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Enhancement of productivity of recombinant α-amidating enzyme by low temperature culture

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Abstract

We have produced a recombinant C-terminal α-amidating enzyme (799*Bgl*IIα-AE) derived from *Xenopus laevis* by culturing a CHO cell line named 3μ-1S. Recently, we demonstrated that culturing 3μ-1S cells at a temperature below 37 °C led to the following phenomena: inhibited cell growth with high viability, enhanced cellular productivity (maximally at 32 °C), and suppressed medium consumption and release of impurities from the cells. Therefore, it is suggested that the 799*Bgl*IIα-AE production will be increased by culturing a sufficient number of the cells at a low temperature (especially at 32 °C). To assess this effect on batch and perfusion cultures, the culture temperature was shifted from 37 to 32 °C in the mid-exponential phase in the case of batch culture and from 37 to 34 °C when the cell density became high enough in the case of perfusion culture. Application of the low temperature culture to batch and perfusion cultures was effective in comparison with the culture at 37 °C: the productivity per medium and the productivity per time were increased severalfold with enhanced cellular productivity at a low culture temperature. The low temperature culture also increased the relative content of 799*Bgl*IIα-AE in the supernatant and reduced the glucose consumption. The method presented here would contribute to production of bioactive proteins using other recombinant cell lines.

Introduction

Culture temperature is an important factor to be studied whenever animal cells are cultured, because it would affect such cellular events as cell growth, viability, protein synthesis and metabolism. In addition, because the response of cells to culture temperature would not depend on the culture scale; the optimization of culture temperature would greatly contribute to animal cell culture on an industrial scale.

Currently, many bioactive proteins are produced by culturing recombinant cells on an industrial scale; however, knowledge of the effects of culture temperature on recombinant cells is insufficient. Although animal cells are most commonly cultured at 37 °C, several groups have reported the advantages of culturing recombinant cells at low temperature (below 37 °C). Rasmussen (1991) reported that the production of recombinant FVIII derivatives was increased severalfold by culturing several different cell lines at a low temperature (especially at 27 °C). Weidemann et al. (1994) demonstrated that culturing a recombinant BHK cell line at low temperature reduced the growth rate and glucose metabolism without a decrease in the cellular productivity of antithrombin III in batch and repeated batch cultures. Chuppa et al. (1997) demonstrated that the perfusion culture of a recombinant CHO cell line at 34 °C led to a decrease in the growth rate, glucose and glutamine metabolism, oxygen uptake rate, and proteolytic activity while maintaining the cellular productivity of the desired protein and the product quality. In these papers, however, enhanced productivity at low temperature is not demonstrated.

On the other hand, it has been shown that culturing

some non-transformed cell lines at low temperature enhances the cellular productivities of interferon(s) (Giard and Fleischaker, 1980; Giard et al., 1982; Kojima and Yoshida, 1974; Vilček and Havell, 1973) and tissue plasminogen activator (Takagi and Ueda, 1994). In the case of recombinant cell lines as well, if the cellular productivity can be enhanced by culturing at low temperature, the production process by low temperature culture will be further improved.

We have studied the effects of culture temperature on a recombinant CHO cell line producing a C-terminal α-amidating enzyme (799*Bgl*IIα-AE) and demonstrated that culturing the cells below 37 °C enhanced the cellular productivity (maximally at 32 °C) while suppressing the cell growth with high viability, medium consumption, and the release of impurities from the cells (Furukawa and Ohsuye, 1998). These phenomena strongly suggest that the production and productivity of 799*Bgl*IIα-AE will be enhanced by culturing at a low temperature (especially 32 °C) after obtaining a sufficient number of the cells by culturing at an adequate temperature to promote the cell growth (36–37 °C). In this report, we describe that applying this method to batch and perfusion cultures is useful for efficient production of 799*Bgl*IIα-AE.

Materials and methods

Cell line, maintenance, and product

A recombinant CHO cell line, 3μ-1S, secrets a recombinant C-terminal α-amidating enzyme (799*Bgl*IIα-AE) derived from *Xenopus laevis* and can grow in suspension with a serum-free medium (Furukawa et al., 1993). The enzyme catalyzes the conversion of glycine extended prohormone substrates to bioactive C-terminal 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). In the 3μ-1S cell, the 799*Bgl*IIα-AE and the DHFR genes are respectively transcribed under the control of the SV40 early promoter and amplified with increasing concentration of methotrexate (MTX, Sigma). 3μ-1S cells were maintained in siliconized 300-ml flasks while gently shaking at 37 °C with the serum-free medium (pH 7.2) which was Ham's F-12 (Ajinomoto, Japan) supplemented with 1.0 μM of MTX, 60 mg/l of kanamycin sulfate (Meiji, Japan), 600 μg/ml of polyvinyl alcohol, 1.2 g/l of sodium bicarbonate, 15 mM of HEPES (Sigma), and 5 μg/ml each of bovine insulin and transferrin (Intergen).

