Fed-Batch CHO Cell t-PA Production and Feed Glutamine Replacement to Reduce Ammonia Production

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Industrial therapeutic protein production has been greatly improved through fed-batch development. In this study, improvement to the productivity of a tissue-plasminogen activator (t-PA) expressing Chinese hamster ovary (CHO) cell line was investigated in shake flask culture through the optimization of the fed-batch feed and the reduction of ammonia generation by glutamine replacement. The t-PA titer was increased from 33 mg/L under batch conditions to 250 mg/L with daily feeding starting after three days of culture. A commercially available fed-batch feed was supplemented with cotton seed hydrolysate and the four depleted amino acids, aspartic acid, asparagine, cysteine, and tyrosine. The fed-batch operation increased the generation of by-products such as lactate and ammonia that can adversely affect the fed-batch performance. To reduce the ammonia production, a glutamine-containing dipeptide, pyruvate, glutamate, and wheat gluten hydrolysate, were investigated as glutamine substitutes. To minimize the lag phase as the cells adjusted to the new energy source, a feed glutamine replacement process was developed where the cells were initially cultured with a glutamine containing basal medium to establish cell growth followed by feeding with a feed containing the glutamine substitutes. This two-step feed glutamine replacement process not only reduced the ammonia levels by over 45% but, in the case of using wheat gluten hydrolysate, almost doubled the t-PA titer to over 420 mg/L without compromising the t-PA product quality or glycosylation pattern. The feed glutamine replacement process combined with optimizing other feed medium components provided a simple, practical, and effective fed-batch strategy that could be applied to the production of other recombinant therapeutic proteins. © 2012 American Institute of Chemical Engineers

Keywords: Chinese hamster ovary cells, tissue plasminogen activator, fed-batch, ammonia, glutamine, plant hydrolysate, pyruvate

Introduction

The demand for mammalian cell based therapeutic proteins and antibodies are expected to continue increasing, along with a need for reduced costs. To improve productivity, research has focused on the choice of cell line, clone selection, expression vector design, medium optimization, metabolic engineering, and bioreactor operational conditions. Optimization of cell culture medium along with fed-batch operation has become the most effective way to improve productivity, especially for monoclonal antibodies. As a result, maximum fed-batch recombinant protein yields have increased from ~50 mg/L to over 10 g/L in the past 15 years.

A systematic approach has been able to increase productivity, increase fed-batch product titers, and reduce reactor volumes and cost of goods. Notably, a number of studies have reported iterative methods where basal media are supplemented with concentrated nutrient solutions and in some cases supplements are added according to the uptake rate of the cells. A model based on stoichiometric nutritional demands for animal cell growth was developed to increase cell growth and product titers by minimizing toxic

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A number of strategies have been proposed to reduce the detrimental generation and accumulation of ammonium in mammalian cell cultures (Table 1) such as maintaining low levels of glutamine in the culture media,26–28 culturing cells in perfusion mode,29 removing ammonium using a cation exchange column, electrodialysis, hydrophobic membranes, or hollow fiber modules30 or through the coculture with hepatoma cells.31 Cell lines have also been genetically engineered to be able to convert ammonia to other nontoxic products,32 or to reduce ammonia production.33,34 Although, in many cases these strategies enhanced the productivity, ammonium levels were only partially reduced and complicated the process in ways not likely to be adopted in the biopharmaceutical industry. More practical approaches to reduce ammonia production have focused on replacing glutamate with glutamate,23 pyruvate,35,36 or asparagine.37 For example, the adaptation of BHK or HEK293 cells to a glutamate-based medium38,39 or the substitution of glucose and glutamine by galactose and glutamate in Chinese hamster ovary (CHO) cell culture40 have reduced ammonia by 60–90%. Using stabilized glutamine-containing dipeptides (alanyl-glutamine or glycyl-glutamine) has also reduced ammonium generation41,44 and Gillmeister et al.42 reported that the galactosylation of tissue-plasminogen activator (t-PA) was increased by more than 10%. Plant protein hydrolysates have also been considered as an alternative source of glutamine that can also enhance both cell growth and productivity45 despite their lot-to-lot variability and not being chemically defined.15

