Effect of culture temperature on a recombinant CHO cell line producing a C-terminal α -amidating enzyme

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Abstract

In order to seek an efficient method for producing a recombinant protein by using animal cell culture, we investigated various effects of the culture temperature on a recombinant CHO cell line (3 μ -1S), producing a C-terminal α -amidating enzyme (799BgIII α -AE) originating from *Xenopus laevis*. The results revealed that a low culture temperature (below 37 °C) led to the following phenomena: [1] inhibited cell growth, [2] enhanced cellular productivity of the recombinant protein, [3] maintained high cell viability, [4] suppressed medium consumption, and [5] suppressed release of impurities from the cells. These findings indicate that a quite simple method, the culture at low temperature, will contribute to the total improvement of the industrial process for the production of the recombinant protein, 799BgIII α -AE.

Introduction

Animal cells are most commonly cultured at 37 °C. However, since the culture temperature, as well as pH (Borys et al., 1993; Hosoi et al., 1991; Ozturk and Palsson, 1991) and DO (Lin et al., 1993; Ozturk and Palsson, 1991; Reuveny et al., 1986), would affect such cellular events as growth, viability, protein synthesis and metabolism, it is an important factor which needs to be studied to realize an efficient process for protein production by animal cell culture. Many bioactive proteins are currently produced by culturing recombinant cells. Weidemann et al. (1994) have described that a low culture temperature (below 37 °C) for a recombinant BHK cell line suppressed the cell growth and glucose consumption, but did not affect the cellular productivity of recombinant antithrombin III. However, there is not sufficient known about the effect of culture temperature on recombinant cells for the production of recombinant proteins.

On the other hand, there are several reports on the effects of culture temperature on hybridomas producing monoclonal antibodies and on non-transformed

cells producing interferon(s). In the case of hybridomas, while it has been shown that the response to culture temperature varied with the cell line used, it can be summarized that a culture temperature below 37 °C inhibited cell growth, maintained high cell viability for a longer period and suppressed glucose consumption, but it did not enhance the cellular productivity of the monoclonal antibody (Bloemkolk et al., 1992; Borth et al., 1992; Reuveny et al., 1986; Sureshkumar and Mutharasan, 1991). In contrast, it has been shown that a low culture temperature enhanced the yield of the interferon produced by non-transformed cells which had been induced by certain reagents or viruses (Giard and Fleischaker, 1980; Giard et al., 1982; Kojima and Yoshida, 1974; Vilček and Havell, 1973). Furthermore, in the recent report of Takagi and Ueda (1994), there is data which indicates that the TPA (tissue plasminogen activator) productivity was increased by culturing human embryo lung cells at a low temperature.

Therefore, we hypothesized that some recombinant cell lines should show increased cellular productivity of the recombinant protein at a low culture temperature. If enhanced cellular productivity, as well as main-

tained high cell viability and suppressed glucose consumption, could be achieved by culturing recombinant cells at a low temperature, the process for the protein production with the cells would be greatly improved.

In the present study, we examined the effect of culture temperature on a recombinant CHO cell line from the following aspects: the growth, viability, cellular productivity of a recombinant protein, nutrient consumption and the recombinant protein secreted into the medium, in order to establish more efficient processes. The cells used here can grow in suspension with a serum-free medium, and secrete a recombinant C-terminal α -amidating enzyme (799BgIII α -AE) from *Xenopus laevis* (Furukawa et al., 1993). This enzyme catalyzes the conversion of glycine-extended prohormone substrates to biologically active α -amidated peptide hormones such as human calcitonin (Bradbury et al., 1982; Eiper et al., 1983; Kato et al., 1990; Mizuno et al., 1987; Mizuno et al., 1986; Ohsuye et al., 1988).