Culture system

For culture experiments, we used 1-liter cell culture systems which were established as described in our previous report (Furukawa and Ohsuye, 1998). This system enabled the culture temperature, pH, and DO to be controlled. For perfusion culture, the circulation and perfusion lines were attached to the culture system as shown in Figure 4. To separate the cells, we used a centrifuge, a Centritech Lab System (Sorvall).

Experiment in batch culture

3μ-1S cells were inoculated into three 1-liter spinner flasks (Corning) of the systems at 2×10^5 cells/ml in 650 ml (final volume) of the fresh medium without MTX. The culture temperatures of vessels 1 and 2 were controlled to 37 and 32 °C, respectively. The culture temperature of vessel 3 was first controlled to 37 °C in order to grow the cells and then shifted to 32 °C at 48 h. The pH was controlled to 7.2, and DO was kept above 60% air saturation by continuously supplying 100 ml/min of air. The agitation speed of each vessel was adjusted to 100 rpm. The cells were cultured for 9 days, the total and viable cell densities being determined daily by the trypan blue dye exclusion method in a hemacytometer. The glucose concentration in the supernatant was measured with a Glucose analyzer ST-1 (Oriental Electric, Japan).

Experiment in perfusion culture

The perfusion culture was performed using the culture system shown in Figure 4. 3μ-1S cells were inoculated into a 1-liter spinner flask (Corning) of the culture system and precultured at 37 °C until the cell density reached 6×10^6 cells/ml. The culture was then divided into two portions, and each culture volume was adjusted to 800 ml. The culture temperature of one of the vessels was shifted to 34 °C when the cell density again reached 6×10^6 cells/ml. The parameters of both cultures were equally controlled except for temperature. In each vessel, the perfusion rate was raised to 4.5 volume/day according to the increase in the cell density and adjusted to 4.5 volume/day after the point of the temperature shifting. The pH and DO were controlled to 7.2 and 60% air saturation, respectively. The daily sampling and measurement were carried out in the same way as the batch culture.

Analysis of 799BglIIα-AE

The assay for α-AE activity in each culture supernatant was carried out as reported previously with Ac-[^{125}I]-Tyr-Phe-Gly as a substrate (Mizuno et al., 1986). One unit of enzymatic activity is defined as the amount of enzyme that gives a 50% conversion of Ac-Tyr-Phe-Gly to Ac-Tyr-Phe-NH_2 under the standard assay conditions. The cellular productivity of 799*Bgl*IIα-AE was calculated as described in our previous report (Furukawa and Ohsuye, 1998).

SDS-PAGE analysis

Aliquots of each culture supernatant corresponding to 45 units of α-AE activity were electrophoresed in a 10% polyacrylamide gel containing SDS. The proteins separated on the gel were stained with a Silver Stain II Kit Wako (Wako Pure Chemical Industries, Japan). As a molecular weight marker, Wide-Range SDS-PAGE Protein Standard (Tefco) was used.

Results and discussion

Application of low temperature culture to batch culture

In order to assess the effects of low temperature culture on batch culture, the culture temperature was shifted from 37 to 32 °C, at which the highest cellular productivity of 799*Bgl*IIα-AE was exhibited, in the mid-exponential phase (48 h).

Figure 1 shows the effects of the temperature shift on cell growth, viability and glucose concentration in the medium. As shown in Figures 1A and B, in the 37 °C-culture, the cells grew exponentially to 1×10^6 cells/ml, and thereafter the cell growth stopped, exhibiting a remarkable decrease in the viability (31% on day 9). In addition, although the cell density in the 32 °C-culture decreased slightly, the viability was kept above 85% throughout the culture period. On the other hand, in the temperature shift-culture, the cell growth was nearly equal to that in the 37 °C-culture until 24 h after the temperature shift, with the cell density reaching 6.5×10^5 cells/ml. As well as the 32 °C-culture, although the cell density then tended to decrease, the viability was maintained at 80% or above.

The glucose concentration of the temperature shift-culture was maintained at a higher level than that of the 37 °C-culture (Figure 1C). By shifting the temperature to 32 °C, the glucose consumption rate, which was calculated from the glucose concentration and the viable cell density (Figure 1A), was reduced from 74 μg/10^5 cells/day to 42 μg/10^5 cells/day within 2 days after the temperature shift. In the 37 °C-culture, the glucose consumption also decreased after 96 h of the culture; however, this is considered to be caused by a remarkable decrease in the viable cell density.

