論文 / 著書情報
Article / Book Information

Title	Analysis of Cell-Cycle Dependent Production of Tissue Plasminogen Activator Analogue (Pamiteplase) by CHO cells
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Journal/Book name	Biochem. Eng. J.,, Vol. 39, No. , pp. 297-304
Issue date	2008, 1
URL	http://www.sciencedirect.com/science/journal/1369703X
DOI	http://dx.doi.org/10.1016/j.bej.2007.09.016
Note	このファイルは著者（最終）版です。 This file is author (final) version.

Title: Cell Cycle Dependent Production Characteristics of CHO Cells Producing Tissue Plasminogen Activator Analogue (YM866) .

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Abstract

Understanding of the relationships between recombinant protein production and cell cycle is meaningful to develop an optimal animal cell culture production process of recombinant tissue-plasminogen activator analogue (YM866). A non-linear relationship between YM866 specific production rate and specific growth rate was observed under varied serum concentration in batch culture of recombinant CHO cell, clone PM200. Clone PM200 and its derivative clone PM400, which are resistant to 200nM and 400nM methotrexate (MTX) respectively, showed difference in their cell sizes but similar linear increase in their cell size from G1 to G2/M phase in synchronized culture in medium containing growth factor solution, GF-21 made from bovine serum. Regarding the clone PM400, there were two peaks in specific production rate profile during a cell cycle transition, one small peak in early S phase and one large peak in late S to G2/M phase. Clone PM200 had similar specific production rate profile but did not have clear peaks and the expression level was lower than that of clone PM400. Difference of GF-21 concentration took effect immediately from the beginning of synchronized culture on specific production rate of YM866. Specific production rate and cell cycle time responded to the GF-21 concentrations independently. Specific production rate varied more dramatically than cell cycle time depending on the GF-21 concentration. Whereas cell cycle time attained saturated time at lower concentration of GF-21, specific production

rate increased up to higher concentration of GF-21. Designing of process and medium for this type of CHO cell should be proceeded on the basis that specific production rate attains maximum at G2/M phase and varies more dramatically than cell cycle time depending medium conditions, and both parameters are affected independently.

1. Introduction

The production of human therapeutic proteins through recombinant animal cell cultures is a most growing area of drug industry. It is well known that animal cells have three basic phases of the cell cycle, *i.e.* G1, S, and G2/M phase. Understanding the relationship between cell cycle and protein expression is critical to the optimization of medium and environmental conditions for successful commercial operation of animal cell culture process. In the case of growth associated production, cell culture process needs to cell proliferation process to produce the protein, but in the case of non-growth associated production, it is possible to develop processes focusing on cell retention system. Although many previous studies on cell cycle have been done on both endogenous and foreign genes [1, 6, 9, 10, 14, 16], the relationship between the cell cycle and protein production under varied medium condition is still unclear. Cell cycle related productivity could vary with cell line, the nature of the recombinant gene expressed or the promoter/enhancer used to generate product expression. Almost studies focused on the specific production rate and production phase of target protein.

It is also important to take into consideration that overall process productivity depends on specific production rate and cell cycle time. There is no study investigating the effect of medium on both specific production rate and cell cycle time. On the other hand, the use of expression plasmids amplified by the presence of the gene for DHFR is one popular route to creating and maintaining a high gene copy number in dhfr deficient host cells. This gene is amplified in the presence of methotorexate (MTX). There is no studies on the differences of cell cycle dependency between parental and its derivative DHFR-amplified CHO cells.

A novel t-PA analogue (YM866) [15, 23] gene was introduced into CHO cells, and several clones were isolated in the process of stepwise increase of MTX concentration. The aim of this work is to determine the cell cycle dependent recombinant protein production profile and the effects of medium condition on the production profile and cell cycle time as well as the comparison of cell cycle dependent expression between parental and its derivative clone being resistant to increased MTX concentration.