Materials and Methods

Cell line and maintenance

A recombinant CHO cell line, 3 μ -1S, which produced 799BgIII α -AE derived from *Xenopus laevis*, was obtained as described in our previous report (Furukawa et al., 1993). In this cell, the 799BgIII α -AE gene and DHFR gene are respectively transcribed under the control of the SV40 early promoter and amplified with increasing concentration of methotrexate (MTX, Sigma). The 3 μ -1S cells can be grown in suspension with a serum-free medium. The medium was Ham's F-12 (Ajinomoto, Japan) supplemented with 1.0 μ M of MTX (Sigma), 60 mg/l of kanamycin sulfate (Meiji, Japan), 600 μ g/ml of polyvinyl alcohol, 1.2 g/l of sodium bicarbonate, 15 mM of HEPES (Sigma) and 5 μ g/ml each of bovine insulin and transferrin (Intergen). The pH of the medium was adjusted to 7.2, and the cells were maintained in siliconized 300-ml flasks while gently shaking at 37 °C.

Culture system

We established an 1-litre cell culture system, which enabled the temperature, dissolved oxygen (DO) and pH to be controlled, 1-litre spinner flasks (Corning) being used as culture vessels. The temperature could be controlled to a set value within ± 0.1 °C by rubber-coated heater, DO to within $\pm 3\%$ air saturation by

air, O₂ or N₂, and pH to within ± 0.03 by CO₂ or an alkaline solution (7% w/v of sodium bicarbonate), all gases being supplied to the medium through a Teflon membrane. This system enabled six kinds of culture to be performed independently at the same time.

Cell culture for testing

As a seed culture, the 3 μ -1S cells were inoculated to a 3-litre spinner flask (Bellco) at 2×10^5 cells/ml in 1500 ml of the serum-free medium without MTX, and cultured at 37 °C in a 5% CO₂/air humidified incubator for 3 days. The cells obtained from the seed culture were then inoculated into each culture vessel of the system at 2×10^5 cells/ml in 750 ml (final volume) of the fresh medium. The culture temperature for each test was controlled to 30, 32, 33.5, 35, 36 and 37 °C, respectively, with the pH controlled to 7.2, and the agitation speed adjusted to 100 rpm. Oxygen was supplied by continuously supplying 100 ml/min of air through the Teflon membrane. We confirmed that DO could be maintained above 60% air saturation under these culture conditions. The cells were cultured for 5 days, the total and viable cell density being determined daily by the trypan blue dye exclusion method in a hemacytometer. All samples, which included the cell suspension, cells and supernatant, were stored at -20 °C until required.

Assay for α -AE activity

The assay for α -AE activity in each culture supernatant was carried out as reported previously with Ac-[¹²⁵I]-Tyr-Phe-Gly as a substrate (Mizuno et al., 1986). One unit of activity is defined as the amount of enzyme that gives a 50% conversion of Ac-Tyr-Phe-Gly to Ac-Tyr-Phe-NH₂ under the standard assay conditions.

Northern blot analysis

Total RNA was isolated from the cells with TRIzol™ reagent (Gibco BRL). 20 μ g of total RNA was electrophoresed in a 1% agarose gel containing 9% formaldehyde and a 10 mM phosphate buffer (pH 7.4), followed by vacuum transfer to a nylon membrane (Hybond-N+, Amersham) in 20 $\times$ SSC and UV cross-linking. The ³²P-labeled 799BgIII α -AE DNA probe was synthesized by the Megaprime™ DNA labelling systems (Amersham), using the insert DNA (2.35 kb *Eco*RI fragment) isolated from the plasmid vector, pKD799BgIII (Furukawa et al., 1993). The RNA blots

on the membrane were hybridized to the ^{32}P -labeled probe at 42 °C for 16 h in 50% formamide, $5 \times \text{SSC}$, 0.5% (w/v) SDS, $5 \times \text{Denhardt's solution}$, 100 $\mu\text{g/ml}$ of sheared and denatured herring sperm DNA, and 5×10^5 cpm/ml of the probe. After hybridization and stringency washes, the blots were analyzed by imaging plate (IP)-autoradiography with Bio-imaging analyzer (BAS2000II, Fuji Photo Film, Japan). The intensity of each 799Bg/III α -AE mRNA band was quantitated as the photo-stimulated luminescence (PSL) value. Subsequently, the 799Bg/III α -AE probe hybridized was removed by boiling the membrane with a 0.5% (w/v) SDS solution for 30 min, in order to re-hybridize to a ^{32}P -labeled β -actin DNA probe. This probe was synthesized by using human β -actin cDNA (Clontech) as the template. Hybridization and analysis were carried out in the same way as that just described.