Figure 2 indicates the accumulation of 799*Bgl*IIα-AE in the culture supernatant. The production of 799*Bgl*IIα-AE in the 37 °C-culture leveled off at 1500 units/ml in 96 h of the culture. In contrast, the production in the temperature shift-culture reached 4900 units/ml on day 9, and it still maintained an increasing trend. In the case of the 32 °C-culture, the production reached 2900 units/ml. The time course of the cellular productivity is shown in Figure 3. The cellular productivity in the temperature shift-culture was elevated to about 2-fold after the temperature shift, and then it was maintained at a high level. In the 32 °C-culture, the increase in cellular productivity was more remarkable. On the other hand, the productivity in the 37 °C-culture rapidly decreased after day 4.

As described above, applying the low temperature culture to batch culture, i.e., shifting the culture temperature from 37 to 32 °C, effectively enhanced the productivity of 799*Bgl*IIα-AE. In comparison with the repeated batch culture, the temperature shift-culture increased 5.68-fold in productivity per volume and 2.36-fold in productivity per time (Table 1).

However, it is considered that the optimization of the temperature shift-culture should be needed hereafter. In the batch culture, because the culture conditions vary at every moment with consumption of the medium and accumulation of toxic metabolites, the progress of the culture would exhibit not only an increase in the cell number but also a decrease in the cellular productivity of 799*Bgl*IIα-AE. Therefore, in this experiment, the culture temperature was shifted to 32 °C at 48 h in the mid-exponential phase of the culture. The determination of an optimal point of the temperature shift will enable realization of a more effective process for 799*Bgl*IIα-AE production in the batch culture.

Application of low temperature culture to perfusion culture

When the perfusion culture becomes stable, active cell growth and metabolism would not be required, because those factors would cause useless medium consumption. Several papers have shown that the cell

