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NOTE

The Effect of Glutamine on Production of Tissue-type Plasminogen Activator from Recombinant Human Melanoma Cells in Glutamine-limited Fed-batch Cultivation

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Under glutamine-limited condition, 2×10^6 (viable cells/ml) of maximum cell density and 13.5 (μg/ml) of tissue-type Plasminogen Activators (tPA) production were maintained by spike feeding fresh medium in fed-batch cultivation of human recombinant melanoma cells. It showed that tPA production was much seriously affected than cell growth according to initial glutamine concentrations. Above 3.4 (mmol/l) of glutamine concentration both cell growth and tPA production were not much affected by increasing initial glutamine concentration. Glutamine depleted situation was occurred at latter periods of batch and fed-batch cultivations below 5.4 (mmole/l) of initial glutamine concentration. It also showed that maximum glutamine consumption and ammonia evolution rates were closely related to initial glutamine concentrations. Maximum specific tPA production rate was estimated as 8.1×19^{-6} (μg/cells/h) at 3.4 (mmol/l) of glutamine concentration, which is higher than that from other batch and fed-batch processes.

There have been many reports on the production of tissue-type Plasminogen Activators (tPA's) from animal cells (1, 4). TPA proves to be effective in treating acute myocardial infraction and deep vein thrombolysis and are also known to be the first biopharmaceuticals appeared on the markets from cell cultures (15). Several kinds of recombinant and normal mammalian cells are cultivated to secrete tPA's into the medium through batch or mostly perfusion culture processes (8, 14). For the production of tPA, a perfusion cultivation process has been proven to be most effective process since it can maintain high density of mammalian cells by removing accumulated ammonia and lactate in the medium (7, 10). However, there is still controversial in economic feasibility between batch and perfusion systems due to the complexity and fragility and the limits of scaling-up in the perfusion process (16, 18). Fed-batch cultivation can be an alternative to overcome the problems encountered in both batch and perfusion cultivations in small and large vessels (6). It is also known that a glutamine in the medium plays key role in energy metabolism like glutamolysis compared to glycolysis, especially at low glucose levels such as transient period when most often occurred in batch and fed-batch processes (2, 5, 9, 13). It can affect the protein production in fed-batch cultivation. However, there has been less studied in cultivating animal cells by fed-batch system because it has high possibility of contaminations and the requirement of intensive monitoring, etc. (12). Glutamine-limited experiment has not been much carried out, compared to glucose and serum-limited conditions even though a glutamine is an essential nutrient in mammalian cell cultures (9, 13). In this work, the effect of glutamine on mass production of tPA is to be analyzed for glutamine-limited fed batch process by cultivating human recombinant cell line. It will also be used for minimizing initial glutamine concentrations since excess glutamine could evolve harmful ammonia into the media (9).

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Key words: tPA, human melanoma cells, glutamine-limited, fed-batch cultivation

Cell Line and Culture Condition

A recombinant human melanoma cell line (CRL 9607, ATCC) was grown in EMEM basal medium with 10% FBS (GIBCO, USA) in a 75 cm^2 T-flask. When the cell density reached to 1×10^4 total cells/ml, it was inoculated into a bioreactor (working volume 2 liters, Celligen, USA) for fed-batch cultivation. Prewashed 2.5 (g/l) of microcarriers (Cytodex III) was also inoculated before inoculating the cells. The initial concentration of glutamine in the fresh medium was varied as 1.3, 3.4 and 10.5 mM for glutamine-limited experiments. pH and

dissolved oxygen concentrations were automatically maintained as 7.1 and 30% of air saturation by a microprocessor in the bioreactor. The agitation speed was set to 40 rpm. For fed-batch operation, 300 ml of fresh medium containing 1 % serum was fed by a peristaltic pump, up to 2 liters of final volume. The medium was added when the cell density was exponentially increased, by about five day intervals.