2. Materials and methods

2.1. Cells and Cell Culture Methods

Two transformed cell lines, PM200, PM400 were used for synchronization studies. CHO dhfr-negative cells [8] were transfected with a plasmid (pKSV10) carrying YM866 and

mouse dhfr gene. Each gene is controlled by a different promoter, the SV40 early promoter and the ALMP promoter, respectively. The dhfr gene was used for gene amplification under selective pressure of up to 400 nM methotrexate (MTX). The amplified sequences were integrated into the CHO genome. Both cell lines contained an insert for YM866 under the control of the SV40 promoter/enhancer which was co-amplified with the DHFR gene under the control of ALM promoter by the presence of MTX. YM866 is a modified recombinant t-PA that contains a finger domain, growth factor domain, kringle-2 domain, and serine protease domain, as well as a point mutation at the kringle-2-serine protease linkage site (del 92 – 173, ^{275}Arg $\rightarrow$ Glu) [23]. Cell lines have been selected stepwise for resistant to MTX [18]. Cell line PM200 is stably resistant to 200nM MTX. Cell line PM400 was cloned from cells which had been passaged several times from clone PM200 being adapted in the medium containing 400nM MTX. Cells were maintained as monolayer cultures in 75 cm2 T-flasks containing 10 mL of α(-) Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), and the T-flasks were incubated in the humidified 5% CO2 incubator at 37 ℃. The viable cell concentration was estimated by using an automated cell-counter (Vi-CELL XR 2.03, Beckman Coulter) and trypan-blue exclusion method.

2.2 Batch cultivation method of clone PM200

Seed cells were maintained as monolayer cultures in 75 cm2 T-flasks containing 10 mL of α (-) MEM supplemented with 10% FBS. Seed cells were inoculated in 60 mm dishes at a concentration of 7.5 × 10^5 cells/ml containing 5 ml of α (-) MEM supplemented with 0.3, 1, 3, 10% FBS, respectively, and the dishes were incubated in the humidified 5% CO2 incubator at 37 ℃. Medium was changed with fresh medium every day. Before the assay, spent medium was drained and 2 ml fresh medium was re-fed, and incubated for 2 – 3 h, then supernatant was harvested and assayed for the determination of YM866 concentration by ELISA, cells were trypsinized and counted using a cell counter (SYSMEX Corporation). Two dishes of each serum concentration were applied for assay every day so that the reported concentration of cells and YM866 are the average of two dishes.

2.3 Synchronization by mitotic cell selection

Clone PM400 cells were allowed to grow to approximately 50% confluence in 75-cm^2 T-flasks and then subjected to mitotic selection. This was performed according to the method of Mariani *et al*. (1981) [1] with the following modifications. At 50% confluence, medium was drained, 5 mL of fresh medium was added, and the flask was tapped 5 timed on each side. The medium containing dislodged mitotic cells was collected in a centrifugal tube (Falcon)

and placed in ice to stop the cell cycle progression, and T-flask was re-fed with fresh medium and placed in the humidified 5% CO2 incubator at 37 ℃. This procedure was repeated three successive times about 1hr interval to collect mitotic cells sufficient for cell cycle studies. Medium containing mitotic cells was centrifuged and cells were suspended with medium for synchronization study and spread to 35 mm dishes (Falcon) at around 3×10^5 cells/dish with 1mL medium. Medium GIT100, GIT30 and GIT15 for synchronization studies were prepared from GIT medium (Nihon Pharmaceutical Co., Ltd.) and Daigo's T medium (Nihon Pharmaceutical Co., Ltd.). T medium is chemically defined basal medium. GIT medium is a mixture of T medium and Daigo's GF-21 solution (WAKO CHEMICALS)) which contains serum factors. In this paper, GIT100, GIT30, GIT15 means mixture of GIT medium and T medium at the ratio of 100 : 0, 30 : 70, 15 : 85, respectively. Dishes containing mitotic cells were incubated in the humidified 5% CO2 incubator at 37 ℃. Periodically, dishes were sacrificed to determine viable cell densities and YM866 concentrations. Cells were tripsinized and suspended in T medium and counted by the automated cell counter and trypan-blue exclusion method. Culture supernatants were aliquoted and kept frozen at -80 ℃ for later analysis.

2.4. YM866 quantification

Enzymatic activity of the secreted YM866 in culture medium was determined by chromogenic assay. YM866 was incubated in 200 μL of 0.3 mM CH_3SO_2-(D)-HHT-Gly-Arg-pNA・AcOH (Pentapharma) in microtitration plate. The change in absorbance at 405 nm was measured (n=3). Absorbance was converted to YM866 concentration (mg/L) by interpolation from the standard curve. Regarding to the batch culture using serum containing medium, aliquots of the culture supernatants were harvested, and t-PA was quantified by a t-PA ELISA. Shortly, ELISA plate were incubated for 1 h at room temperature (RT) with coating buffer (200 μl, 50 mM phosphate buffer, pH 7.5) containing a goat anti-t-PA IgG antibody. A second incubation step followed with buffer containing 0.5% bovine serum albumin for 30 min (RT). Supernatant samples were diluted in coating buffer, loaded into ELISA plates, and incubated for 1 h (RT). Thereafter a peroxidase-conjugated anti-t-PA antibody was added and incubated for 1 h at RT. After a wash, the peroxdase substrate was added for 30 min, and the absorbance at 405 nm was determined in an ELISA.