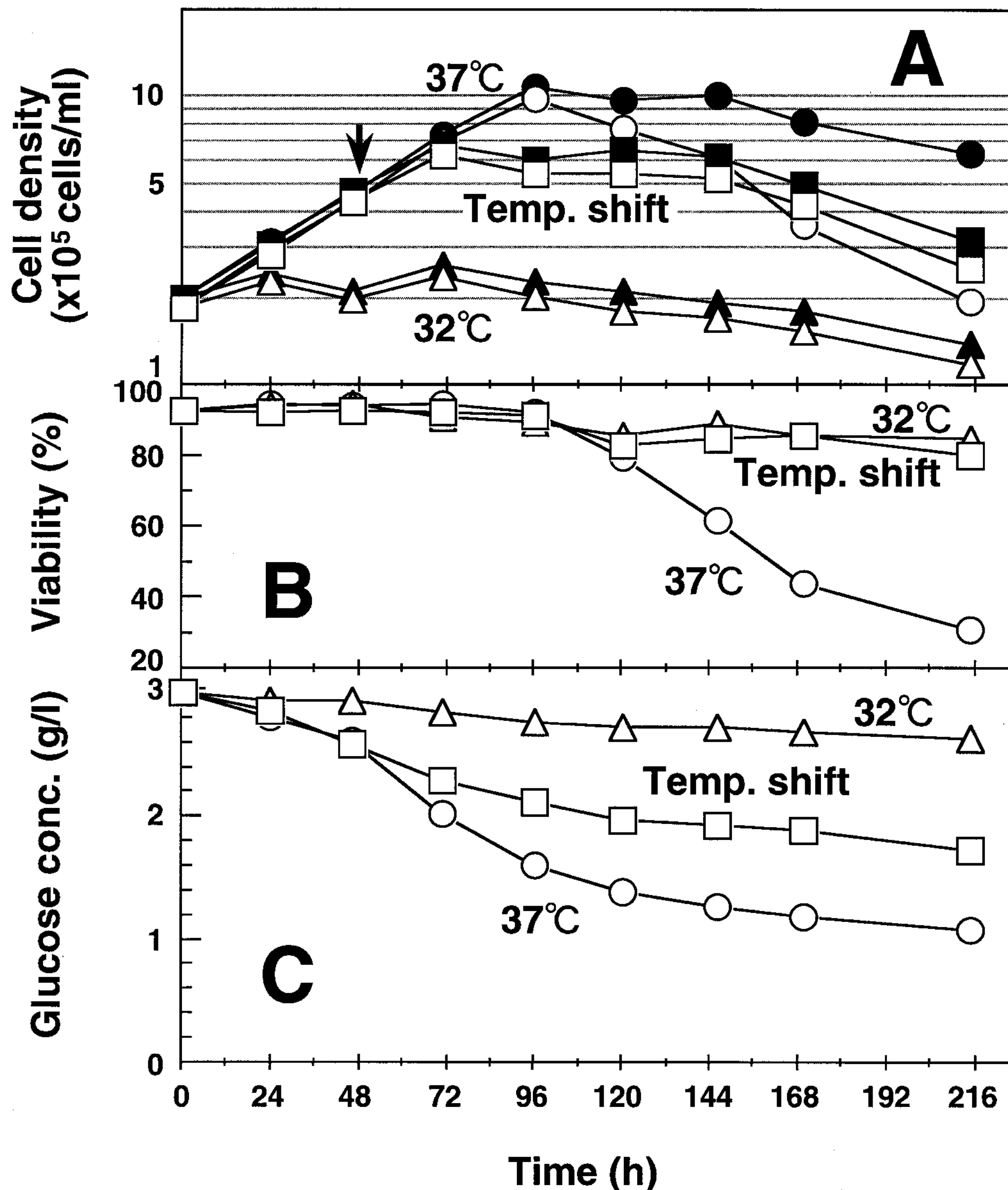

Figure 1. Effects of temperature shift on batch culture. (A) Growth curves of 3μ-1S cells. The total cell densities (closed symbols) and the viable cell densities (open symbols) were determined by the trypan blue dye exclusion method in a hemacytometer. In each culture, the pH was controlled to 7.2 by CO_2 or 7% 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 arrow indicaties the point of the temperature shift (37 $\rightarrow$ 32 °C). (B) Time course of viability in each culture. (C) Time course of glucose concentration in the supernatant of each culture. The glucose concentration was analyzed by a glucose analyzer ST-1.

growth rate and/or glucose metabolism are reduced by culturing below 37 °C (Borth et al., 1992; Reuveny et al., 1986; Sureshkumar and Mutharasan, 1991; Weidemann et al., 1994). Therefore, it is expected that the low temperature culture would contribute to the maintenance of the cells in perfusion culture. Recently, Chuppa et al. (1997) demonstrated that the cellular productivity of a recombinant protein was hardly changed by lowering the culture temperature and that perfusion culture at 34 °C was successfully carried out. Because 3μ-1S cells exhibit higher cellular productivity of 799*Bgl*IIα-AE at the low culture

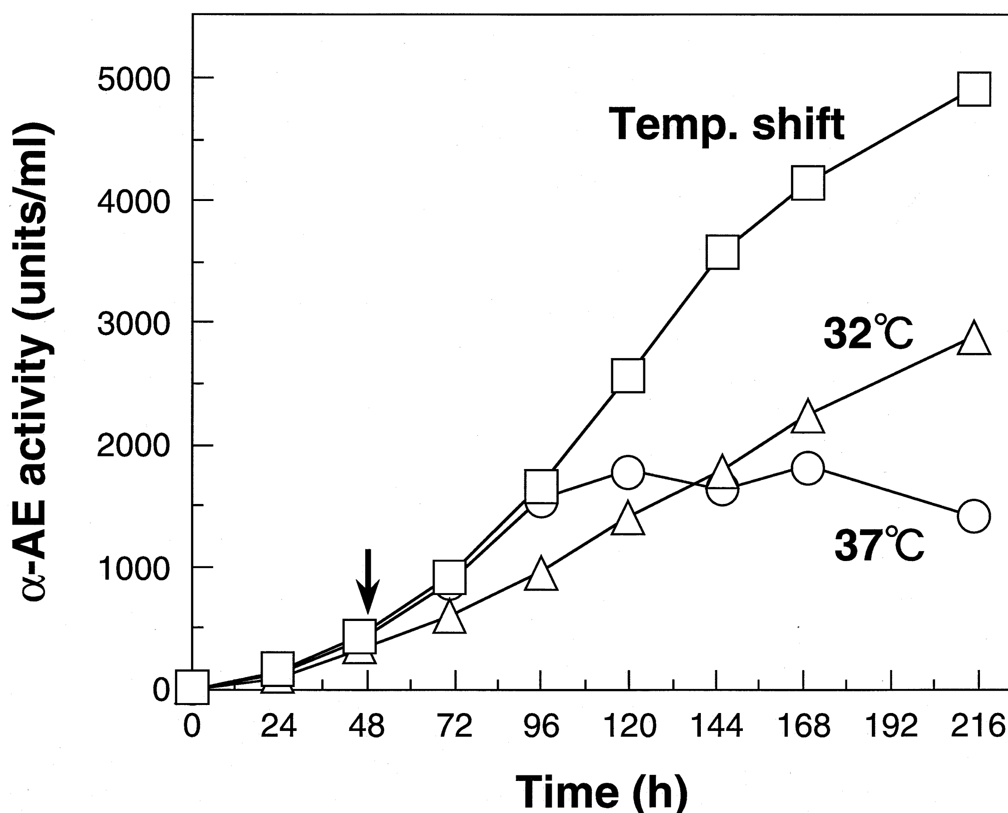

Figure 2. Enhancement of 799*Bgl*IIα-AE production by temperature shift-culture. The enzymatic activity accumulated in the cultured medium was determined as described in the Section 'Materials and methods'. The arrow indicates the point of the temperature shift.

temperature, a perfusion culture at low temperature for 799*Bgl*IIα-AE production would be more effective.