Measurement of Cell Density and Growth Parameters

The viable cell density was measured by a trypan blue dye exclusion method everyday. tPA concentration in the medium was also measured by a ELISA kit (American Diagonistics, USA) and its activity was measured by fibrin agar plate method (17). The specific activity of the purified tPA from spent medium was calculated as 130,000 (IU/mg). The concentrations of glucose and lactate in the medium was also measured by a glucose-lactate analyzer (YSI, USA). The concentrations of ammonia and glutamine were estimated by calorimetric method using Wako kit (Japan) and HPLC (Waters, USA), respectively. Specific substrate consumption and production rates were calculated in spike fed-batch cultivation, assuming that the concentration of the substrate in the reactor was at the approximately calculated concentration (11).

As the initial glutamine concentration was increased, the cell growth rate also increased at 0.14 (1/day) of maximum specific growth rate in 1.37 (mmol/l) and at 0.22 (1/day) of maximum specific growth rate in 10.5 (mmol/l). The cell density was temporarily dropped when fresh medium was added, then gradually increased up to 2×10^6 (viable cells/ml) by the third feeding of fresh medium in 3.4 (mmole/l) of glutamine. However, the dramatic increase of the cell density was not observed by adding 10.5 (mmol/l) of glutamine, compared to that by 3.4 (mmol/l). No more glutamine-limited condition can not be occurred in adding above 10.5 (mmol/l) of initial glutamine concentration by spike feeding fresh medium (Fig. 1).

The effect of initial glutamine concentrations on tPA production was also observed by adding various concentrations of glutamine as shown in Fig. 1. At 1.4 (mmole/l) of initial glutamine concentration, the production of tPA was not much improved even though the cell density was relatively increased. tPA production was greatly increased by adding 3.4 (mmol/l) of glutamine, while the production rate was not much increased in 10.5 (mmol/l) of glutamine. It also tells that glutamine-limited condition is maintained in adding lower than 10.5 (mmole/l) of initial glutamine concentration for this fed-batch cultivations. Maximum tPA production was obtained as 16.5 (μg/ml) and 13.8 (μg/ml) in 10.5 (mmol/l) and 3.4 (mmol/l) of initial glutamine while only 3.5 (μg/ml) of tPA was secreted in 1.37 (mmol/l). It shows that a glutamine plays an impotant role in producing proteins than in growing cells under glutamine-limited fed-batch cultivation. It can also be confirmed by the result that the metabolic rates can be changed by medium exchange schedule (3) (Fig. 2).

Glutamine consumption rate seems to be strongly correlated to initial glutamine concentrations in the medium. The uptake rate was linearly increased as initial glutamine concentration increased up to 5.8 (mmole/l). At more than this concentration, the uptake rate was no longer increased due to possibly no more glutamine-limited condition. It tells that the glutamine-limited con-

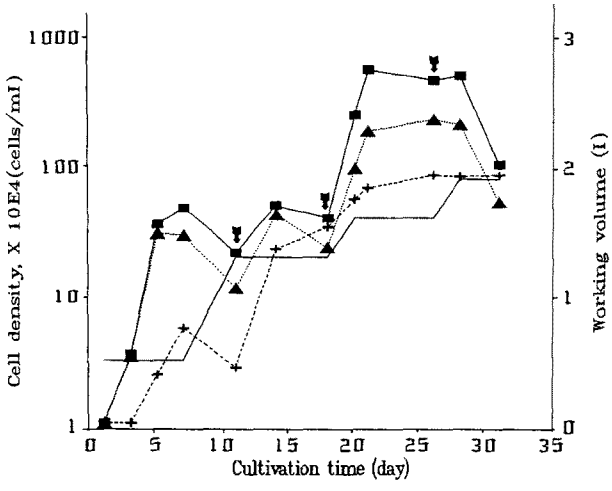

Fig. 1. The growth of human recombinant melanoma cells in fed-batch cultivation at various initial glutamine concentrations.
Arrows indicate the points of feeding fresh medium. —■—, 10.5 (mmole/l); —▲—, 3.4 (mmole/l); —+—, 1.4 (mmole/l).

