accepted that oxygen does not directly bind to HIF–1 complex, but redox condition of HIF-1 through the oxidation of sulfhydryl group is important for the binding of HIF-1 complex to DNA (Wang *et al*., 1995; Huang *et al*., 1996).

Under pseudo-steady state condition, the following equations are developed.

$$k_{+1}[\text{DNA}][\text{HIF-1}]-(k_{-1}+k_{+2})[\text{DNA HIF-1}]=0 \quad (1)$$

$$k_{+3}[\text{HIF-1}][O_2]-k_{-3}[\text{HIF-1}_{\text{inactive}}]=0 \quad (2)$$

$$[\text{DNA}]_0 = [\text{DNA}]+[\text{DNA HIF-1}] \quad (3)$$

$$[\text{HIF-1}]_0 = [\text{HIF-1}]+[\text{HIF-1}_{\text{inactive}}] \quad (4)$$

where [ ] and the subscript 0 indicate concentration and the initial values, respectively. From Eqs. (1) to (4), the following equation can be obtained. Finally the transcription rate of laxZ can be found.

$$\frac{d[\text{mRNA}_{\text{lacZ}}]}{dt} =$$

$$\frac{k_{+2}[DNA]_0[HIF-1]_0}{K([O_2]/Ki + 1) + [HIF-1]_0} \quad (5)$$

where $mRNA_{lacZ}$ is mRNA of β-galactosidase, K is $(k_{-1}+k_{+2})/k_{+1}$, and Ki is k_{-3}/k_{+3}.

As to HIF-1 production, the following equations are considered.

$$DNA_{HIF-1} \xrightarrow{k_{+0}} mRNA_{HIF-1}$$

$$DNA_{HIF-1} + O_2 \underset{k_{-4}}{\overset{k_{+4}}{\rightleftarrows}} DNA\ O_2$$

where DNA O_2 is a complex of DNA in which the HIF-1 gene is coded, DNA_{HIF-1}, and oxygen. Under a pseudo-steady state condition, Eqs. (6) and (7) are assumed.

$$k_{+4}[DNA_{HIF-1}][O_2] - k_{-4}[DNA\ O_2] = 0 \quad (6)$$

$$[DNA_{HIF-1}]_0 = [DNA_{HIF-1}] + [DNA\ O_2] \quad (7)$$

From these equations, $[DNA_{HIF-1}]$ can be found as

$$[DNA_{HIF-1}] = \frac{[DNA_{HIF-1}]_0}{[O_2]/Ks + 1} \quad (8)$$

where Ks is k_{-1}/k_{+1}.

Moreover, when HIF-1 as well as HIF-$1_{inactive}$ are assumed to be decomposed according to the first order reaction, the following equation is yielded.

$$\frac{d[HIF-1]_0}{dt} = \frac{K_{+0}[DNA_{HIF-1}]_0}{[O_2]/Ks + 1} - k_{-5}[HIF-1]_0 \quad (9)$$

where k_{-5} is decomposition rate constant of both HIF-1 and HIF-$1_{inactive}$.

Equation (9) can be analytically solved under an initial condition ($[HIF\text{-}1]_0 = 0$, at $t = 0$).

$$[HIF-1]_0 = \frac{\beta}{K_{-5}([O_2]/Ks + 1)}\{1 - \exp(-k_{-5}t)\} \quad (10)$$

where β is $k_{+0}[DNA_{HIF-1}]_0$.

Finally Eqs. (10) and (5) and the hypothesis that β-galactosidase is decomposed according to the first order reaction yield

$$\frac{d[\beta - galactosidase]}{dt} = \frac{\alpha\beta\{1 - \exp(-k_{-5}t)\}}{k_{-5}K[(O_2]/Ki + 1)([O_2]/Ks + 1) + \beta\{1 - \exp(-k_{-5}t)\}} - k_{-6}[\beta - galactosidase] \quad (11)$$