In this study, a model CHO cell line expressing t-PA was used to investigate glutamine replacement strategies to reduce ammonia production and to improve recombinant protein productivity in fed-batch culture. t-PA levels in
fed-batch cultures have been reported up to 45 mg/L using serum-free medium\textsuperscript{40,44} and up to 250 mg/L of t-PA using serum containing medium.\textsuperscript{45} Recently, we have reported over 500 mg/L fed-batch production using inhibition of autophagy by 3-methyl adenine.\textsuperscript{46} Here we report, the development of a fed-batch feed media supplemented with plant hydrolysates and specific amino acids (AAs) along with the benefits to be obtained from combinations and sequencing of alternative medium glutamine replacement strategies. Glutamine was replaced by pyruvate, glutamine containing dipeptides, glutamate, or wheat gluten hydrolysate in both the initial basal medium and the feed medium.

Materials and Methods

Cell line

CHO cells,\textsuperscript{47} CHO540/24 derived from the parental CHO/dhfr- cell line and expressing recombinant human t-PA were used as a model system in this study. The cells were cryopreserved at $\sim 1 \times 10^5$ cells/mL in 1 mL vials.

Cell culture

Frozen cells in 1 mL vials were thawed rapidly into 20 mL of basal medium, CD-CHO (Invitrogen, Burlington, ON) supplemented with 4 mM glutamine (Invitrogen, Burlington, ON) in a 75 cm$^2$ T-flask. The cells were maintained at 37°C in 5% CO$_2$ in a humidified incubator. After 24 h, the cells were transferred to a 125 mL shake flask containing 30 mL of CD-CHO supplemented with 4 mM glutamine and maintained at 37°C in a 5% CO$_2$ humidified orbital shaking incubator at 136 rpm (Infors HT, Bottmingen, Switzerland). After 3 to 4 days when the cells reached a viable cell density of $\sim 2 \times 10^6$ cells/mL, the cells were regularly passaged to 2–3 $\times 10^5$ cells/mL. After a minimum of three passages, batch and fed-batch studies were initiated in 125 mL shake flasks at 0.2 $\times 10^5$ cells/mL in 30 mL of basal medium. Fed-batch cultures in the shake flask were operated for 14–16 days and terminated after cell viability dropped below 60%. Feeding was started after two days of culture time or when the cell density had reached 1 $\times 10^6$ cells/mL.

The various feeds containing plant hydrolysates were tested in 10 mL bioreactors (Micro-24 or M24 Microreactor System, MicroReactor Technologies, Mountain View, CA). The M24 uses a 24-deep-well plate (or cassette) as cylindrical vessels whose operation has been previously described.\textsuperscript{48,49} Briefly, for fed-batch operation, each 10 mL bioreactor was operated with 5 mL of exponentially growing cells from shake flask cultures at $\sim 4 \times 10^5$ cells/mL in basal medium plus 1 $\mu$L/mL antifoam C (Sigma, St. Louis, MO) with feeding beginning after two days of culture.

All batch and fed-batch cultures were performed in duplicate with 1 mL sampling every 1 or 2 days and replaced with 1 mL of a specified feed, maintaining a constant volume. Samples were stored at 4°C until analyzed.

Batch and fed-batch feed media

The initial basal medium used for the batch and fed-batch studies was CD-CHO medium (Invitrogen) supplemented with either 4 mM L-glutamine (Gln; Invitrogen) or one of the following four glutamine substitutes (See Table 2): 2 mM glutamine containing dipeptides (GCD) (GlutaMAX\textsuperscript{TM} 1 supplement, Invitrogen); 5 mM sodium pyruvate (Pyr; Invitrogen); 4 mM glutamate (Glu; Sigma); or 1.7 mg/mL wheat gluten hydrolysate (WGH) (HyPep 4601, Kerry Bio-Science, Hoffman Estates, IL). The glutamate was initially dissolved at a concentration of 100 mM in 1 N NaOH and wheat gluten hydrolysate was initially prepared at a concentration of 100 g/L in distilled water.