To evaluate the effect of the low temperature culture on a perfusion culture of 3μ-1S cells, we established two culture systems as illustrated in Figure 4. The results of the perfusion culture are shown in Figure 5. Before the point of shifting temperature, the viable cell density, the production per vessel per day and the cellular productivity in each vessel varied in the same way. After that point, the viable cell density in the temperature shift-culture became steady at a slightly lower level than that in the 37 °C-culture. On the contrary, the production and the cellular productivity of 799*Bgl*IIα-AE were enhanced 1.6-fold and 2.0-fold, respectively (Figures 5B and C). In addition, the glucose consumption during the culture at 34 °C decreased in comparison with that at 37 °C (data not shown).

Based on these results, the perfusion culture of 3μ-1S cells at low temperature (34 °C) led to a higher productivity of 799*Bgl*IIα-AE than that at 37 °C. As shown in Table 1, the productivity per volume and the productivity per time were respectively raised 1.64-fold and 1.60-fold by culturing at 34 °C.

However, it should be mentioned that the perfusion culture presented here could not be performed successfully. Under the conditions of this experiment, because the circulation rate became too high, the cells suffered significant physical damage. As a result, the growth rate notably declined during the culture at 37 °C. The growth of 3μ-1S cells depends on culture temperature: it attains a maximal level at 36–37 °C and is completely inhibited below 32 °C in batch culture (Furukawa and Ohsuye, 1998). In this perfusion culture, if the culture temperature was shifted to 32 °C, which was the optimal temperature for enhancing the cellular productivity of 799*Bgl*IIα-AE, there was a possibility of a decrease in the viable cell density during the culture at 32 °C. In order to maintain the viable cell density at low temperature, the culture temperature had to be shifted to 34 °C, not to 32 °C, in this experiment. Further increase in the cell density and productivity of 799*Bgl*IIα-AE would be achieved

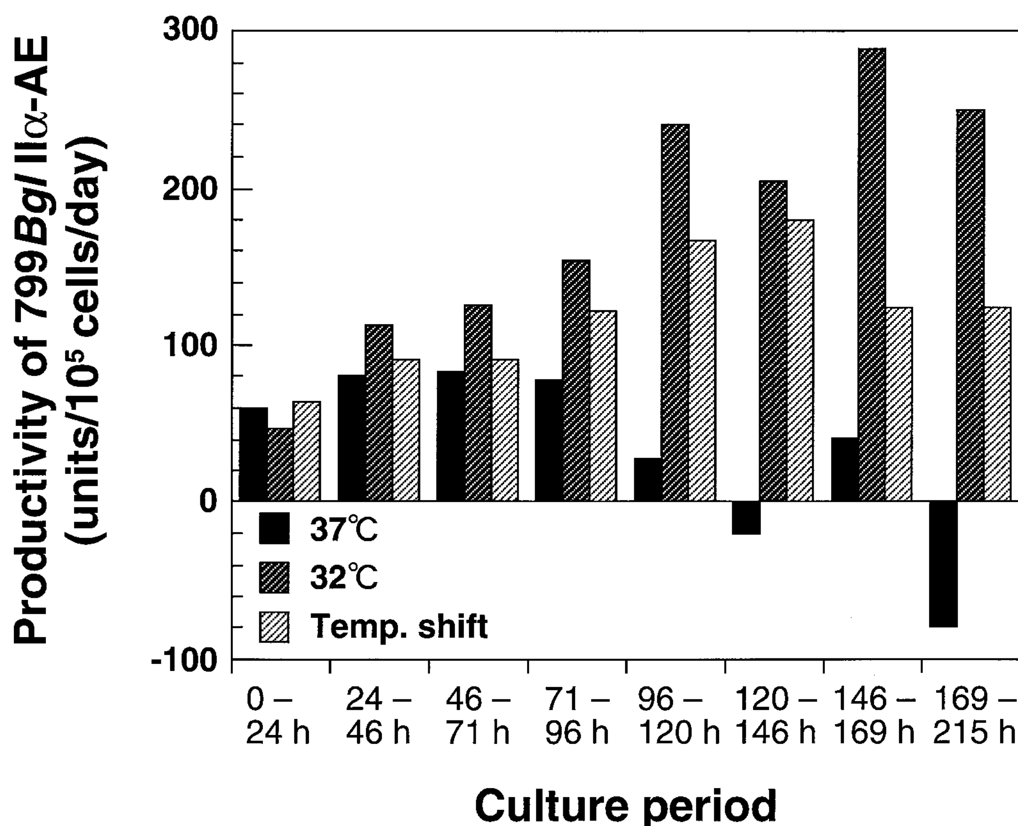

Figure 3. Time course of cellular productivity of 799*Bgl*IIα-AE in batch culture. The productivity was calculated from the viable cell density (Figure 1A) and the 799*Bgl*IIα-AE production (Figure 2).

by improving the basal conditions of the perfusion culture.

