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PRBI-10863; No. of Pages 9
ARTICLE IN PRESS
Process Biochemistry xxx (2016) xxx–xxx
Contents lists available at ScienceDirect
Process Biochemistry
journal homepage: www.elsevier.com/locate/procbio

LongR3 enhances Fc-fusion protein N-linked glycosylation while improving protein productivity in an industrial CHO cell line

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A R T I C L E   I N F O

Article history:
Received 9 June 2016
Received in revised form
30 September 2016
Accepted 28 November 2016
Available online xxx

Keywords:
Cell culture
Chinese Hamster Ovary (CHO)
Glycosylation
PCR array
Protein production
Sialic acid
Neu2

A B S T R A C T

Glycosylation is a critical quality attribute for many therapeutic glycoproteins. Numerous studies in academia and industry have sought to understand and control protein glycosylation in order to obtain consistent glycoform profiles in production. In many cases, the resulting enhancement of protein glycosylation comes at the expense of protein productivity. Here we examine the impact of insulin-like growth factor-I analogue, LongR^3 (LR3), on an Fc-fusion producing Chinese Hamster Ovary (CHO) cell process. We observe that LR3 improves protein productivity and significantly increases protein N-linked glycosylation. The beneficial effects can be seen at low concentrations (micrograms/L), which allows LR3 to be a cost-efficient additive for improving cell culture performances. The recombinant protein examined in this study has both higher sialic acid content and lower percent of asialylated species of N-linked glycans in the presence of LR3. Finally, CHO-specific glycosylation Real-Time Reverse Transcription (RT^2) PCR Arrays combined with an enzymatic assay demonstrate that LR3 significantly down-regulates cytosolic sialidase gene *Neu2* and hexosaminidase-D gene *Hexdc*, decreases extracellular sialidase activity, and therefore prevents recombinant glycoprotein desialylation.

1. Introduction

Chinese Hamster Ovary (CHO) cells have become the dominant mammalian host for production of recombinant therapeutic proteins because of their human-like glycosylation, extensive characterization, and familiarity by regulatory agencies [1,2]. Since the first CHO-produced biologic was commercialized in 1987, over 70% of biologics in the clinic have been produced by this host, and yields have improved from milligrams per liter to grams per liter [1,2]. Despite these improvements, increasing market demands and the need for efficient delivery of new medicines to patients drive further optimization of culture performance. Significant progress in this regard has been made through media development and bioprocess optimization, resulting in increased specific and volumetric productivity [3].

One key aspect of media formulation has been a shift from media containing serum to chemically defined, serum free media. In doing so, it has been necessary to supplement media with recombinant protein growth factors (GF) to improve process robustness and achieve desired cell density, viability, and productivity. Insulin, transferrin and albumin were the first recombinant alternatives to serum to be developed and widely used for mammalian bioprocessing, and once optimized showed equivalent or improved performance compared to serum up to commercial scale. Recombinant insulin-like growth factor (IGF) has also been explored for the same purpose [4]. To date, some studies have shown that different growth factors and growth factor concentrations may be needed to achieve optimal performance for each cell line and process [5,6], although many CHO bioprocesses are still relying solely on insulin in media formulations to prevent cell death and maintain high cell viability [7].

LongR^3 (LR3) is a recombinant fusion protein analogue of IGF-I [8]. It comprises two parts; the first portion contains human IGF-I peptide sequence in which a glutamic acid residue at position 3 is replaced by arginine, and the second segment has 11 N-terminal amino acids of methionyl porcine growth hormone. Compared with authentic IGF-I, this analogue has a 3.2-fold lower binding affinity for IGF-I receptors and 689-fold lower binding affinity for IGF-binding proteins (IGFBPs). Subsequently its biological potency

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http://dx.doi.org/10.1016/j.procbio.2016.11.018
1359-5113/

Please cite this article in press as: Y. Qian, et al., LongR3 enhances Fc-fusion protein N-linked glycosylation while improving protein productivity in an industrial CHO cell line, Process Biochem (2016), http://dx.doi.org/10.1016/j.procbio.2016.11.018

is elevated when used in cell cultures secreting IGFBPs because of higher bioavailability [8]. LR3 has been shown to inhibit cell death among different types of cells [5,9–12]. For CHO cells specifically, it was demonstrated to be able to sustain higher cell viability than insulin [5,13], although a more recent study showed that the responses varied among clones [14].

Here we assess the impacts of LR3 on an industrial CHO cell culture process. In addition to promoting cell growth and extending cell viability, we observe improvements to product quality as well. Specifically, the recombinant Fc-fusion protein produced by the CHO cultures in the presence of LR3 has higher sialic acid content and a lower percentage of asialylated species of N-linked glycans. This is the first report of LR3 impacting the glycosylation of a biologic. Furthermore, we identify expression of the gene encoding cytosolic sialidase (NM_00124, neuraminidase 2, Neu2) to be significantly down-regulated, and extracellular sialidase activity to be lowered by LR3, and conclude that the observed reduction in recombinant glycoprotein desialylation is a direct result of decreased Neu2 expression in response to LR3.