Analysis of glucose and amino acids

The glucose and amino acid concentrations in the culture supernatant were measured by Glucose analyzer ST-1 (Oriental Electric, Japan) and Amino acid analyzer L-8500 (Hitachi, Japan), respectively. The consumption rate was calculated as described later.

Analysis of cell lysis

The level of cell lysis during the culture was evaluated by a lactate dehydrogenase (LDH) assay (Goldblum et al., 1990; McQueen et al., 1987; Petersen et al., 1988; Wu et al., 1992), the LDH activity being measured by determining the initial rate of oxidation of β -NADH in the presence of pyruvate. For this assay, the cell suspension was diluted with a fresh culture medium to an equivalent cell density of $1\text{--}5 \times 10^5$ cells/ml. A 450- μl portion of the supernatant or diluted cell suspension was mixed with a 50 μl of 10% Triton X-100 (Sigma) to lyse the cells in the suspension. The sample (100 μl) was added to 600 μl of a reaction solution made up from a 50 mM potassium phosphate buffer (pH 7.4) containing 1.9 mM of sodium pyruvate (Sigma). 3.5 mM β -NADH (20 μl ; Sigma) was then added to the reaction solution to a final concentration of 0.1 mM, and the absorbance of the sample at 340 nm was continuously recorded by a Spectrophotometer UV-240 (Shimadzu, Japan). The β -NADH oxidation rate, corresponding to the LDH activity, was obtained as a slope of decreasing absorbance. The cell lysis ratio was calculated as follows:

Cell lysis ratio (%) =

$$\left(\frac{\text{LDH activity of the supernatant}}{\text{LDH activity of the cell suspension}} \right) \times 100$$

SDS-PAGE and Western blot analysis

Aliquots of each culture supernatant corresponding to 45 units of α -AE activity were electrophoresed in duplicate in a 10% polyacrylamide gel containing SDS. The proteins separated on one gel were stained with Coomassie brilliant blue R-250, and those on the other gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) for the Western blot analysis. The membrane was blocked with 3% gelatin/0.1% Tween 20-PBS, and incubated with rabbit anti-799Bg/III α -AE polyclonal antibody conjugated biotin. After having been washed with 0.1% Tween 20-PBS, the membrane was treated with horseradish peroxidase avidin D (Vector). The immunoreactive bands were visualized with a POD immunostain set (Wako Pure Chemical Industries, Japan). As molecular weight markers, wide-range SDS-PAGE protein standards (Tefco) for SDS-PAGE and SeeBlue™ pre-stained standards (Novex) for Western blotting were used.

Data analysis

On the assumption that the viable cell density varied exponentially during each period between measurement points, the cellular productivity of 799Bg/III α -AE (q_p ; units/ 10^5 cells/day) and consumption rate of glucose or amino acids (q_c ; $\mu\text{g}/10^5$ cells/day) were respectively calculated by the following equations (Merten, 1988; Mochizuki et al., 1993):

$$q_p = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \times \frac{P_2 - P_1}{X_2 - X_1} \quad (\text{for } X_1 \neq X_2)$$

$$q_p = \frac{1}{X_1} \times \frac{P_2 - P_1}{t_2 - t_1} \quad (\text{for } X_1 = X_2)$$

and

$$q_c = - \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \times \frac{C_2 - C_1}{X_2 - X_1} \quad (\text{for } X_1 \neq X_2)$$

$$q_c = - \frac{1}{X_1} \times \frac{C_2 - C_1}{t_2 - t_1} \quad (\text{for } X_1 = X_2)$$

where X_1 and X_2 are viable cell densities ($\times 10^5$ cells/ml), P_1 and P_2 are α -AE activities accumulated in the medium (units/ml), and C_1 and C_2 are concentrations of glucose or amino acids (μ g/ml) on days t_1 and t_2 , respectively.

