

Temperature Effects on Product-Quality-Related Enzymes in Batch CHO Cell Cultures Producing Recombinant tPA

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Culture conditions that affect product quality are important to the successful operation and optimization of bioreactors. Previous studies have demonstrated that enzymes, such as proteases and sialidases, accumulate in batch bioreactors. These enzymes are known to be detrimental to the quality of recombinant glycoproteins. Bioreactor temperature has been used to control cell growth and recombinant protein production rates. However, the effect of culture temperature on the production of proteases and sialidases has not been investigated. In this study, Chinese hamster ovary cells were cultured with a temperature profile that decreased from 37 to 34 °C over 8 days and with a constant temperature of 37 °C. Analysis of extracellular protease activity indicated that two major proteases were present (50 and 69 kDa). The 50 kDa protease activity decreased similarly with time for both culture conditions. The 69 kDa protease activity increased with time for both culture conditions. The constant-temperature cultures had significantly lower 69 kDa protease levels compared to the ramped-temperature cultures in the early stationary phase. Intracellular sialidase activity was present in both cultures. The intracellular sialidase activity increased dramatically for both culture conditions immediately after the cells were inoculated into fresh medium. The initial peak in intracellular sialidase activity was followed by a first-order decay. The intracellular sialidase activities for the two culture conditions were not significantly different. The production of recombinant tissue type plasminogen activator was not significantly different for the two culture conditions. Thus, the previously hypothesized advantages that lower culture temperatures have reduced protease activity and improved productivity do not appear to be universal.

Introduction

Reduced culture temperatures are hypothesized to improve recombinant protein production via cell arrest (1). Several groups have demonstrated that there is a shift in the proportion of cells from the S to the G₁, or arrested, phase of growth at lower culture temperatures (2–5). Also, it has been observed that, at lower culture temperatures, the growth rate, glucose consumption, lactate production, and ammonia production are reduced (3–7). Increased protein production rates are not the only benefit attributed to lower culture temperatures. Product quality has been observed to be temperature dependent as well (3, 8). Higher product quality at lower culture temperatures has been attributed to lower protease activity and lower activity of other deleterious temperature-sensitive enzymes (3, 8). However, no significant data have been presented specifically correlating culture temperature and protease levels. Another measure of product quality is the degree of sialylation, or proportion of glycans with terminal sialic acid residues. It has been proposed that a reduction in temperature may increase sialylation by decreasing sialidase activity in the super-

natant. For lower temperature cultures, apoptosis, the primary mechanism of programmed cell death, is lower (5, 9–11). The primary mechanism for sialidase release into the cell culture medium has been hypothesized to be cell lysis (12). Thus, lower temperature cultures should lead to lower sialidase activity in the supernatant via reduced cell lysis.

In this study, the effects of low-temperature cultivation on recombinant protein productivity, extracellular protease activity, and intracellular sialidase activity in Chinese hamster ovary (CHO) cells were examined. Recombinant CHO cells expressing tissue plasminogen activator (tPA) were cultured at a constant temperature (37 °C) and with a ramped temperature profile. For the ramped-temperature culture, the temperature was decreased from 37 to 34 °C over an 8 day period. The objective of this study was to determine whether changes in protease and sialidase activities correlated with changes in culture temperature, specifically lower temperatures, and resulted in improved protein production.

Materials and Methods

CHO cells (ATCC CRL-9606, CHO 1–15₅₀₀) expressing recombinant human tPA were maintained in 1 L suspension spinner flasks in 5% CO₂ at 90 rpm. The medium used was IS-CHO V (Irvine Scientific, Santa Ana, CA) supplemented with 1.75% (v/v) fetal bovine serum (FBS) (Gibco, Carlsbad, CA), 6 mM L-glutamine (Irvine Scien-