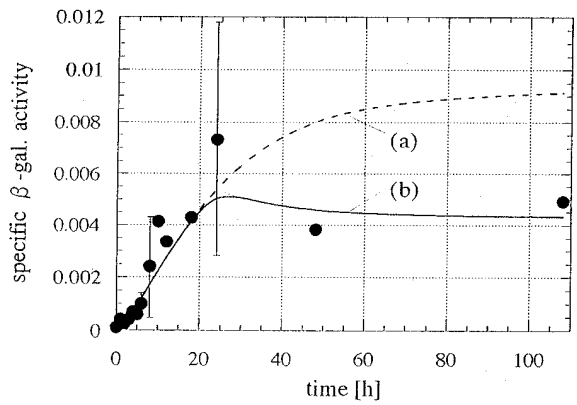

Figure 2. Effect of hypoxic duration on the β-galactosidase production. (including simulation results): When cell growth was in the middle of an exponential growth (5×10^5 cells/ml), air with 5% CO_2 supplying to the culture flasks was switched to nitrogen gas with 5% CO_2 to give a hypoxic condition. At definite intervals the culture flask was removed from the tail of the series for analyzing β-galactosidase activity and other components concentrations.

where α is $k_{+2}[DNA]_0$, k_{-6} is the first order rate constant of β -galactosidase decomposition.

The change in β-galactosidase concentration in the culture duration under a hypoxic conditions can be simulated by solving Eq. (11). In this research Eq. (11) was numerically solved by a Runge Kutta method.

ATP regeneration and consumption rates

Under an assumption that the P/O ratio is 3 in the oxidative phosphorylation, the specific ATP regeneration rate, q_{ATP} is calculated by the following equation.

$$q_{ATP} = q_{lac} + 6q_{O2} \quad (12)$$

where q_{lac} and q_{O2} are the specific production and oxygen uptake rates, respectively. The specific ATP consumption rate is estimated by the following equation.

$$\left(\frac{d[ATP]}{dt}\right)_{accum.}/X = q_{ATP} - \left(\frac{d[ATP]}{dt}\right)_{consum.}/X \quad (13)$$

where $(d[ATP]/dt)_{accum.}$ is ATP accumulation rate, $(d[ATP]/dt)_{consump.}$ is ATP consumption rate and X is the cell concentration.

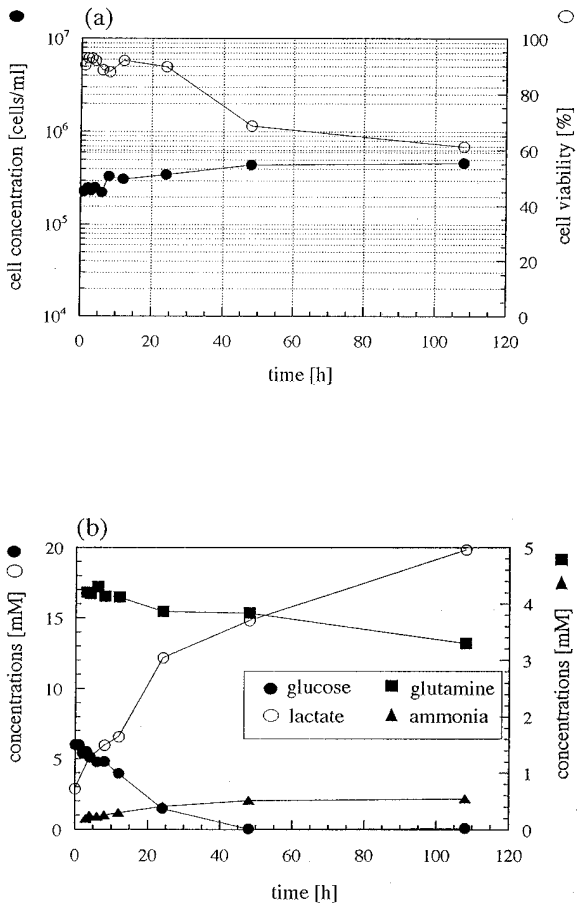

Figure 3. Cell concentration and viability (a) and medium composition changes (b) in batch culture in flasks under hypoxic conditions given by nitrogen gas introduced.