2.5. Curve fitting and non-linear regression analysis

Curve fitting and non-linear regression analysis were performed using SigmaPlot scientific graphing software (SYSTAT Software Inc.).

3. Results and discussion

3.1. Batch cultivation with varied FBS concentration

Specific growth rate was in correlation with concentration of FBS (Fig. 1(A)) and that of each culture condition kept constant over the entire culture duration. Whereas specific production rate of YM866 also increased with concentration of FBS, decreased with elapsed days (Fig. 1(B)) in every condition, and decreased more rapidly at the higher concentration of FBS. From the dependency of the specific production rate on the FBS concentration, synthesis of recombinant protein is thought to be stimulated by several factors in FBS, but worsening culture condition between medium changes might bring the decrease of the specific production rate. Relationship between specific growth rate and specific production rate seemed to be in positive correlation at least, but non-linear correlations were observed (Fig. 1(C)). This is not coincident with previous report (22), which showed linear correlation between specific production rate of r-IFN and specific growth rate at least during logarithmic growth phase under varied concentration of FBS. This difference was supposed to come from the different condition of each experiment and it would be possible to say that non-linear

relationship between specific growth rate and specific production rate could occur under varied medium conditions. Lots of studies showed that many kinds of recombinant protein expression were growth-associated and most studies reported that specific production rate correlates to the distribution of cell cycle phase. On the other hand, varied intracellular protein expression caused by medium change 7 hours before synchronized culture was reported previously [11]. There is not such a study that investigates the influence of varied medium condition just before synchronized culture on cell cycle and specific production rate.

3.2. Synchronized growth

Cell number of each cell line increased approximately 2-fold between 14 and 20 h after mitotic selection (Fig. 2) indicating that almost all cells traversed a cell cycle via G1, S and G2/M phase. As errors in cell density measurements were considered to be rather big because of many steps; dispersion with tripsinization, pipetting, cell harvesting, dilution and counting, growth curve was smoothed by determining the best-fit regression-line between the observed points [5]. The curve fit for the cell density and time in Fig.2 was based on a logistic function given by:

$$x_v = \frac{a}{\left(1 + e^{-(t-c)/b}\right)} + d \qquad (1)$$

where x_V is the cell density (cells/ml), t is the time (h) from synchronization, a, b, c and d are constants. Using SigmaPlot curve fitting program, the constants a, b, c and d were obtained (Table1). C corresponds to the cell cycle time of each clone and distributed from 16.6 to 18.2 h. As seen in Fig. 4, mean cell diameters at 2 h is smaller than that of 0 h, which can be explained by the presence of large cells just before entering mitosis phase harvested by mitotic selection (Fig. 3). From the distribution of cell diameter and video image (not shown), mitotic selection allowed selection of large cells just entering mitosis phase, tandem connected small cells under mitosis phase and single small daughter cells just after mitosis phase which are all weekly connected to the surface of dishes. After 2 h from the beginning of synchronous culture, the large cells disappeared and all cells had small diameter. That is that large cells harvested from mitotic selection traversed the mitosis phase within 2 h, which means that mitotic selection collected cells from beginning to end of mitosis phase which is spreading in about 2 h duration in a cell cycle duration.

Cell diameter increased continuously from G1 to G2/M phase in each case (Fig. 3). A basic property of cells in culture is that their volume increased approximately 2-fold in the progression from G1 to M. As expected the volume of the G2/M was approximately twice the volume of G1. Direct relationship between cell volume and cell cycle has been reported on several cases [2, 4, 11, 17, 19, 20]. Clone PM400 has smaller and narrower distribution of cell diameter than clone PM200 (Fig. 3). Diameter of clone PM400 decreased along with the

decrease of GF-21 concentration, which differ from the phenomenon that diameter of recombinant CHO producing IFN increased under low concentration of serum [22].