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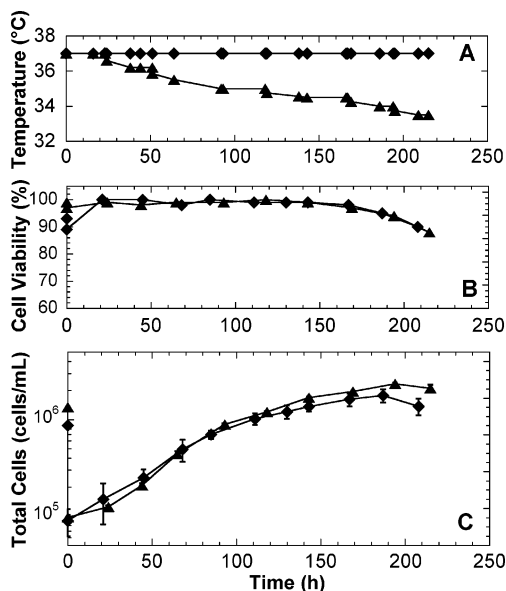


Figure 1. Temperature, cell growth, and cell viability curves: (▲) control, (◆) ramped, (A) temperature, (B) cell viability, (C) total cells. Error bars represent 95% confidence intervals.

tific), 50 mg/L penicillin, 50 mg/L streptomycin, 100 mg/L neomycin (Gibco), and 50 nM methotrexate (Sigma, St. Louis, MO). Duplicate 1 L spinner flasks were used for each of the culture conditions. The control cultures were maintained at 37 °C. The ramped-temperature cultures were initially cultured at 37 °C for 24 h. The incubator cabinet set point was decreased, and heat transfer to the room provided cooling. The actual temperature profiles for the culture conditions are shown in Figure 1. The cell density and viability were determined by trypan blue exclusion. Cells were harvested and centrifuged for 15 min at 500 rpm and 4 °C. Cell supernatants were frozen at -20 °C for tPA and protease activity analysis. The cell pellet was resuspended in ice cold PBS and then centrifuged at 500 rpm for 15 min at 4 °C. The cell pellet was resuspended in 500 μ L of ice cold PBS and disrupted by passage through a 27 $\frac{1}{2}$ gauge needle. The disrupted cells were centrifuged at 12000g for 30 min at 4 °C. The cell lysate was aliquoted and frozen at -20 °C for sialidase analysis.

The tPA activity was determined using the Spectrolyse tPA activity assay kit (American Diagnostica, Stamford, CT) per the kit instructions. tPA activity was normalized, for all cultures, to the first sample point for each culture. tPA was also analyzed by Western blots. An anti-tPA rabbit primary antibody (American Diagnostica) and an anti-rabbit secondary antibody conjugate to streptavidin (BioRad, Hercules, CA) were used. Sialidase activity was assayed by an adaptation of the method described by Berg et al. (13). This method was used by Gramer and Goochee (12) to characterize sialidase activity from CHO cells. They observed the half-life of CHO cell sialidase was 57 h, which indicated a stable enzyme. They also characterized the pH optimum for CHO cell sialidase to be pH 5.5 and demonstrated that the sialidase could hydrolyze sialic acid from a glycoprotein. Sialidase (Glyco, currently Prozyme, San Leandro, CA) from *Salmonella typhimurium*, produced in recombinant *Escherichia coli*, was used to prepare a standard curve for sialidase activity. The linear standard curve was 0.15–3 mU of sialidase activity per 800 μ L of reaction volume. The substrate buffer used was 0.2 M sodium acetate buffer, pH 5.5, with 500 mM NaCl. Each standard or sample, 10 or 100 μ L, respectively, was placed in a 1 mL