2. Materials and methods

2.1. Cell line and media

The cells used in this study were from a proprietary CHO cell line engineered from DG44 parental cells, and cultured in chemically defined, animal component-free basal and feed media. The basal medium contained 1 mg/L recombinant human insulin whereas the feed did not.

2.2. Shake flask experiments

The experiments were performed in 250-mL shake flasks with initial volumes of 100 mL and cell densities of 5×10^5 cells/mL. Flasks were placed on a shaker platform (VWR International), agitating at 150 rpm and maintained at 5% CO_2. Culture temperature was constantly controlled at 37 °C, or initially at 37 °C and then on day 5 shifted to 34 °C for two sets of flasks. Culture pH was measured daily and adjusted using 1 M sodium carbonate if lower than 6.9. Starting from day 3, cells were fed daily with a fixed volume of feed medium that was equivalent to 3.6% of initial culture volume. Measurement of cell density and cell viability was performed offline by trypan blue dye exclusion using an automated Cedex cell counting system (Innovatis AG, Bielefeld, Germany). Culture metabolites and nutrients, such as glucose and glutamine, and pH were analyzed offline using a Bioprofile Analyzer 400 (Nova Biomedical Corporation, Waltham, MA). Product titers were measured via a Protein-A HPLC assay using an Agilent 1100 system with an established reference standard.

2.3. Determination of sialic acid content

Cell culture supernatant was collected periodically by centrifugation and purified using a protein-A column. The recombinant Fc-fusion protein was treated with partial acid hydrolysis to release sialic acids *N*-acetyl-neuraminic (Neu5Ac) and *N*-glycolyl-neuraminic acid (Neu5Gc). The released sialic acids were then separated by reversed phase HPLC to determine the content, which was calculated as the total moles of Neu5Ac and Neu5Gc per mole of the protein product, and reported as normalized sialic acid content. Data normalization was performed by dividing the actual values by the same arbitrary number.

2.4. Determination of N-linked oligosaccharides

N-linked oligosaccharides were profiled with high-pH anion-exchange chromatography with pulsed amperometric detection (PHAEC-PAD) as described in literature [15]. Each separated N-linked oligosaccharide was computed based on the area under the chromatographic peak in proportion to the sum of areas of five species representing different sialic acid content including asialylated, mono-, di-, tri- and tetra-sialylated.

2.5. RNA isolation

Approximately 5×10^6 cells from each sample were washed with 1 x phosphate buffered saline solution. The cell pellets were homogenized in QIAzol Lysis Reagent and total RNA was extracted and purified using miRNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA concentrations were determined and purity was verified by NanoDrop 2000 UV–vis Spectrophotometer (Thermo Scientific, Wilmington, DE).

2.6. Real-time reverse transcription (RT^2) PCR array processing

After genomic DNA elimination step, first strand cDNA was synthesized using RT^2 First Strand Kit (Qiagen). CHO-specific glycosylation RT^2 Profiler PCR Arrays (Qiagen) were then used for transcription profiling of 84 key glycosylation genes. Real-time PCRs were performed in 96-well plate format using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The values of threshold cycle (Ct) for all wells were exported to Excel-based PCR Array Data Analysis Templates and analyzed according to manufacturer's recommendations.

2.7. Sialidase activity assay

Cell culture supernatants were collected on day 10 and stored at −80 °C. Prior to conducting the activity assay, thawed samples were centrifuged at 13,000 rpm for 1 min at 4 °C to further remove cell debris, and diluted 1:25 in culture medium. This dilution was chosen to maintain the assay within the linear range based on preliminary experiments with dilutions 1:5–1:100,000. The assay was performed with the Amplex Red Neuraminidase Assay Kit (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instructions. With this kit, sialidase (neuraminidase) activity is proportional to the amount of resorufin produced, which is a red-fluorescent oxidation product from the Amplex red reagent. Fluorescence was measured on a SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) at an Ex/Em, 530/590 nm.

3. Results and discussion

3.1. Cell growth and recombinant protein productivity

3.1.1. Dose-responses

Previous reports have shown that LR3 is able to maintain cell viability in a concentration-dependent manner [5]. As the first step of the present study, the dose-responses of recombinant CHO cells to LR3 under chemically-defined and animal component-free medium conditions were determined. Compared with the absence of LR3, supplementation with 3 μg/L as a single bolus on day 3 was able to moderately stimulate cell growth (Fig. 1A), and this effect was incremental at 10, 30, and 100 μg/L. A similar dose-dependency of LR3 and cell viability was also seen (Fig. 1B). This was especially prominent at later times. Most likely, it was because the cells lacking LR3 could not last alive long and started to crash quickly at the later points, as shown from viability changes in

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