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Improvement and simplification of fed-batch bioprocesses with a highly soluble phosphotyrosine sodium salt

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ABSTRACT

Fed-batch culture bioprocesses are currently used predominantly for the production of recombinant proteins, especially monoclonal antibodies. In these cultures, concentrated feeds are added during cultivation to prevent nutrient depletion, thus extending the cellular growth phase and increasing product concentrations. One limitation in these bioprocesses arises from the low solubility or stability of some compounds at high concentrations, in particular amino acids. This study describes the synthesis and evaluation of a phosphotyrosine disodium salt as a tyrosine source in fed-batch processes. This molecule is highly soluble in concentrated feeds at neutral pH. Mechanistic studies demonstrated that the molecule is cleaved in the cell culture supernatant after processing by released phosphatases, leading to phosphate and free L-tyrosine which can be taken up by the cells. No intact phosphotyrosine was detected intracellularly or incorporated into the sequence of the monoclonal antibody. The use of this new molecule allows the simplification of fed-batch processes in large scale manufacturing via the implementation of neutral pH, highly concentrated feeds.

1. Introduction

Cultivation of Chinese hamster ovary cells (CHO) has been widely used to produce recombinant proteins like monoclonal antibodies (mAbs) in the pharmaceutical industry (Wurm, 2004). During cell culture process development, the main goal is to achieve high titers and a defined product quality of the recombinant molecule. This can be achieved through cell line engineering, media optimization and bioprocess development (Huang et al., 2010; Li et al., 2010). For the production of mAbs, high performing fed-batch processes have been developed in which the basal medium supports the initial cell growth and one or multiple feeds are added to increase the culture duration and to prevent nutrient depletion while increasing the cell specific productivity.

L-Tyrosine (Tyr) is a key amino acid for both cellular metabolism and protein synthesis and its depletion in fed-batch processes has been correlated with a drop in specific productivity (Yu et al., 2011) and with protein sequence variants (Feeney et al., 2013). To supply required amino acid concentrations to cells, earlier developed media formulations used animal, yeast or plant derived hydrolysates which represent rich sources for both free amino acids and bioactive peptides with the disadvantage of higher lot to lot variability (Mosser et al., 2013; Nyberg et al., 1999). To improve the robustness of manufacturing processes and to comply with regulatory standards, the current trend suggests a shift from undefined components like hydrolysates to completely chemically defined culture media and feeds. One issue in the development of chemically defined feeds arises from the extremely low solubility of L-tyrosine, especially at neutral pH (Carta and Tola, 1996; Hitchcock, 1924). The use of concentrations of tyrosine di-sodium salts above 1 g/l in feeds induces precipitation and increases the risk of media instability, mainly through co-precipitation of other amino acids (unpublished data). This could lead to suboptimal performance due to the insufficient supply of nutrients and finally to low performing processes.

This challenge is also known in the field of parenteral nutrition where the low solubility of L-tyrosine has led to the development of chemically modified tyrosine like acetyl-tyrosine or tyrosine containing di- or multi-peptides. Successful utilization of N-acetyl-tyrosine, glycyl-tyrosine or alanyl-tyrosine as sources of L-tyrosine in medical products like infusion solutions or parenteral nutrition has been described (Daabees and Stegink, 1979; Neuhauser et al., 1985). Bioavailability of N-acetyl-L-tyrosine was linked mainly to deacetylation by kidney enzymes (Neuhauser et al., 1985). Investigations regarding the mechanism of action of dipeptides indicated no cellular uptake and proposed the involvement of cytosolic peptidases released into the culture medium in

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the extracellular hydrolysis of the molecule (Christie and Butler, 1994).

To overcome the challenge of L-tyrosine solubility, several groups have developed multiple feed processes where L-tyrosine and L-cysteine are solubilized at alkaline pH (Feeney et al., 2013; Yu et al., 2011). These separate feeds are commonly established in the industry but imply complex pH control strategies especially at small scale to reduce the pH spikes during feed addition. This can include, for example in spin tubes, the concomitant use of a slight acidic main feed (pH 5–6). In bioreactors, the alkaline feed can be added slowly while monitoring the pH continuously and PID settings need often to be adjusted to reduce the CO_2 sparging in response to pH spikes. In large scale manufacturing, even if pumps can allow a very slow addition of the separate feed, precipitation is likely to occur after contact with the neutral pH medium in particular when residual foam is present thus inducing variability and potential loss of performance.

The following study summarizes the evaluation of a phosphotyrosine di-sodium salt ($PTyr2Na^+$) as a source of L-tyrosine in CHO fed-batch processes. The new molecule proved to be soluble at high concentrations in complex neutral pH feeds containing already more than 100 g/l amino acids. The performance in fed-batch applications showed at least a comparable cell growth and specific productivity as in the control process. Scale up experiments with a single feed strategy also containing stable cysteine demonstrated the ease of use of such a process and finally led to performance improvements.