SDS-PAGE analysis of secreted 799BglIIα-AE

The purity of the product in the culture supernatant is important for the purification process. To access the purity, i.e., the relative content of 799*Bgl*IIα-AE secreted in the medium, we carried out SDS-PAGE analysis of the supernatant from the batch and the perfusion cultures. The supernatant containing 45 units of 799*Bgl*IIα-AE was applied in each lane. The results are shown in Figure 6.

In the case of the batch culture (Figure 6A), the relative contents of 799*Bgl*IIα-AE from the temperature shift-culture and the 32 °C-culture remarkably increased. On day 9 of both cultures, 799*Bgl*IIα-AE became the main protein in the supernatant. On the contrary, in the case of the 37 °C-culture, the intensities of impurity bands elevated with the progress of the culture. The result from the perfusion culture is shown in Figure 6B. In this figure, there are many cellular proteins due to the cell damage mentioned above. However, in the 34 °C-culture, the intensities of these impurity bands were apparently reduced.

For purification, the quality of the product is also important. Chuppa et al. (1997) have demonstrated that the culture temperature (34–37 °C) does not strongly affect the product quality (glycosylation and molecule integrity). The 799*Bgl*IIα-AE is also a glycoprotein with three glycosylation sites, and it has 75 and 81 kDa molecular species which may be caused by different modifications of the sugar chains. As shown in Figures 6A and B, no change in either molecular weight or the intensities of these bands was recognized, implying that the specific activity and the glycosylation of 799*Bgl*IIα-AE were not changed by the low temperature culture. This was also confirmed by Western blot analysis (data not shown).

Therefore, the low temperature culture would contribute to cost reduction for the purification of 799*Bgl*IIα-AE.

Table 1. Comparison of productivity between culture methods

Culture method	Productivity per volume		Productivity per time	
	units/ml	ratio	units/day[a]	ratio
37 °C, repeated batch	866	1.00	1.85×10^5	1.00
Temp. shift (37 → 32 °C), batch	4917	5.68	4.37×10^5	2.36
37 °C, perfusion	739	1.00	2.52×10^6	1.00
Temp. shift (37 → 34 °C), perfusion	1213	1.64	4.04×10^6	1.60

[a] Each value indicates the productivity per time which can be obtained by 800-ml culture.