centrifuge tube with 710 or 620 μ L of substrate buffer, respectively. Samples were centrifuged at 14000g for 5 min to remove any precipitants. Samples were transferred to fluorimetry cuvettes. An 80 μ L sample of 0.5 mM artificial substrate 4-methylumbelliferyl-*N*-acetyl- α -D-neuraminic acid (Sigma) in substrate buffer was added to the reaction tube and gently aspirated. The samples were then incubated at 37 °C for 45 min. The fluorescent signal ($\lambda_{\text{ex}} = 366$ nm and $\lambda_{\text{em}} = 460$ nm) was recorded using a Shimadzu fluorometer. Zymograms were used to quantify protease activity per the method of Harcum and Bentley (14). Gelatin and glycerol were purchased from Sigma. All other reagents were purchased from Bio-Rad. Gels were scanned using a GS-700 densitometer and quantified with the QuantityOne software (Bio-Rad). The protease activity for both the constant-temperature and temperature-ramped cultures was reported as the normalized protease activity. Samples were normalized to fresh medium. Total protein was determined using a BCA protein assay kit (Pierce, Rockford, IL) per the manufacturer's instructions.

Results and Discussion

CHO cells expressing recombinant human tPA were cultured in parallel 1 L spinner flasks. Two control cultures were maintained at 37 °C, and the two experimental cultures were ramped from 37 to 34 °C over an 8 day period. Figure 1 shows the cell growth and viability curves for the two culture conditions. The cell densities were initially $(1 \pm 0.1) \times 10^5$ cells/mL (95% CI). The average doubling time for all four cultures was 29 ± 2.9 h (95% CI). Cell viabilities were similar for both conditions and greater than 85% throughout both of the cultures. Only at the end of the cultures were the cell densities slightly higher for the ramped-temperature culture compared to the control cultures; however, the cell viabilities were identical.

In this study, it was observed that the change in temperature from 37 to 34 °C over 8 days was not sufficient to cause significant changes in the dynamics of the CHO cell cultures. Literature reports on the effect of culture temperature on cell growth and viability for mammalian cell cultures are mixed. Sureshkumar and Mutharasan (1) demonstrated that growth and production in a mouse–mouse hybridoma were significantly different for a 6 °C temperature difference. They observed the highest and lowest cell densities at 33 and 39 °C, respectively. Chuppa et al. (3) observed that, for a long-term CHO cell perfusion system, the growth rate was dependent on temperature. The growth rate at 34 °C was approximately 10% lower than at 35.5 or 37 °C. Ludwig et al. (15) reported that, for an anchorage-dependent BHK cell line, the growth rate decreased 40% for cells cultivated at 33 °C compared to cells grown at 37 °C. In contrast, Ducommun et al. (6) saw nearly identical growth rates for CHO cells maintained in a packed bed cultured at 37 and 33.5 °C. It was also reported that final cell viabilities decreased with increasing culture temperature for these mammalian cell cultures (1, 3–5, 8, 15). The CHO cell viabilities observed in this study were not significantly different, regardless of the culture temperature. It is possible that the slowly ramped temperature profile did not significantly alter the cell metabolism, as compared to a step change in temperature. These results indicated that a slow temperature decrease does not always result in increased cell viability.

One factor that has not been considered is increased oxygen solubility, which may play a part in these mixed observations, since it was unclear in most of the reports

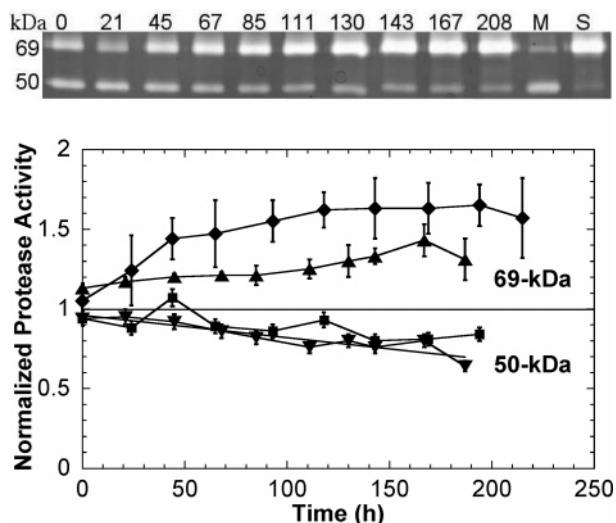


Figure 2. Data for extracellular proteases (50 and 69 kDa) are shown. (A, top) Zymogram. Lanes are labeled with culture times (h) or with M for fresh medium and S for seed culture. (B, bottom) Protease activity was quantified for both proteases: 69 kDa, control (▲), ramped (◆); 50 kDa, control (▼), ramped (■). A linear fit of the 50 kDa experimental data is represented by the solid line. Error bars represent 95% confidence intervals.