Cell cycle time of clone PM400 was shorter than that of parental clone PM200 (Table1). Generally, the higher resistance to MTX leads to the lower specific growth rate [13, 22], but in this case they were in inverse relation. As wide distribution of the relationship between MTX resistance and specific production rate was reported [3], it would be possible to select a clone having both higher specific growth rate and higher production rate in the course of increasing MTX resistance. Clone PM400 showed the same cell cycle time when cultured with medium GIT100 or GIT30, but showed the prolonged time when cultured with medium GIT15, which is as same as the one of clone PM200 with medium GIT100 (Fig. 2).

3.3. Specific Productivity

Continuous increase in cell size (Fig. 3) means that cell cycle traversed as typical way [2, 4, 17, 20, 21]. YM866 concentration in culture medium increased continuously from G1 phase to G2/M phase and final concentration differed among clones and GF-21 concentrations (Fig. 2). Velocity of increase in YM866 concentration was not constant. Secretion of YM866 was observed at every cell cycle phase of both recombinant clones, PM200 and PM400, but not host cells (data not shown). Specific production rate was calculated from differences of

YM866 concentration between adjacent sample time and cell density calculated from smoothed curve fittings (Fig. 4). The specific growth rate (μ) in G2/M phase of synchronized culture was obtained using equation (2) and (3).

$$\mu = \frac{1}{Xv} \bullet \frac{dXv}{dt} \qquad (2)$$

$$\frac{dXv}{dt} = \frac{a}{b} \bullet \exp\left(\frac{c-t}{b}\right) \bullet \left(1 + \exp\left(\frac{c-t}{b}\right)\right)^{-2} \qquad (3)$$

Widths of distribution of specific growth rate in G2/M phase were narrower in decreased GF-21 concentration, which showed better synchronization. According to studies under similar conditions [12, 14], the duration of G1, S and G2/M phase were supposed to approximately 5 h, 8 h and 5 h, respectively, and production phase was possibly located mainly from S to G2/M phase with both clones and every conditions, and the highest peak of specific production rate was located at G2/M phase of every conditions (Fig. 4). A low peak of specific production rate in the early S phase was observed with clone PM400, which is clearly observed with GIT100 and GIT15 medium, whereas such a peak is ambiguous with clone PM200. According to the previous report with similar materials [16], recombinant DHFR under the control of AML promoter was synthesized particularly in G1 and early S phase and constantly synthesized during other cell cycle phases. On the contrary, secretion rate of recombinant t-PA under the control of SV40 early promoter measured by 2h incubation with cells collected by flow cytometry from each cell cycle phase showed following order,

late-S > early-S > G2/M > G1. This previous study depended on samples with rather long sampling interval, so it is not clear about detailed specific production rate profile. Relative production rate of t-PA (late-S / G1) of this study was higher than this previous study [16], particularly with clone PM400. Several studies reported that expression phases of endogenous protein and recombinant protein depend upon promoters [14]. It is reported that SV40 early promoter and CMV promoter promoted strongly at S-phase and AML promoter promoted strongly mainly at G1-phase [7, 16]. Whereas the expression phase of this study , having two-phase expression profile between early-S phase to G2/M phase, differed from those of previous studies using recombinant cells, resembled to a expression pattern of amplified endogenous DHFR activity which expressed strongly at early S and G2/M phase [11].

Clone PM400 was considered to be superior to clone PM200 from two aspects, namely specific production rate and cell cycle time. Specific production rate responded in short time to the change of GF-21 concentration (Fig. 4). It is notable that the specific production rate of clone PM400 was lower with GIT30 than with GIT100, but cell cycle time was almost the same with both conditions (Table1, Fig. 4). In the case of GIT15, in addition to decrease in specific production rate, increase in cell cycle time which is as same as clone PM200 with GIT100 was observed, that is disadvantageous to production of recombinant protein. From the above observations, it is supposed that in a batch culture, worsening of medium conditions induces a decrease of the height of peaks of specific production rate particularly in the S and

G2/M phase at first, and additional worsened medium conditions leads to a prolonged cell cycle time resulted in decreased production rate. This phenomenon consists well with the data of batch culture (Fig. 1(A)-(C)). From the aspect of development of improved medium for high productivity, namely increasing the nutrients or growth factor concentrations, cell cycle time reached the minimum duration at GF30 (saturation point), but specific production rate showed the tendency of increase up to GF100. Consequently, in the case of recombinant protein production of which recombinant protein expression depends on S phase or G2/M phase, medium development would have room to improve specific production rate largely, beside shortening of cell cycle time would meet the limit immediately. It is also obvious that poorly change of medium condition has influence on specific production rate immediately.