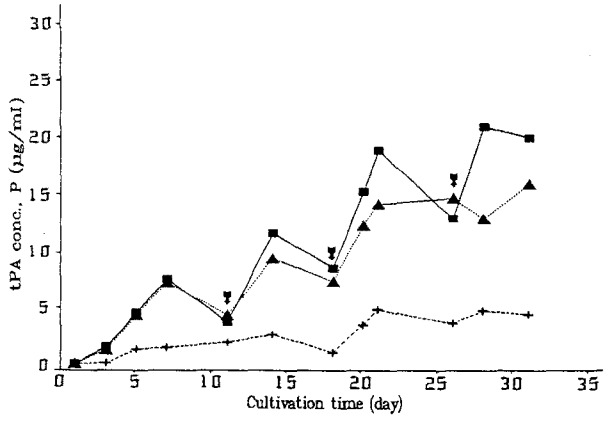

Fig. 2. The production of tPA from human recombinant melanoma cells in fed-batch cultivation at various initial glutamine concentrations.
Arrow indicate the points of feeding fresh medium. —■—, 10.5 (mmole/l); —▲—, 3.4 (mmole/l); —+—, 1.4 (mmole/l).

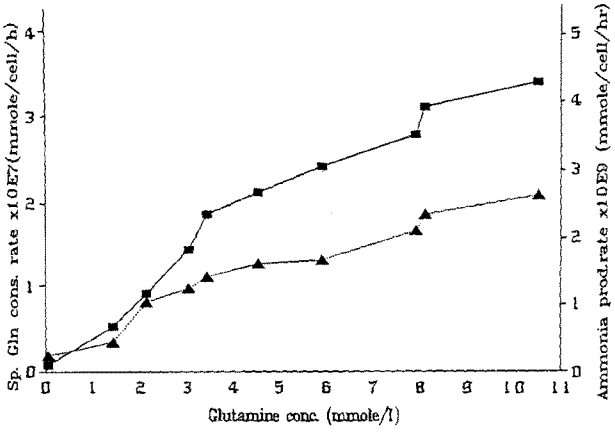

Fig. 3. The relationship between initial glutamine concentrations and specific glutamine consumption and specific ammonia production rates in fed-batch cultivation.
—■—, Gln consumption; —▲—, Ammonia production.

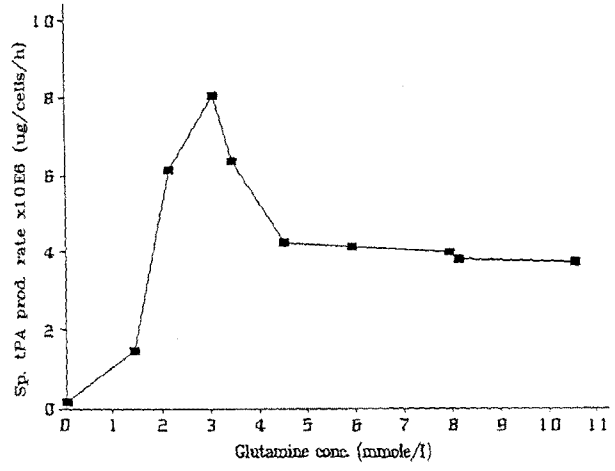

Fig. 4. The production rate of tPA from recombinant melanoma cells at various initial glutamine concentrations.

dition can only be maintained up to that concentration. Maximum NH_3 evolution rate is also closely related to initial glutamine concentrations. The molar ratio of glutamine to ammonia was less than one which can be observed in conventional perfusion cultivations (9, 13). Most of consumed glutamine was used to generate energy by evolving less amounts of ammonia since glutamolysis was dominated under glutamine-limited condition. The carbon sources for energy production was not altered by adding fresh medium in fed-batch cultivations. It also shows that the transamination process in TCA cycle can be affeced by pulse-adding medium (Fig. 3).

Above 5.8 (mmol/l) of initial glutamine concentrations, tPA production rate was not much affected by glutamine concentrations. At lower initial glutamine concentrations, specific tPA production rate was increased up to 8.2×10^{-6} (μg/cell/h). This tPA production rate seems to be higher than that from conventional batch and fed-batch cultivations, such as 2.1×10^{-6} (μg/cells/h) from CHO cells and 1.24×10^{-7} (μg/cells/h) from recombinant Bowes melanoma cells, etc. (13, 14). It implies that higher tPA production can be obtained in glutamine-limited condition than glucose-limited for fed-batch cultivation (Fig. 4).

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