The fed-batch feed was CHO CD EfficientFeed\textsuperscript{TM} A (Feed A) (Invitrogen). Initially, this Feed A, was supplemented with 4 mM L-glutamine then tested with 1.7 mg/mL of plant hydrolysates. The plant hydrolysate supplement was WGH (HyPep 4601, Kerry Bio-Science), soy protein hydrolysate (SPH; HyPep\textsuperscript{TM} 1510), or cotton seed hydrolysate (CSH) (HyPep\textsuperscript{TM} 7504) or all three hydrolysates combined for a total of 1.7 mg/mL. Each hydrolysate was initially prepared at a concentration of 100 g/L in distilled water and stored at 4°C. Based on optimal hydrolysate and limiting amino acid analyses, two fed-batch feeds were developed: (i) FB-1 where Feed A plus glutamine was supplemented with 1.7 mg/mL of CSH and (ii) FB-2 where FB-1 was supplemented with four limiting AA: 12 mM l-asparagine (Sigma), 1.6 mM l-cysteine disodium salt hydrate (MP Biochemical LLC, Solon, OH), 2.1 mM l-aspartic acid (Invitrogen), and 1.8 mM L-tyrosine (Invitrogen). The following stock solutions were prepared and stored at 4°C: 180 mM of l-asparagine in distilled water, 40 mM of L-cysteine in distilled water, 125 mM of L- aspartic acid in 1 N NaOH, and 100 mM of L-tyrosine in 1 N NaOH.

In the glutamine replacement studies, two feeding strategies were studied: the first was “Complete glutamine replacement” where the glutamine supplement was replaced in both the CD-CHO basal medium and in the fed-batch feed (FB-2), and the second was, “Feed glutamine replacement” where glutamine was retained in the CD-CHO basal medium but replaced in the fed-batch feed (FB-2). The supplement concentrations (Table 2) were based on either preliminary experiments (data not shown) or published data. Since the feeding was begun only after cell densities had reached $\sim 1 \times 10^6$ cells/mL, the feeding was initiated at different times for different supplements. Finally, glucose (Sigma) was added to the fed-batch cultures to maintain $\geq 3$ g/L\textsuperscript{44,46,50} on an as needed basis.

### Analytical methods

**Cell Density and Viability.** Viable cell density was determined by first mixing equal volumes of sample with 0.25%
trypsin-EDTA solution (Invitrogen) at 37°C for 10–15 min to disperse cellular aggregates then analyzed using a Cedex automated cell counter (Innovatis AG, Bielefeld, Germany). This device uses an automated trypan blue dye exclusion method and video imaging to count the cells.

**t-PA Titer and Cell Productivity.** The t-PA titer was determined using a colorimetric enzymatic activity assay as described previously. A conversion factor of 630,000 U/mg converted the activity units to concentrations. The overall cell specific productivity (qp) was determined from the slope of plots of integrated viable cell density versus t-PA concentration.

**Glucoser, Lactate, Ammonia, and Amino Acid Concentrations.** A Multiparameter Bioanalytical System YSI 7100 (YSI Life sciences, Yellow Springs, OH) was used to measure glucose, lactate, glutamine, and glutamate concentration in the culture supernatant. Amino acid and ammonia concentrations were determined by the AccQ-tag method followed by separation on a Waters (Milford, MA) C-18 reverse-phase column using a Waters HPLC system (600S controller and Millenium) according to Dowd et al. 2000.

**Glycan Analysis.** An in-gel method was used for t-PA purification, in tubes treated with Sigmacote, omitting treatment with formic acid to not disturb glycan sialylation. The glycans were cleaved from the core protein and fluoresced by Normal Phase-HPLC. The profiles obtained for all peptides were compared with determine whether the pyruvate-containing medium (Figure 1A) followed by slow growth and a low maximum cell density, the t-PA productivity was considerably increased compared with the control culture supplemented with glutamine. The t-PA titer was increased by feeding the culture with CHO CD EfficientFeed™ Feed A. This feed is a chemically defined supplement for CD-CHO medium that contains a carbon source, concentrated AAs, vitamins, and trace elements. Plant hydrolysates were added to Feed A to increase cell densities and product titers by providing mixtures of low and high molecular weight oligopeptides, nutrients, and survival factors. Three different plant hydrolysates were tested: SPH, WGH, and CSH.