if oxygen levels were maintained or controlled. Since the objective of this study was to determine the effects of lower culture temperatures on sialidases, protease, and productivity, elevated culture temperatures were not investigated. To fully understand the effect of culture temperature on sialidase, proteases, and productivity, well-controlled bioreactor studies are needed, where the oxygen concentration is controlled.

Zymograms were used to detect proteases in the medium. Two distinct extracellular proteases were observed in the culture supernatants. Figure 2 shows a representative zymogram. The proteases detected had apparent molecular masses of 50 and 69 kDa. Both proteases were present in the fresh medium at measurable levels as indicated by lane M in Figure 2, which indicated that the FBS was also a source of these proteases or proteases with similar size. The 50 kDa protease activities decreased with time, and the 69 kDa protease activity increased with time. The protease activities from the zymograms were quantified and are also shown in Figure 2. The initial 50 kDa protease activities for both culture conditions were not statistically different from those in fresh medium ($p \leq 0.05$, two-tailed t -test). The 50 kDa protease activities decreased linearly for both culture conditions. The decay rate of the 50 kDa protease was determined to be the same for both culture conditions ($p \leq 0.05$, two-tailed t -test). The half-life of the 50 kDa protease was measured to be 21 ± 5.7 days (95% CI).

The 69 kDa protease activities were quantified and are shown in Figure 2. The initial 69 kDa protease activities for both conditions were not significantly different from each other ($p \leq 0.05$, two-tailed t -test); however, the initial 69 kDa protease activities for both cultures were $9 \pm 4\%$ (95% CI) greater than those in the fresh medium. The 69 kDa protease activities at the start of the cultures were greater than the protease activity in the fresh medium, most likely due to carryover from the seed cultures. The 69 kDa protease activities observed in both the control and ramped-temperature cultures increased throughout the experiment and were significantly greater than the 69 kDa protease activities in the fresh medium ($p \leq 0.05$, one-tailed t -test). The 69 kDa protease activity

in the temperature-ramped cultures was significantly higher than that in the control cultures after 2 days and remained significantly higher for 3 days ($p \leq 0.05$, one-tailed t -test). At the end of the cultures, the 69 kDa protease activities in the control cultures approached the levels observed in the ramped-temperature cultures; however, the control culture protease activity was significantly lower than those of the ramped-temperature cultures ($p \leq 0.05$, one-tailed t -test).

Kratje et al. (16) conducted a comprehensive study of CHO proteases. It was observed that the cell-specific protease composition changed, depending on the culture environment; however, they did not examine temperature effects on the protease composition. Kratje et al. (16) observed that protease activity spiked immediately following a medium exchange, similar to the sialidase activity spike observed in this study. It is commonly thought that increased extracellular protease and sialidase activity would be harmful to recombinant products and should be avoided; however, the results of this study have demonstrated that increased extracellular protease activity in a culture does not necessarily result in decreased recombinant protein productivity. Additionally, lower culture temperatures do not always result in lower protease levels, as demonstrated by the 69 kDa protease activity profile for the ramped-temperature cultures.

It is important to note that the zymograms, in this study, were all incubated at 37 °C. Thus, the amount of protease activity in a sample was not a measure of the protease activity in the cultures, but reported the amount of protease present in the sample. The 69 kDa protease content was observed to be higher for the ramped-temperature cultures compared to the constant-temperature cultures; however, the 69 kDa protease activities in the ramped-temperature cultures may have been lower than that in the control due to the Arrhenius effect on enzyme activity; however, how much lower was not determined. Therefore, the actual 69 kDa protease activity in the cultures may have been similar, but additional protease mass was required to maintain the optimal amount of protease activity in the ramped-temperature cultures.