2. Materials and methods

2.1. Synthesis of phosphotyrosine di-sodium salt, solubility and stability in chemically defined media and feed

Phosphotyrosine was synthesized from tyrosine and orthophosphoric acid by addition of phosphorus pentoxide according to the literature (Alewood et al., 1983; Plimmer, 1941). The disodium salt was subsequently formed by addition of a sodium ethoxide solution. Purity was assessed by ultra high performance liquid chromatography (UPLC) coupled to UV or MS detection and quantitative ^1H nuclear magnetic resonance spectroscopy and was above 95%. Detected contaminants corresponded to substrates of the reaction (3–4% of tyrosine and remaining solvents). The maximum solubility was assessed in water at room temperature through preparation of a saturated solution. After sedimentation, the solution was dried using infrared and the residual mass was determined. For solubility experiments in feeds, increasing concentrations of phosphotyrosine were solubilized in Merck Millipore proprietary feed at pH 7.0 until reaching the maximum solubility. Stability of phosphotyrosine was assessed at several concentrations (1, 4.5 and 12.4 mM) during 6 months in Merck Millipore proprietary medium at pH 7.0 and compared to the stability of tyrosine di-sodium salt at 1 mM concentration. PTyr and tyrosine concentrations were determined by UPLC to follow the potential spontaneous hydrolysis of PTyr or eventual degradation or complexation reactions. The compounds were considered stable if the standard deviation of the measured concentration over 6 months was below 10%.

2.2. Cell culture

For fed-batch cultures, two CHO cell lines expressing a human monoclonal antibody (mAb) were seeded at 2×10^5 cells/ml in Merck Millipore proprietary medium containing tyrosine di-sodium salt. This medium was chemically defined and contained a recombinant protein. In the control condition, the main feed was added at days 3, 5, 7, 9 or 10, and 14 at following V/V ratio (3, 6, 6, 6 and 3%) whereas 300 mM tyrosine di-sodium salt was added in a separate feed at pH 11 (0.3, 0.6, 0.6, 0.6 and 0.3%). In the tested conditions, phosphotyrosine di-sodium salt was solubilized in the main feed at pH 7.0 and added at the same ratio and days as the control. Glucose was quantified daily and adjusted on demand to 4 g/l with a 400 g/l solution. The feeding strategy resulted in the addition of 33% and 40% v/v of feed in spin tubes and bioreactors, respectively. This dilution had an impact on the final titer but not on the specific productivity (see calculation of the specific productivity).

For spin tube experiments, the starting culture volume was 30 ml and the incubation was performed at 37 °C, 5% CO_2, 80% humidity and agitation at 320 rpm.

For bioreactor experiments, pH was controlled at 7.0 ± 0.02 (Fig. 3) or 6.95 ± 0.15 (Fig. 6) whereas dissolved oxygen was controlled at 50% air saturation by sparging with air and pure oxygen gas via an open pipe sparger. Temperature and agitation were set to 37 °C and 140 rpm, respectively. For the experiment presented in Fig. 6, a temperature shift was performed at day 5 with reduction of the process temperature to 33 °C. The small scale experiments were performed using DasGIP 1.2 l glass bioreactors.

2.3. Offline analysis

Cell growth and viability were monitored using Beckman Coulter ViCell® (Beckman Coulter, Fullerton, CA). Metabolites and IgG concentrations were determined by spectrophotometric and turbidometric methods using the Cedex bio HT® (Roche Diagnostics, Mannheim, Germany). The amino acid analysis was performed using a pre-column derivatization employing the AccQ Tag® Ultra reagent kit. Derivatization, chromatography and data analysis were performed according to the supplier recommendations (Waters, Milford, MA). Phosphate concentration and phosphatase activity were determined using Enzcheck® kits (Invitrogen, Carlsbad, CA) according to the supplier instructions.

2.4. Intracellular amino acid quantification

To quantify intracellular amino acids, cells were washed three times in cold PBS and further lysed either with a freeze-thaw method (Tran et al., 2010) or using a phosphatase inhibitor containing extraction buffer. Briefly, for the freeze-thaw method, 12×10^6 cells were lysed in 100 μl milli-Q water and stored on ice. Cells were then subjected to four freeze-thaw cycles, each consisting of 20 min freezing at −20 °C and 20 min thawing at +37 °C. For chemical lysis, 12×10^6 cells were lysed in 100 μl of phosphoSafe reagent (Merck Millipore, Darmstadt) containing four phosphatase inhibitors: sodium fluoride, sodium vanadate, β-glycerophosphate and sodium pyrophosphate. The amino acid concentrations were determined using the UPLC method described above and data were normalized with the total protein concentrations determined using the Bradford method.

2.5. Specific productivity

The productivity per cell per day was calculated each day by dividing the titer by the integral viable cell density corrected to take into account the dilution resulting from feeding. The overall specific productivity in the run was determined by calculating the slope from the linear regression between titer and corrected integral viable cell density.

2.6. mAb purification and characterization

IgG were purified from the supernatants using pure proteome protein A magnetic beads (Merck Millipore, Darmstadt, Germany) and eluted with 90% 200 mM glycine pH 3 and 10% 1 M Tris pH