**Effect of supplementing fed-batch medium with plant hydrolysates.**

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**Results and Discussion**

**Batch studies with glutamine replacement by pyruvate**

Batch shake flask cultures with basal medium (CD-CHO) supplemented by either 4 mM glutamine or 5 mM sodium pyruvate were initially compared with determine whether replacement of glutamine by pyruvate would reduce the ammonia production and potentially increase t-PA titer. Although there was a long lag phase as the cells adapted to the pyruvate-containing medium (Figure 1A) followed by slow growth and a low maximum cell density, the t-PA productivity was considerably increased compared with the control culture supplemented with glutamine. The t-PA titer was increased by feeding the culture with CHO CD EfficientFeed™ Feed A. This feed is a chemically defined supplement for CD-CHO medium that contains a carbon source, concentrated AAs, vitamins, and trace elements. Plant hydrolysates were added to Feed A to increase cell densities and product titers by providing mixtures of low and high molecular weight oligopeptides, nutrients, and survival factors. Three different plant hydrolysates were tested: SPH, WGH, and CSH.

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only CSH significantly improved the t-PA productivity (maximum titer of 278 mg/L and \(q_p\) of 8.3 pg/cell/day). All other combinations of hydrolysates with CSH resulted in similar productivities. Based on these results, an optimized hydrolysate supplement ratio was calculated by the Design-Expert software to be 21% WG and 79% CS hydrolysates. However, since using this ratio was expected to only marginally improve t-PA titers and since it is best to have fewer complex medium component sources, Feed A plus glutamine was only supplemented with 1.7 mg/mL of CSH in subsequent fed-batch cultures. The higher t-PA production observed with CSH supplementation could be due to free AAs or minerals being in a more balanced ratio than in the other hydrolysates tested. For example, mineral ratios of magnesium and phosphorus in CSH are 5–9 times higher than in SPH (Product guide from Kerry Bio-Sciences). With addition of CSH to the feed, high cell viability (\(>80\%\)) was maintained for greater than nine days thereby potentially reducing the release of intracellular proteases that could degrade or inactivate the secreted t-PA. Furthermore, the hydrolysate may also provide components that promote stability of t-PA in culture supernatants resulting in further increases in active t-PA titer. It is, therefore, possible that the high t-PA yields that are reported in this study are due to combined stimulatory and stabilizing effect of hydrolysates in the feed. These results are novel compared with most of the work published on the use of hydrolysates that directly supplement the basal culture medium rather than the feeds.53–58

Table 3. Effect of Hydrolysate Supplements on Maximum t-PA titer, Maximum Viable Cell Density, and Cell Specific Productivity in Fed-Batch M24 Cultures (Average of Two Runs)

<table>
<thead>
<tr>
<th>Hydrolysate Supplement</th>
<th>Max. t-PA titer (mg/L)</th>
<th>Max. Viable Cell Density ((\times 10^6) cell/mL)</th>
<th>(q_p) (pg/cell/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed-Batch Control</td>
<td>68 ± 10</td>
<td>6.3 ± 1.9</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>SPH (%) WGH (%) CSH (%)</td>
<td>72 ± 6</td>
<td>7.9 ± 2.4</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>100 – – –</td>
<td>71 ± 4</td>
<td>6.6 ± 1.8</td>
<td>2.4 ± 2.5</td>
</tr>
<tr>
<td>– 100 – –</td>
<td>278 ± 8</td>
<td>7.4 ± 1.0</td>
<td>8.3 ± 1.0</td>
</tr>
<tr>
<td>– – 100</td>
<td>81 ± 15</td>
<td>8.1 ± 2.2</td>
<td>2.2 ± 0.9</td>
</tr>
<tr>
<td>50 – 50 –</td>
<td>263 ± 78</td>
<td>8.1 ± 0.1</td>
<td>8.1 ± 2.3</td>
</tr>
<tr>
<td>– 50 – 50</td>
<td>272 ± 8</td>
<td>9.2 ± 0.1</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>50 – 50 – 50</td>
<td>195 ± 10</td>
<td>8.2 ± 1.3</td>
<td>5.3 ± 2.6</td>
</tr>
</tbody>
</table>

CSH, Cotton seed hydrolysate; SPH, Soy protein hydrolysate; WGH, Wheat gluten hydrolysate.
Additional feed development