Industrially, CHO cells are cultured below 37 °C to improve product titer. Chuppa et al. (3) measured the presence of CHO cell proteases with the artificial peptide substrate S-2288. They observed that the protease activity at 37 °C was higher than those at 34 and 35.5 °C, although statistical significance was not addressed. Also, they observed decreased recombinant protein production with increased fermentation temperatures. Presumably, the decrease in recombinant protein productivity was due to increased proteolytic degradation of the peptide backbone. CHO cells may secrete higher levels of recombinant proteins at lower culture temperatures. The data presented here indicated that protease levels may also increase as well. Specifically, the 69 kDa protease level was higher for the ramped-temperature cultures compared to the control cultures. These results demonstrated an important factor in culture dynamics not reported previously.

Both intracellular and extracellular sialidase activities were analyzed; however, only the intracellular sialidase activity was detected. Gramer and Goochee (12) were able to detect extracellular sialidase activity in CHO cell culture medium, only after significant sample concentration of industrial samples, so the lack of detectable extracellular sialidase activity in the nonconcentrated sample was expected. The intracellular sialidase activity, in this study, was determined for the seed cultures,

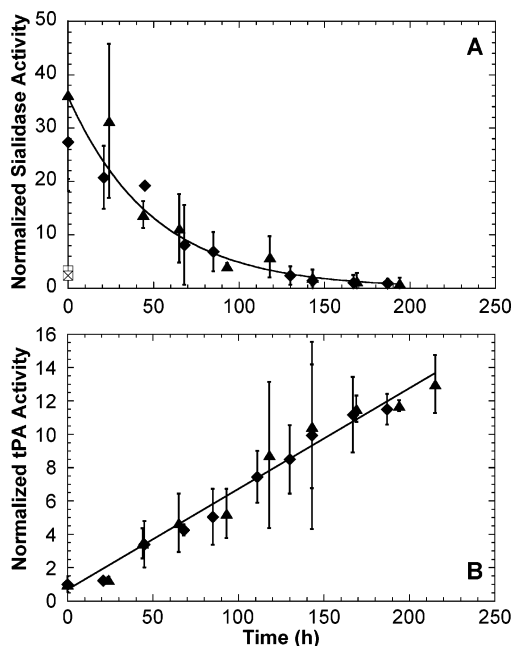


Figure 3. Intracellular sialidase enzyme activity and extracellular recombinant tPA activity: control (▲), ramped (◆). (A) The sialidase activities in the seed cultures are shown as open symbols. A first-order decay rate is represented by the solid line. (B) The experimental tPA data are represented by a linear fit, shown as a solid line. Error bars represent 95% confidence intervals.

control cultures, and temperature-ramped cultures. Figure 3 shows the intracellular sialidase activity for these culture conditions. The intracellular sialidase activity was the same for the control and the temperature-ramped cultures ($p \leq 0.05$, two-tailed t -test). The time zero point represented the sialidase activity in the seed cultures, which was low relative to that of the freshly inoculated cultures. Intracellular sialidase activity for both culture conditions was 18 ± 5.1 -fold (95% CI) higher than that for the seed cultures. The intracellular sialidase activity was observed to have an exponential decay, which indicated a first-order rate expression. The intracellular sialidase activity had an average *in vivo* half-life of 39 ± 2.9 h (95% CI) for the four cultures.