Results

Cell growth and viability

In order to evaluate the effects of culture temperature on the recombinant CHO cell line named 3 μ -1S producing 799Bg/III α -AE (Furukawa et al., 1993), the cell line was cultured at various temperatures (30–37 °C) for 5 days (see the 'Materials and Methods' section).

As shown in Figure 1, the cell growth indicated a dependency on the culture temperature: it attained a maximal level at 36 °C and was completely arrested below 32 °C. On the other hand, the cell viability tended to decline with increasing culture temperature. To assess the level of cell-death during the culture, the cell lysis ratio was determined by LDH assay (Goldblum et al., 1990; McQueen et al., 1987; Petersen et al., 1988; Wu et al., 1992). As shown in Figure 2, although no significant difference in the cell lysis ratio was observed until 72 h after the inoculation (11–14%), the ratio was notably elevated at higher temperature by 120 h (30 °C, 15%; 37 °C, 32%).

These results indicate that culturing at a low temperature (below 37 °C) had the advantage of not only the maintaining a high cell viability for a longer period, but also of suppressing the release of proteins from the dead cells to the culture medium.

Production of 799Bg/III α -AE

The α -AE activity of the culture supernatant was subsequently measured with the synthetic substrate, Ac-[¹²⁵I]-Tyr-Phe-Gly (Mizuno et al., 1986).

Figure 3A shows the cumulative activity of 799Bg/III α -AE secreted into the medium. The production of 799Bg/III α -AE also exhibited temperature dependency. The maximal production was observed with culturing at 35 °C, and the α -AE activity attained 2000 units/ml, in comparison with 1100 units/ml at 37 °C, during the whole culture period.

The cellular productivity of 799Bg/III α -AE was calculated from the enzymatic activity (Fig. 3A) and the viable cell density (Fig. 1). As shown in Figure 3B, while the cellular productivity reduced

at lower temperature (30 °C, 37 units/ 10^5 cells/day; 37 °C, 62 units/ 10^5 cells/day) during the initial period (0–21.5 h), the productivity thereafter at 30 °C and 32 °C increased remarkably and attained 130 and 154 units/ 10^5 cells/day by the 5th day, respectively. In contrast, the increasing rate of the productivity above 33.5 °C decreased with progress of the culture, moreover the productivity itself decreased. This tendency was more remarkable at higher culture temperature; i.e. the productivity at 37 °C decreased from 62 units/ 10^5 cells/day on the 1st day to 30 units/ 10^5 cells/day on the 5th day.

These results reveal that the highest production and cellular productivity of 799Bg/III α -AE were obtained by culturing at 35 °C and 32 °C, respectively.

Analysis of 799Bg/III α -AE mRNA

The cellular productivity of 799Bg/III α -AE increased at low culture temperature. It would be quite reasonable for a low culture temperature to affect the expression of the 799Bg/III α -AE gene, so to clarify this, we carried out Northern blot analysis of the transcribed mRNA by using Bio-imaging analyzer.

The total RNA samples were isolated from the cells cultured at various temperatures and subjected to Northern blotting. To accurately measure the content of 799Bg/III α -AE mRNA, the RNA blots containing 20 μ g of the total RNA per lane were hybridized to a 799Bg/III α -AE DNA probe and re-hybridized to a human β -actin DNA probe. The signals of the bands hybridized to the probes were respectively quantitated as PSL values. Since we had confirmed that the expression of β -actin mRNA was not affected by the culture temperature (data not shown), β -actin mRNA could be used as an internal control for the analysis. As shown in Figure 4, the relative content of 799Bg/III α -AE mRNA, which was the PSL value of the 799Bg/III α -AE mRNA band normalized by that of the β -actin mRNA band, increased with lowering the culture temperature: the content at 30 °C or 32 °C was 1.8–2.4-fold higher than the content at 37 °C. In addition, we also analyzed 799Bg/III α -AE DNA by Southern blot hybridization, but no detectable difference was apparent (data not shown).