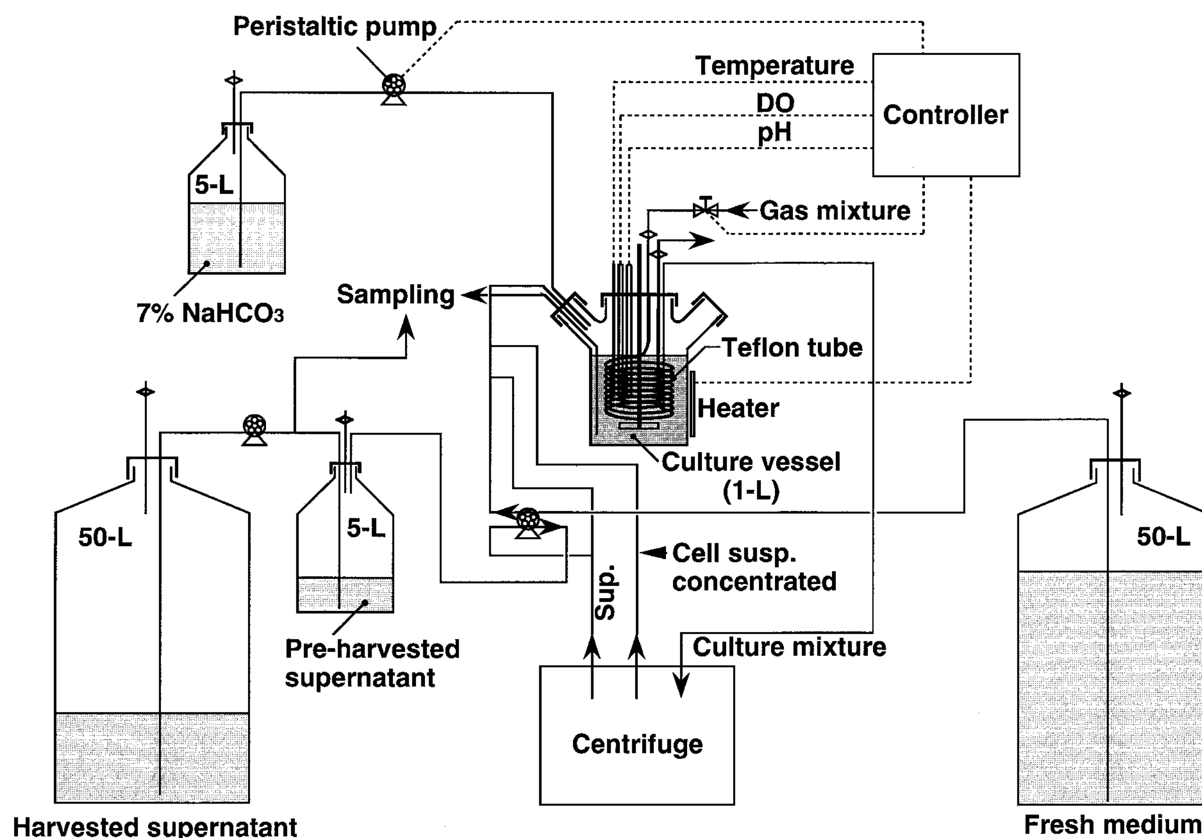

Figure 4. Flowchart for perfusion culture. Using a centrifuge (Centritech lab system), the circulation and the perfusion lines were set to a 1-liter culture system.

Conclusions

We demonstrated here that low temperature culture of 3μ-1S cells could be successfully applied to batch and perfusion cultures, although optimization of the culture should be needed in the future. The total production, as well as the cellular productivity, of 799*Bgl*IIα-AE was significantly increased by shifting to the low temperature. In parallel, the low temperature culture of 3μ-1S cells reduced the glucose

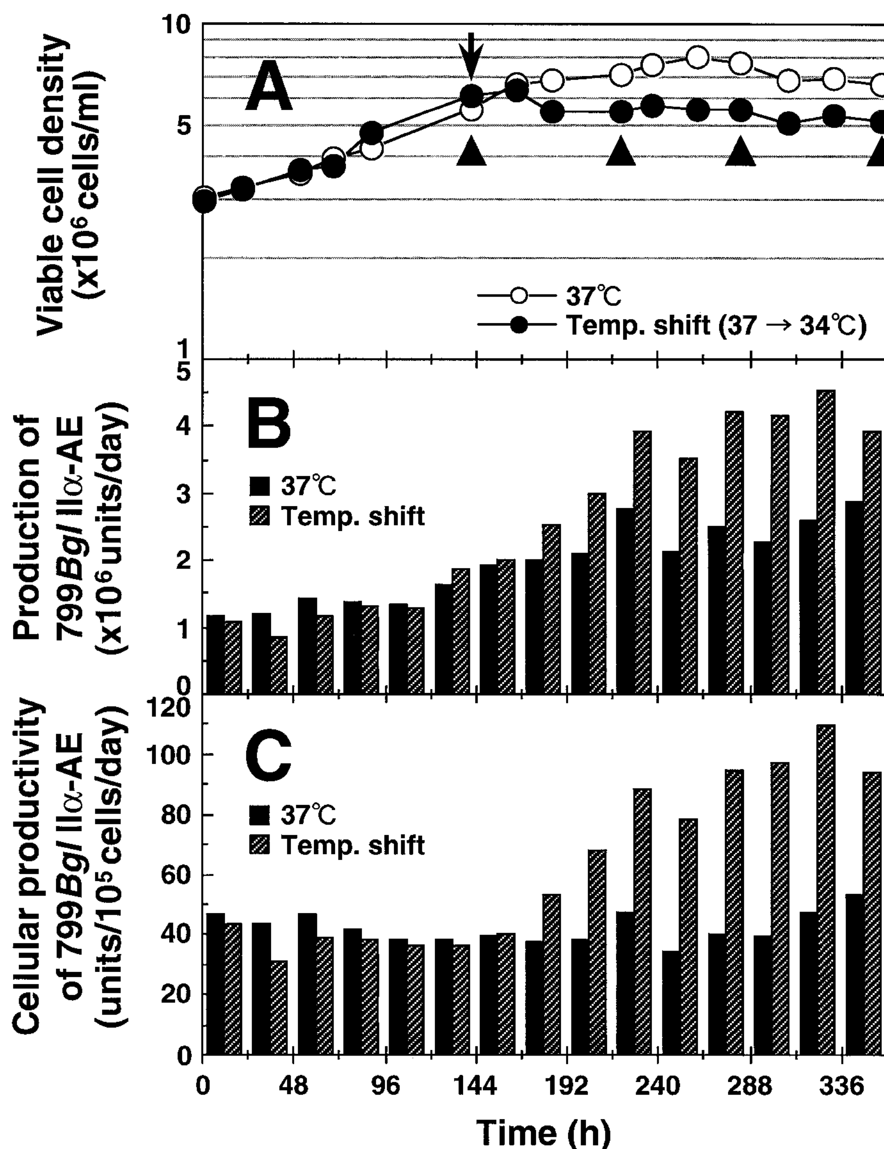

Figure 5. Effects of temperature shift on perfusion culture. (A) Transition of viable cell density. The perfusion cultures were carried out as described in the Section 'Materials and methods'. The open circles and the closed circles indicate the viable cell densities in the 37 °C-culture and the temperature shift-culture, respectively. The arrow indicaties the point of the temperature shift (37 → 34 °C). The black triangles are the sampling points of the supernatant for SDS-PAGE analysis (Figure 6B). (B) Enhancement of the daily production of 799*Bgl*IIα-AE by the temperature shift-culture. (C) Enhancement of the cellular productivity of 799*Bgl*IIα-AE by the temperature shift-culture.