Previous study reported that there was a linear correlation between γ-IFN productivity with CHO cells and the duration time of G1 phase [9]. It is considered that productivity is influenced by not only the distribution of cell cycle phase but also varied specific production rate at each cell cycle phase caused by medium conditions which might have large portion of variance of productivity. This idea is consistent with other study [5]. The relationship between cellular constituent protein and recombinant protein is considered to be non-parallel. Synthesis of endogenous constituent protein is strictly controlled by cell cycle transition, synthesized to double amount between a cell cycle, on the other hand, synthesis of recombinant protein depends on cell cycle phase but the synthesis rate strongly depends on

copy number of the product gene and medium condition. In the designing of medium components for optimal production, both specific production rate and cell cycle time to be considered.

4. Conclusion

In batch culture study using recombinant CHO cells expressing t-PA analogue, YM866, specific growth rate was maintained constant over the entire culture at rate in proportion to serum concentrations. Non-linear relationship was observed between specific growth rate and specific production rate. The phenomenon was explained by the data of synchronized cultures. Recombinant clone PM200 resistant to 200nM MTX and its derivative clone PM400 resistant to 400nM MTX had production phase mainly from early S to G2/M phase, that is that cells showed growth-associated production in batch culture. Clone PM400 had two production phase, small peak in early S phase and large peak in from late S to G2/M phase, and both peaks showed their heights in proportion to GF-21 concentrations immediately from the beginning of synchronized culture. Specific production rate and cell cycle time responded to the medium conditions independently. Some extent of decrease of GF-21 concentration introduced only the decreased specific production rate, and more decrease of GF-21

concentration introduced prolonged cell cycle time and decreased specific production rate with lowered both production peaks in the cell cycle. These findings can explain the non-linear relationship between specific growth rate and specific production rate and suggest that development of medium and culture process for improved productivity of cells having similar production characteristics like this study depends on mainly how stimulate the potential of specific production rate and prevent from worsening medium condition with maintaining minimum cell cycle time.

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Legends to figures

Figure 1(A): Growth of clone PM200 in batch culture.

Clone PM200 was cultured with medium containing 0.3, 1, 3, 10% FBS, respectively. Cell density is a mean of two dishes. Curve fitting was applied with $y = ax^b$.

Figure 1(B): Specific production rate of YM866 in batch culture.

Specific production rates were measured by medium change with 2ml fresh medium followed by 2-3 h incubation at 37℃, and calculated from the YM866 concentration in supernatant divided by the cell density. Specific production rate is a mean of two dishes.

Figure 1(C): Relationship between specific growth rate and specific production rate

Specific growth rates were calculated from fitting curve in Fig. 1.

Figure 2: Cell growth, YM866 concentration and cell diameter in synchronized culture.

Cell growth, YM866 concentration and cell diameter in batch cultures synchronized by mitotic selection; (A) Clone PM200 with GIT100 medium; (B) Clone PM400 with GIT15 medium; (C) Clone PM400 with GIT30 medium; (D) Clone PM400 with GIT100 medium; ○ cell density; ◆ YM866 concentration; ● cell diameter; ------- curve fit for the cell density

Figure 3: Distribution of cell diameter in synchronized culture.

3D and contour graphs of distribution of cell diameter in batch cultures synchronized by mitotic selection; (A) Clone PM200 with GIT100 medium; (B) Clone PM400 with GIT15 medium; (C) Clone PM400 with GIT30 medium; (D) Clone PM400 with GIT100 medium.

Figure 4: Profiles of specific growth and production rates in synchronized culture.

Specific growth and production rates in batch cultures synchronized by mitotic selection. ; (A) Clone PM200 with GIT100 medium; (B) Clone PM400 with GIT15 medium; (C) Clone PM400 with GIT30 medium; (D) Clone PM400 with GIT100 medium.; ● specific production rate; ------- specific growth rate.

Table 1: Constants of sigmoid formulation.

Using the SigmaPlot curve fitting program, the constants a, b, c and d were obtained.