Decreased cell viability has been reported to be the primary mechanism for the introduction of sialidases into the culture medium (12, 17, 18). The primary mechanism for cell lysis has been reported to be apoptosis (5, 9–11). In this study, intracellular sialidase activity was measured as the potential for removal of the terminal sialic acid residue from a glycan and as the source of extracellular sialidase activity. Interestingly, the intracellular sialidase activity was observed to dramatically increase following a medium exchange for both culture conditions, and then decrease exponentially. The intracellular sialidase exponential decay rate was lower than the growth rate of the cells. This indicated that sialidase production was greater than the dilution effect due to cell growth. Sialidases in human red blood cells have been observed to be localized in vesicles (lysosomes) (19); therefore, it is possible that CHO cell sialidases are also associated with lysosomes. Lysosomes, known as secretory lysosomes, have been shown to secrete proteins under nutrient stress (20). Therefore, the decreased intracellular sialidase levels may be considered indirectly to have indicated increased extracellular sialidase levels. Further work is needed with respect to CHO cell sialidases to address these observations with regard to the intracellular sialidase dynamics.

The tPA was assayed by Western blots and activity. The Western blots confirmed the presence of tPA (data not shown). The tPA activities for the two culture conditions are shown in Figure 3. Initially, the tPA activity in the cultures was low, as expected. As the cells grew, tPA activity increased in the cultures with apparent zero-order kinetics. The tPA activities were identical for both culture conditions. The rate of accumulation for tPA was 1.4 ± 0.14 (95% CI) relative tPA activity units per day. The tPA activity in the cultures increased 14 \pm 2.3-fold (95% CI) over the course of the cultures.

Several authors have reported that decreasing the temperature of a culture increased productivity, decreased protease activity, increased cell densities, increased viabilities, and decreased nutrient utilization rates in mammalian fermentations (1, 3–5, 8). Suzuki and Ollis (21) were able to demonstrate mathematically that higher productivities were attainable by arresting cell growth in G_1 . They suggested that suitable experimental stimuli for arresting cells could be growth-inhibiting drugs, confluence, and temperature; however, their mathematical model did not predict the magnitude of the stimulus needed to arrest cells (21). Additionally, the effects of temperature on culture productivity and growth have been shown to be variable (1, 7, 8).

Several authors have observed that, at lower culture temperatures, the product titer was higher (1, 3–6, 8). Interestingly, Sureshkumar and Mutharasan (1) measured higher product titer after 10 days of culturing. At that point, the cell viability for all of the cultures had decreased, for some cultures more than others. Thus, it was unclear if the lower temperature, higher nutrient levels, or higher cell viability produced the higher product titer. For culture times of 5 days, when the cell viability of all of the cultures was still high, all cultures produced approximately the same amount of the Mab (1). Therefore, the increased cell viability at the end of the lower temperature cultures was probably the significant factor in the increased production of Mab. Kaufmann et al. (8) monitored T-flask conditions for CHO-K1 subjected to a temperature shift from 37 to 30 °C. Contrary to Sureshkumar and Mutharasan, (1) and others (3–5), Kaufmann et al. (8) observed significantly lower cell densities in the lower temperature cultures. The results of this study indicated there was no significant difference between the two conditions with respect to the production of tPA. These results may be indicative of the slow transition from 37 to 34 °C, or of oxygen solubility differences. Therefore, the data on protein productivity overall are mixed (1, 3–5, 7, 8).

In summary, for mammalian cell batch cultures, it is often suggested that lower culture temperatures can be used to improve recombinant protein productivity and reduce deleterious enzyme accumulation. In this study, parallel cultures were examined for recombinant protein productivity, protease activity, and sialidase activity. Cultures were maintained at a constant 37 °C or were ramped from 37 to 34 °C. The constant-temperature and ramped-temperature cultures had similar tPA productivity and intracellular sialidase activity levels and dynamics. Two proteases (50 and 69 kDa) were detected in the cultures. The 50 kDa protease activity decreased similarly with culture time between the two culture conditions, while the 69 kDa protease activity increased with culture time for both culture conditions. The amount of 69 kDa protease was observed to be significantly higher for the ramped-temperature cultures compared to the control cultures. The only significant difference that can be attributed to the ramped-temperature profile is the

higher 69 kDa protease levels. Therefore, even though the 69 kDa protease was observed to be higher in the ramped-temperature cultures, the recombinant protein titer was the same for the two culture conditions. Thus, the lower culture temperature did not improve recombinant protein titer via reduced protease activity.

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