The fed-batch feed FB-1 (Feed A plus glutamine supplemented with 1.7 mg/mL of CSH) developed using the M24 microbioreactor system was tested in shake flask culture with daily feeding at ~3% of the initial working volume beginning after two days of culture. This reduced feeding volume (compared with 20% in the M24 study previously described) was adopted to minimize the culture volume changes. Twelve days of culture (Figure 2) resulted in a maximum cell density of $1.29 \times 10^6$ cells/mL, a maximum t-PA titer of 172 mg/L and a $q_p$ of 2.2 pg/cell/day. This was a considerable improvement over the eight-day batch run (maximum cell density of $7 \times 10^5$ cells/mL, maximum t-PA titer of 33 mg/L, $q_p$ of 1.2 pg/cell/day) and was similar to fed batch improvements achieved in the M24 microbioreactor. The superior results of the CSH fed-batch run in the M24 microbioreactor was probably due to the greater feeding rate and/or the pH control. In addition, the comparability of the M24 and fed-batch cultures was not optimized.

To increase t-PA productivity further during the exponential and early stationary phases, the above FB-1 feed was further supplemented with limiting nonessential AAs. To determine what AAs should be supplemented, the spent medium from fed-batch cultures were analyzed and aspartate, asparagine, cysteine, and tyrosine were found to approach limiting levels during the exponential and stationary phases (i.e., 50–200 h of culture time). Therefore, the FB-1 feed was further supplemented with aspartate, asparagine, cysteine, and tyrosine in fed-batch feed FB-2. Supplementing the fed-batch shake flask cultures with AAs (Figure 2) had little effect on the viable cell density but significantly increased t-PA titer and productivity (maximum cell density of $1.28 \times 10^6$ cells/mL, maximum t-PA titer of 237 mg/L, and a $q_p$ of 3.6 pg/cell/day).

However, while incorporating AAs and hydrolysate into the feed was beneficial for t-PA production, there was a concomitant increase in the generation of by-products such as lactate and ammonia. In both runs with FB-1 and FB-2, the lactate concentration approached 42 mM and ammonium reached toxic levels of over 25 mM. Since ammonia is generated in cultures from glutamine, asparagine, and glutamate metabolism, minimizing glutamine or asparagine could be considered as an option to reduce ammonia production. However, reducing the glutamine, although it reduced...
ammonia production also reduced growth and t-PA production and, therefore, glutamine was maintained at 4 mM for optimal t-PA production. In addition, it has been reported by others that asparagine depletion led to reductions in t-PA production. In addition, it has been demonstrated that several AAs, including asparagine and glycine allow hybridoma and CHO cells to tolerate high pCO₂ and osmolality typically found in fed-batch cultures, resulting in increased recombinant protein productivity. Therefore, although asparagine appears to be necessary to maintain high t-PA production it may need to be controlled at levels that not only maximize t-PA production but also minimize ammonia production.

Fed-batch studies with glutamine replacement with alternate supplements and timing

Glutamine limitation has been shown to reduce ammonium ion accumulation in culture. However, maintaining low glutamine levels during fed-batch culture may not be practical or robust at industrial scales since it complicates the process and can reduce cell growth and product titers and may also induce autophagy in the cells. An alternative approach to reduce ammonia production, would be to use substitutes that reduce or even eliminate the need for glutamine in the medium. Thus, four substitutes were tested: glutamine-containing dipeptides (GCD), pyruvate, glutamate, or WGH in an effort to reduce the ammonia production and thereby increase cell growth and t-PA productivity.

First, a “complete glutamine replacement” strategy was tested where the glutamine was completely replaced in both the CD-CHO initial basal medium and in the FB-2 feed. As previously shown in the batch studies with pyruvate, cell adaption to media with glutamine substitutes was not required to maintain specific t-PA productivity. To adjust for the lag in adapting to the glutamine substitutes, the feeding was initiated only after the cell densities reached ≥1 x 10⁶ cells/mL. Thus, depending on the substitute, the feeding was begun at different times as indicated in Figure 3. Complete glutamine replacement led to a significant reduction in ammonia production ranging up to 60 to 65% in the cases of Pyr/Pyr and WGH/WGH replacement compared to the FB-2 fed-batch control (Table 4). However, the overall t-PA titers fell up to 50% likely due to lower cell densities especially in the case of WGH/WGH replacement. It was only in the case of the GCD/GCD replacement, which had a slight lag phase and maximum cell densities and productivities similar to the FB-2 control, that a slight increase in the maximum t-PA titer was observed (275 vs. 237 mg/L; Table 4). The Pyr/Pyr and Glu/Glu replacements resulted in long lag phases of more than five days after which the cell densities steadily increased to levels similar to the FB-2 control of over 1 x 10⁷ cells/mL by day 14, but ultimately resulting in lower t-PA titers. Only, in the case of WGH/WGH replacement was the maximum cell growth severely reduced to 2.5 x 10⁶ cells/mL but in that case the increased qₚ of over 8 pg/cell/day maintained the maximum t-PA titer at 168 mg/L.