Therefore, the increase in 799Bg/III α -AE mRNA content would have been one of the important factors enhancing the productivity of 799Bg/III α -AE at the low culture temperature.

Figure 1. Growth curves of 3 μ -1S cells at various culture temperatures. The total cell density (●) and the viable cell density (○) were determined by the trypan blue dye exclusion method in a hemacytometer. In each culture pH was controlled to 7.2 by CO₂ or 7% (w/v) of sodium bicarbonate, and DO was kept above 60% air saturation by continuously supplying 100 ml/min of air. The agitation speed was adjusted to 100 rpm. The samples obtained here were subjected to subsequent examinations.

Consumption of nutrients

To evaluate the effect of culture temperature on the consumption of nutrients, we analyzed the consumption rates of glucose and amino acids.

As shown in Figure 5, the glucose consumption rate depended on the culture temperature, decreasing from 80 μ g/10⁵ cells/day at 37 °C to 24 μ g/10⁵

cells/day at 30 °C. Table 1 presents the amino acid consumption rates at 32, 35 and 37 °C. As indicated in this table, the consumption rates of most of amino acids decreased with lowering the culture temperature, while those of aspartate and alanine increased, and those of glutamate, glycine, methionine, leucine and isoleucine hardly changed. Lowering the culture temperature from 37 °C to 32 °C, at which the highest

Figure 2. Effect of culture temperature on the lysis of 3 μ -1S cells. The cell lysis ratio was obtained as the proportion of lactate dehydrogenase (LDH) activity in the culture supernatant to that in the cell suspension (the culture itself). The LDH assay was performed as described in the 'Materials and Methods' section.

cellular productivity was exhibited, caused a 48% and 25% reduction in the consumption rates of glucose and total amino acids (except for the amino acids being produced), respectively.

Thus, culturing at a low temperature suppressed the consumption of most of nutrients with the high productivity of 799Bg/III α -AE.

Analysis of Secreted 799Bg/III α -AE

SDS-PAGE and Western blot analyses were carried out to assess the 799Bg/III α -AE protein secreted into the culture medium (Figure 6). Aliquots of each supernatant from the day 5 culture, corresponding to 45 units of α -AE activity, were subjected to both analyses.

The result of Western blotting is shown in Figure 6A. Two major immunoreactive 75 and 81 kDa bands, corresponding to 799Bg/III α -AE with complete length, were observed. The difference in the molecular weight of these bands would have been caused by different modification of the sugar chains, because 799Bg/III α -AE is a glycoprotein and has three glycosylation sites. As shown in this figure, no change in either the molecular weight or the intensity of these

bands is apparent, implying that the culture temperature (30–37 °C) had no effect on the specific activity or glycosylation of the 799Bg/III α -AE protein. Figure 6B shows the result of the SDS-PAGE analysis. The two bands just below the 75 kDa band of 799Bg/III α -AE are transferrin that was added to the medium. It is apparent that the intensity of the residual bands, corresponding to the cellular proteins, decreased with lowering culture temperature. This result indicates that culturing at the low temperature enhanced the relative content of 799Bg/III α -AE secreted into the medium.

Discussion and Conclusions

As a final purpose of production of a bioactive protein by animal cell culture is isolation of the protein, it is necessary to consider not only enhancing the productivity, but also reducing the impurities derived from metabolites of the cells and/or cellular contents. In this study, we investigated the various effects of culture temperature on a recombinant CHO cell line (3 μ -1S) and found that culturing at a low temperature would

Figure 3. Effect of culture temperature on the 799Bg/IIα-AE production. (A) Accumulation of α-AE activity in each culture supernatant. (B) Cellular productivity of 799Bg/IIα-AE at each culture temperature. The productivity was calculated from the viable cell density (Fig. 1) and α-AE activity (A) as described in the 'Materials and Methods' section.