Results and discussion

Batch culture under a normal oxygen condition

F6D2 cells showed an exponential growth with the cell doubling time of 45 hours. In the batch culture less glutamine consumption was found compared with glucose consumption, indicating that glucose is a main energy source for the F6D2 cells. The oxygen uptake rate of the F6D2 cell was 3.44×10^{-10} mmol/cell/h. The specific glucose consumption rate and the specific lactate production rate were 1.3×10^{-10} mmol/cell/h and 1.5×10^{-10} mmol/cell/h, respectively. The ATP regeneration rate is found to be 2.21×10^{-9} mmol/cell/h using Eq. (12).

The effect of hypoxic duration on the β-galactosidase production

Figure 2 shows change in specific β-galactosidase activity after nitrogen gas was supplied, confirming that β-galactosidase is more produced under a hypoxic condition. The specific β-galactosidase activity was calculated by dividing β-galactosidase activity in a test tube by the viable cell concentration in it. It is seen from Figure 2 that the β-galactosidase production rate first increases exponentially, and is reduced after around 8 hours of the hypoxia. Finally no accumulation of β-galactosidase is found after 10 hours. Figure 3(a) shows changes in viable cell concentration and cell viability during hypoxic duration. No growth is found under a hypoxic condition. Although the cell viability is dropped after 24 hours of the hypoxic from 80% to 60%, cell concentration is stabilized during whole the hypoxic duration. Figure 3(b) shows changes in glucose, glutamine, lactate and ammonium ih the culture medium during the hypoxic duration. Glucose is depleted at 48 hours of the hypoxia. After 48 hours, glutamine should be an energy source for maintaining the cell and synthesizing β-galactosidase. The slope of lactate accumulation should be the specific lactate production rate because the cell concentration is not changed under whole the hypoxic condition. The specific lactate production rate is changed at 24 hours of the hypoxia. The specific lactate production rates from 0 to 24 hours are 2.8×10^{-10} mmol/cell/h and 0.7×10^{-10} mmol/cell/h after that. Under this hypoxic conditions, no oxygen exists in the culture medium, and the specific ATP regeneration rate is calculated only from the specific lactate production rate by Eq. (12). Up to 24 hours, the specific ATP regeneration rate is 2.8×10^{-10} mmol/cell/h and 0.7×10^{-10} mmol/cell/h after that. ATP concentration in the cell during the hypoxic condition. The ATP consumption rate should be the same as the ATP regeneration rate because of no change in ATP concentration in the cell.

Figure 4 shows a semi-logarithmic relationship between β-galactosidase activity and the culture time under hypoxic condition, indicating β-galactosidase decomposition. The slope in Figure 4 indicates the first order decomposition rate constant, yielding 0.053 h^{-1}.

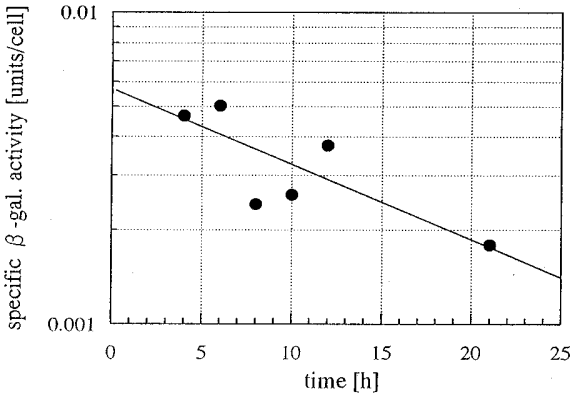

Figure 4. β-galactosidase decomposition during hypoxia. : Decomposition of β-galactosidase was confirmed by examining a change in β-galactosidase activity after any protein synthesis was inhibited by adding 100 μmol cycloheximide.