To minimize the lag phase resulting from complete glutamine replacement, a “feed glutamine replacement” strategy was studied, where glutamine was retained in the CD-CHO initial basal medium and only replaced in the FB-2 feed.

![Figure 4](image_url)  
Figure 4. Growth profiles for fed batch shake flask culture (average of two runs) using the feed glutamine replacement strategy where glutamine was replaced with GCD, pyruvate, glutamate, or WGH in only the FB-2 fed-batch feed. Viable cell density (A) and t-PA titer (B) versus culture time. Arrows indicate initiation of feeding.
Feeding began after three days when the initial 4 mM glutamine had been almost consumed (data not shown). For these cultures, the maximum cell densities were maintained or slightly increased for all glutamine replacements ranging from 10.1 to 13.2 × 10⁶ cells/mL compared with FB-2 control of 12.8 × 10⁶ cells/mL (Figure 4A). The lag phases were reduced compared with the earlier complete glutamine replacement runs (Figure 3A), but the maximum cell densities were still reached later, on day 9–10, compared with day 5 for the FB-2 control. The final ammonium concentrations were decreased by 36–45%. Although 17 mM of ammonium could be toxic, the t-PA production was increased for all feed glutamine replacement cultures compared with the FB-2 control (Figure 4B). In particular, the Gln/WGH replacement yielded the highest final t-PA titer and $q_p$ (422 mg/L; 5.5 pg/cell/day) compared to the FB-2 control (237 mg/L; 3.7 pg/cell/day). This was a significant improvement compared with using the complete glutamine replacement strategy where t-PA titers were not significantly increased or reduced compared to the control. The increased t-PA production was possibly due to eliminating the lag phase of cell growth combined with a reduction in ammonia levels that may have helped increase the maximum cell densities. The effectiveness of replacing the feed glutamine with WGH could be due to this hydrolysate containing a combination of GCD, AAs, peptides, and vitamins, perhaps explaining why it was more effective than either GCD, pyruvate or glutamate alone. Overall, the improvement to the t-PA titers and reduction in ammonium levels was more effective when using a glutamine substitute in only the feed rather than in both the initial basal medium and the feed. This two-step feed glutamine replacement strategy compares well with other approaches to reduce ammonia generation (see Table 1) but differs in that only the glutamine in the feed is modified. This protocol is relatively simple and could be easily adapted to the production of other recombinant proteins.

**Glycan analysis of the t-PA generated by the feed glutamine replacement strategy**

Although the glutamine replacement strategies increased the t-PA titers, it was of interest to determine if the product quality (i.e., glycosylation) of the t-PA had been compromised. Recombinant protein glycosylation is critical since it can affect the stability, potency, absorption, and clearance of therapeutic proteins and high levels of ammonia have been shown to impair galactosylation and sialylation. Thus, it was particularly important to demonstrate that the feed glutamine replacement strategy not only increased production of t-PA, but also did not compromise the t-PA glycan profile. Figure 5 and Table 5 show the HPLC glycan profiles from samples obtained at the end of the feed glutamine replacement cultures compared with the FB-2 fed-batch control. For each sample of t-PA 26 peaks were identified and 23 of the peaks were assigned glycan structures. The peaks that were not considered, due to low levels of detection, typically made up <1% of the overall glycan profile. The