consumption and the release of impurities from the cells. Additionally, it has been demonstrated in our previous report (Furukawa and Ohsuye, 1998) that the consumption of most of the amino acids in 3μ-1S cells is also reduced by lowering the culture temperature. These observations indicate that the low temperature culture greatly contributes to an efficient process for the production of 799*Bgl*IIα-AE.

The response of cellular productivity to culture temperature depends on the cell line used. In order to perform a low temperature culture successfully, it become important to obtain a recombinant cell line which exhibits higher cellular productivity at a low culture temperature, such as 3μ-1S. For example, a suitable cell line would be established by screening transfected cells according to the property as described above. On the other hand, it is substantial to establish

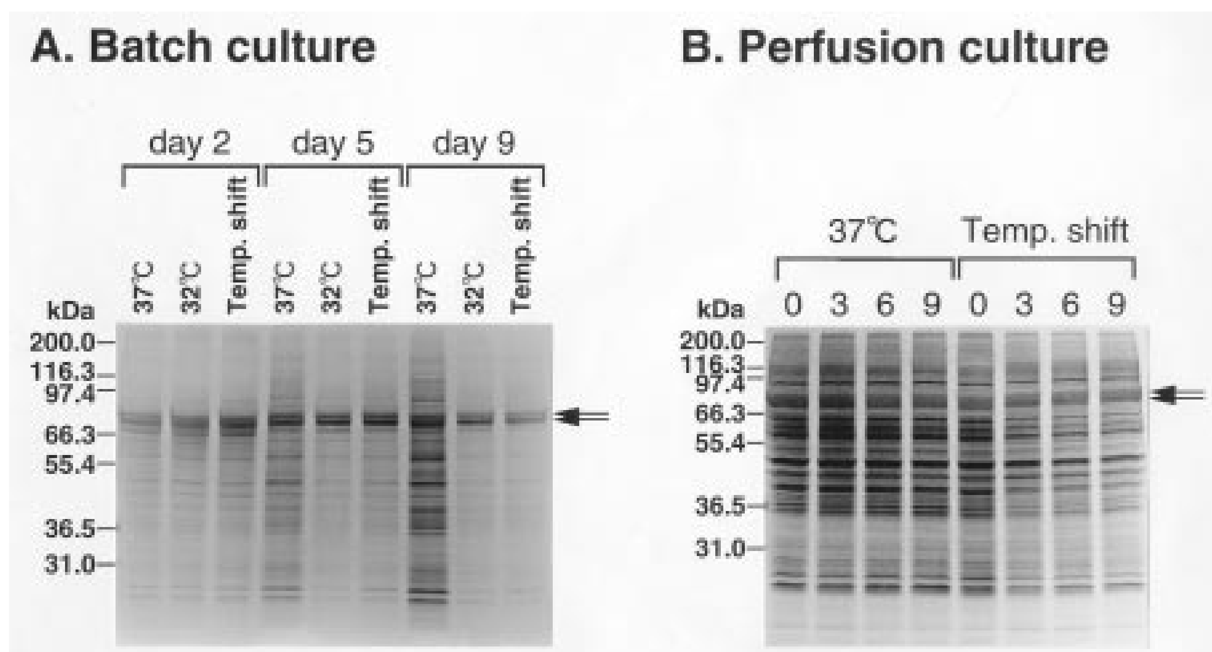

Figure 6. SDS-PAGE analysis of 799*Bgl*IIα-AE secreted into the culture medium. Each supernatant containing 45 units of enzymatic activity from the batch culture (A) and the perfusion culture (B) was subjected to this analysis. The proteins in the gel were visualized by silver staining. The arrows indicate the size of the 799*Bgl*IIα-AE protein (75 and 81 kDa). In (B), the numbers above the lanes, 0, 3, 6 and 9, indicate the days after the point of the temperature shift.

a gene expression system which promotes expression of a desired gene at a low temperature. Concerning such an expression system, the HIV-1 LTR sequences (Chevrier-Miller et al., 1996) could be successfully applied. In addition, investigation of the mechanism of changing the 799*Bgl*IIα-AE productivity by the culture temperature would provide useful information.

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