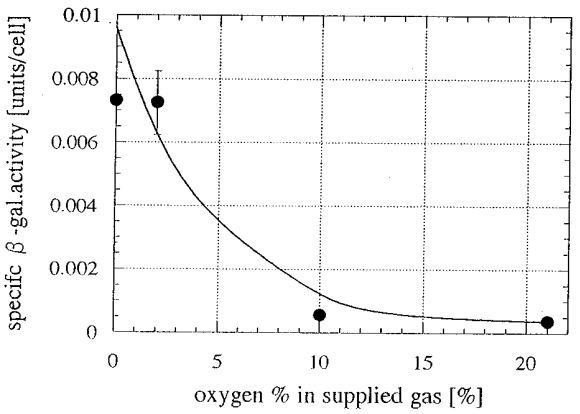

Figure 5. Effect of oxygen concentration on the β-galactosidase production, and a simulation result. : The effect of oxygen concentration on β-galactosidase production was investigated by supplying 2%, 10% and 21% oxygen in nitrogen gas with 5% CO_2, respectively, for 24 hours instead of nitrogen gas with 5% CO_2.

The effect of oxygen concentration on the β-galactosidase production

The effect of dissolved oxygen (DO) concentration in the culture medium on the β-galactosidase production was investigated. Figure 5 shows a relationship between DO and the β-galactosidase activity which was obtained after 24 hours from the gas was switched. The β-galactosidase activity is drastically reduced with increase in oxygen concentration.

Simulation of β-galactosidase production

The F6D2 cell produces β-galactosidase but does not secret from the cell. β-galactosidase is accumulated in the cell. The β-galactosidase productivity is defined as

$$\frac{d\gamma X}{dt} = X\frac{d\gamma}{dt} + \gamma\frac{dX}{dt} \tag{14}$$

where X and γ are the cell concentration and the β-galactosidase content in the cell, respectively. As shown in Figure 3(a), the viable cell concentration was not changed during the hypoxia. Equation (15) is arranged as

$$\frac{d\gamma X}{dt} = X\frac{d\gamma}{dt} \tag{15}$$

d γ/dt is the specific β-galactosidase productivity and equal to Eq. (12). In Eq. (12) α is estimated from the maximum slope in Figure 2 to be 7.84×10^{-4} units/cell/h. The β-galactosidase decomposition rate constant, k_{-6}, is fixed at 0.053 h^{-1} found in Figure 4. Other parameters, K, k_{-5} and β, are estimated so that the calculated result agree with the experimental one shown in Figure 2. A simulation result are also shown in Figure 2. However, the calculated result does not agree with the experimental result at the late stage of the hypoxia (line (a)). The ATP regeneration rate was drastically reduced after 24 hours of the hypoxia, yielding one fourth of that before the 24 hours. This indicaes that less chemical energy would sypply for synthesizing protiens after the 24 hours. In the mathematical model proposed α and β concern the protein syntheses of β-galactosidase and HIF-1, respectively. Here the β value was reduced to one fourth of the original one after the 24 hours. The calculated result using this small β value after 24 hours are shown in Figure 2. The calculated result agree well with the experimental one (line (b)).

The effect of oxygen concentration on the β-galactosidase activity was simulated. The Ki and Ks values were determined so that the calculated result agrees with the experimental one as shown in Figure 5. It is found that the effect of the hypoxic duration as well as that of the oxygen concentration on the β-galactosidase production is simulated based on the following hypotheses;

1. lacZ is transcribed after HIF-1 protein complex is bound to the hypoxic enhancer.
2. β-galactosidase synthesis rate is limited at the transcription of lacZ.
3. HIF-1 becomes inactive form under normal oxygen concentration.

4. Oxygen works as a repressor in the synthesis of HIF-1 protein.
5. Both β-galactosidase and HIF-1 are decomposed according to the first order reaction.

This indicates that the validity of a methematical model proposed is confirmed. It is intriguing about induction of HIF-1α and β mRNA in hypoxia. Wang *et al*. (1995) showed induction of HIF-1α in hypoxia using Hep3B cells or *in vivo* (Wiener *et al*., 1996), but Huang *et al*. (1996) described that no significant induction of HIF-1α and β mRNAs could be seen in hypoxia using Hela cells and Hep3B cells. We have established F6D2 cells using Hep3B cells as host cells. In our model, we considered the induction of HIF-1 mRNAs in hypoxia, which agreed well with our results.

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