Figure 4. Effect of culture temperature on the content of 799Bg/IIα-AE mRNA. Northern blots containing 20 μ g of total RNA per lane were hybridized to the 799Bg/IIα-AE DNA probe, and re-hybridized to the human β -actin DNA probe. Each relative content of 799Bg/IIα-AE mRNA is expressed as the PSL value of the 799Bg/IIα-AE mRNA band normalized by that of the β -actin mRNA band.

radically improve the process for 799Bg/IIα-AE production.

We also investigated the effect of a culture temperature (37–43 °C) on the 3 μ -1S cells. The high temperature caused the following phenomena: inhibited cell growth, decreased viability (the cells became extinct at 43 °C with 24 hours), decreased 799Bg/IIα-AE production, decreased cellular productivity of 799Bg/IIα-AE with progress of the culture, and increased glucose consumption rate maximally at 39 °C (data not shown). Thus, we confirmed that a higher culture temperature than 37 °C did not exhibit any positive effects on the production of 799Bg/IIα-AE.

Figures 1 and 3 indicate that lowering the culture temperature dramatically affected the cell growth and cellular productivity. In the culture at 32 °C, the cellular productivity of 799Bg/IIα-AE attained its maxi-

mal level, and cell growth was completely suppressed with high viability. Therefore, a remarkable increase in 799Bg/IIα-AE production could be expected by culturing at 32 °C after obtaining enough cells by culturing at an adequate temperature to promote the cell growth (36–37 °C). Although 799Bg/IIα-AE accumulated maximally in the medium at 35 °C (Fig. 3A), this result would have been due to the relationship of suppressed growth and enhanced cellular productivity with lowering the culture temperature.

This is the first report indicating that the cellular productivity of a recombinant protein could be enhanced by culturing a recombinant cell line at low temperature. Since Weidemann et al. (1994) have reported that a low culture temperature hardly affected the antithrombin III productivity of recombinant BHK cells, we cannot consider that every recombinant

Figure 5. Effect of culture temperature on the glucose consumption rate. The data are expressed as means of the consumption rates obtained by daily measurement of the glucose concentration and viable cell density.

cell line would show higher productivity at low culture temperature. In the case of recombinant cells, however, it will be possible to obtain a suitable cell line for low temperature culture, like the 3 μ -1S cell line, by screening the cells transformed.

In parallel with the enhanced 799BgIII α -AE productivity by lowering the culture temperature, the 799BgIII α -AE mRNA content in the cells also increased. This result indicates that the low culture temperature caused an increase in either the transcription level of the 799BgIII α -AE gene or in the stability of 799BgIII α -AE mRNA. Since the 799BgIII α -AE productivity decreased with lowering the temperature on the 1st day (Fig. 3B), however, we can hardly consider that transcription was promoted by the low culture temperature. Hence, it is suggested that culturing at low temperature would contribute to the mRNA stability. Vilček and Havell (1973) have demonstrated that human interferon mRNA of human fibroblasts was stabilized by lowering the culture temperature. However they studied this at the protein level; thus, the stabilization of the mRNA by culture temperature has not

yet been confirmed. Despite the content of 799BgIII α -AE mRNA at 30 or 32 °C on the 5th day exhibiting only a 1.8–2.2-fold increase over the content at 37 °C, the cellular productivity of 799BgIII α -AE exhibited a 4.3–5.3-fold increase. Accordingly, it is strongly suggested that the culture temperature affected not only the mRNA content, but also the translation and/or secretion of 799BgIII α -AE.

In contrast to the enhanced 799BgIII α -AE productivity, the consumption rates of glucose and most of amino acids were reduced by culturing at low temperature (Fig. 5, Table 1). Therefore, the low temperature culture would help to reduce the medium cost. However, the aspartate consumption rate clearly increased with lowering the culture temperature. It is possible that aspartate utilization made up for the reduced glucose utilization at the low culture temperature to synthesize energy for either maintaining cell viability or producing 799BgIII α -AE. As previous reports have also shown reduced glucose consumption rate at a low culture temperature (Borth et al., 1992; Reuveny et al., 1986; Sureshkumar and Mutharasan, 1991; Wei-

Figure 6. Analysis of 799Bg/III α -AE secreted into the culture medium. Each supernatant from the day 5 culture, corresponding to 45 units of α -AE activity, was subjected to Western blotting (A) and SDS-PAGE (B). (A) The proteins in the gel were transferred to a PVDF membrane, and reacted with rabbit anti-799Bg/III α -AE polyclonal antibody conjugated biotin and horseradish peroxidase avidin D. Immunoreactive proteins were visualized with a POD immunostain set. (B) The proteins in the gel were stained with Coomassie brilliant blue R-250. In (A) and (B), the arrows indicate 799Bg/III α -AE (75 and 81 kDa).