**Table 5. Summary of Glycans (Predominant Peaks) Released From Feed Glutamine Replacement t-PA Samples Using HPLC**

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>GU</th>
<th>Structure Name</th>
<th>FB-2 Control</th>
<th>Gln/GCD</th>
<th>Gln/Pyr</th>
<th>Gln/Glu</th>
<th>Gln/WGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4.96</td>
<td>F(6)M3</td>
<td>4.14</td>
<td>4.18</td>
<td>3.84</td>
<td>4.1</td>
<td>7.85</td>
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<tr>
<td>4</td>
<td>5.49</td>
<td>A2</td>
<td>8.43</td>
<td>10.26</td>
<td>7.74</td>
<td>7.76</td>
<td>3.14</td>
</tr>
<tr>
<td>6</td>
<td>5.94</td>
<td>A3</td>
<td>9.54</td>
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<td>7.56</td>
<td>8.22</td>
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</tr>
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<td>7</td>
<td>6.22</td>
<td>M5</td>
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<td>20.43</td>
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<td>11.82</td>
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<tr>
<td>12</td>
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<td>A2G1S(6)1</td>
<td>6.24</td>
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<td>2.83</td>
<td>4.63</td>
<td>2.08</td>
</tr>
<tr>
<td>13</td>
<td>7.12</td>
<td>A2G2</td>
<td>7.76</td>
<td>8.88</td>
<td>8.58</td>
<td>8.63</td>
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<td>18</td>
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<td>F(6)A3G(4)3S(3)2</td>
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</table>

**Antennarity Index**

1.94

**Sialylation Index**

1.66

**Fucosylation Index**

0.32

*Refers to % area of total peak area of glycan composition.

A, N-acetylgalactosamine; F, Fucose; G, Galactose; GU, glucose unit; M, Mannose; ND, Not Determined; S, Sialic acid.
glycans were compared based on the antennarity, sialylation, and fucosylation index of the identified glycan structures (Table 5). The antennarity index was based on the number of N-acetylglucosamine residues attached to the core structure. Among all samples, the antennarity index was the highest in the Gln/WGH sample and possibly even higher as some of the higher GU structures could not be identified. Specifically, the fucosylation index appeared to be constant for samples besides Gln/WGH, which was increased by 44% compared with the FB-2 control. The sialylation index seemed to be reduced for the Gln/GCD, Gln/Glu, and Gln/WGH sample. However, the higher GU peaks observed in the t-PA sample from the Gln/WGH replacement culture that were not identified could represent more complex glycan (i.e., higher fucosylation and sialylation index) structures.

Although little has been reported on the impact of specific glycan structures on the activity of t-PA, it has been shown that a high level of sialylation in glycoproteins is important to improve the half-life of t-PA as a therapeutic. In addition, the core fucose of glycoproteins has been shown to have an impact on oligosaccharide conformation important for carbohydrate-protein interactions and to play a role in the activity of some glycoproteins. Overall, the glycan profiles show that the feed glutamine replacement strategy did not significantly affect the glycosylation of the t-PA product. However, in the case of the Gln/WGH replacement culture, the higher GU values may indicate a glycoprotein with an improved activity and half-life.

Conclusions

Although fed-batch processes have significantly increased recombinant protein production, they can also increase the accumulation of metabolic by-products. In particular, ammonia accumulation can compromise the fed-batch advantages. To reduce ammonia generation, the replacement of glutamine under fed-batch conditions was investigated using a CHO cell line that expresses t-PA. Initially, a feed medium, which included hydrolysates and limiting amino acid supplements, was developed to increase t-PA production. Unfortunately, the ammonia generation was also increased and, additional methods were explored to reduce the ammonia generation. Complete glutamine replacement with GCD, pyruvate, glutamate or WGH was shown to reduce ammonia generation while maintaining comparable cell densities, but the t-PA productivity was not substantially improved. This was likely at least in part due to long lag phases required for the cells to adapt to the different media. To shorten the lag phase and allow the cells to reach higher cell densities before adapting them to a glutamine substitute, the cells were initially grown in a basal medium containing glutamine and then glutamine was replaced only in the feed. This feed glutamine replacement both decreased ammonium accumulation and almost doubled the t-PA productivity to a maximum titer of 422 mg/L and a qP of 5.5 pg/cell/day in the case of replacing glutamine with WGH in the feed. In addition, replacing glutamine in only the feed was shown not to affect substantially the t-PA product quality or glycosylation patterns. In conclusion, this feed glutamine replacement process was shown to be an effective strategy to lower ammonia production while maintaining adequate cell growth and increasing productivity in fed-batch culture. This process could be applied to the production of other recombinant proteins at pilot and industrial scale with relatively little modification to the developed fed-batch protocol.

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