Table 1. Amino acid consumption rates at different culture temperatures

Amino acid	Consumption rate ($\mu\text{g}/10^5$ cells/day)		
	37 °C	35 °C	32 °C
Asp	0.67	0.73	0.86
Thr, Gln	10.27	9.04	7.66
Ser, Asn	3.80	3.26	2.84
Glu	-1.67	-1.22	-1.28
Cystein	2.21	2.07	1.42
Gly	-1.36	-1.13	-1.38
Ala	-3.86	-3.05	-1.78
Val	0.65	0.64	0.48
Cystin	0.37	0.15	0.07
Met	0.29	0.29	0.21
Ile	1.13	1.04	1.24
Leu	1.26	1.18	1.18
Tyr	0.40	0.40	0.25
Phe	0.48	0.47	0.31
Lys	0.77	0.75	0.41
His	0.39	0.23	0.16
Trp	0.15	0.16	0.09
Arg	-0.46	-0.57	-0.64
Pro	0.16	0.13	0.00

Each data is expressed as the mean value of the rates obtained in every culture period shown in Figure 3B.

demann et al., 1994), the results reported here would be general.

The quantity and purity of the protein produced in the medium are the keys for efficient purification. The results presented in Figure 6B indicate that the low temperature culture enhanced the relative content, i.e. the purity, of 799Bg/III α -AE secreted into the medium. Furthermore, the concentration of 799Bg/III α -AE could be increased by culturing sufficient number of the 3 μ -1S cells at low temperature (especially 32 °C), as already described. We therefore conclude that culturing the 3 μ -1S cells at low temperature would contribute to the purification of the 799Bg/III α -AE protein from the cultured medium.

Western blot analysis of the culture supernatants (Fig. 6A) revealed that the molecular weight of 799Bg/III α -AE and the intensity of its bands were not changed by culturing at low temperature. It is hence suggested that the specific activity and the glycosylation patterns of 799Bg/III α -AE were maintained in the culture at 30–37 °C. However the relationship between glycosylation and the culture temperature must be further studied, because many previous reports have shown that the culture environment for animal cells affected glycosylation of the protein produced (Borys

et al., 1993; Borys et al., 1994; Goochee and Monica, 1990; Hahn and Goochee, 1992; Hayter et al., 1992; Patel et al., 1992; Tachibana et al., 1996).

The 28 and 53 kDa bands are shown in Figure 6A. We found that the proteins corresponding to these bands were not derived from the 799Bg/II α -AE protein, but from the host CHO cell line (data not shown). As cell lysis was suppressed by lowering the culture temperature (Fig. 2), it is suggested that the release of proteases into the culture medium was also suppressed at the low culture temperature. Additionally, low culture temperature itself could have reduced the activity of the proteases. Hence, the low temperature culture might have the advantage of suppressing the degradation of the protein produced.

We found that the exceedingly simple method, which was only the culture of the 3 μ -1S cells at a low temperature, enhanced the cellular productivity of 799Bg/II α -AE and suppressed the consumption of most nutrients and the release of impurities into the culture medium. Therefore, these findings will greatly contribute to a more economic industrial process for the production of 799Bg/II α -AE. It is also possible that other recombinant cell lines would be applicable to this method. Further studies are underway to establish an efficient process for the production of 799Bg/II α -AE with this method. There is also strong interest in a further investigation of the mechanism involved in changing the metabolism and the 799Bg/II α -AE productivity by the culture temperature.

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