



Cell culture media supplemented with raffinose reproducibly enhances high mannose glycan formation



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ABSTRACT

Glycosylation plays a pivotal role in pharmacokinetics and protein physiochemical characteristics. In particular, effector functions including antibody-dependent cell-mediated cytotoxicity (ADCC) can be desired, and it has been described that high-mannose species exhibited enhanced ADCC. In this work we present the trisaccharide raffinose as a novel cell culture medium supplement to promote high mannose N-glycans in fed-batch cultures, which is sought after in the development of biosimilars to match the quality profile of the reference medicinal product (RMP) also. Up to six-fold increases of high mannose species were observed with increasing raffinose concentrations in the medium of shaken 96-deepwell plates and shake tubes when culturing two different CHO cell lines in two different media. The findings were confirmed in a pH-, oxygen- and CO₂-controlled environment in lab-scale 3.5-L bioreactors. To circumvent detrimental effects on cell growth and productivity at high raffinose concentrations, the media osmolality was adjusted to reach the same value independently of the supplement concentration. Interestingly, raffinose predominantly enhanced mannose 5 glycans, and to a considerably smaller degree, mannose 6. While the underlying mechanism is still not fully understood, minor effects on the nucleotide sugar levels have been observed and transcriptomics analysis revealed that raffinose supplementation altered the expression levels of a number of glycosylation related genes. Among many genes, galactosyltransferase was downregulated and sialyltransferase upregulated. Our results highlight the potential of cell culture medium supplementation to modulate product quality.

1. Introduction

The quality profile of recombinant therapeutic molecules affects its efficacy (Chee Fung Wong et al., 2005; Pacis et al., 2011; Rouiller et al., 2012) and safety (Chugh and Roy, 2014; Jefferis, 2009) *in vivo*. In particular, glycosylation can have a strong impact on protein biological and physiochemical characteristics (Hossler et al., 2009). Glycosylation has been reported to impact protein solubility and stability, enzymatic activity, cellular processing, secretion, clearance and half-life, as well as efficacy and biological activity (Chee Fung Wong et al., 2005; Chugh and Roy, 2014; Goto et al., 2014; Hossler et al., 2009; Kayser et al., 2011; Konno et al., 2012; Pacis et al., 2011; Rouiller et al., 2012; Solá and Griebenow, 2011; Yang et al., 2014). Furthermore, glycosylation

accounts for several pathologies (Abès and Teillaud, 2010; Kanda et al., 2007; Scallan et al., 2007; Wright and Morrison, 1998). It is no surprise that due to its ability to attach complex glycan structures to the protein backbone, the majority of the approved recombinant therapeutics on the market today are being manufactured in mammalian cell lines; in particular in Chinese Hamster Ovary cells (CHO) (Kantardjieff and Zhou, 2014). A significant amount of research has been performed to produce monoclonal antibodies with increased effector functions including antibody-dependent cell-mediated cytotoxicity (ADCC) (Zhou et al., 2008) and cell-dependent cytotoxicity (CDC) (Hodoniczky et al., 2005). It has been shown that the N-linked glycan affected the FcγIIIa receptor binding and ADCC activity of the antibodies (Nimmerjahn and Ravetch, 2008). Like afucosylated glycans,

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high-mannose species induced increased ADCC, thus reflecting their FcγIIIa-binding affinity, nonetheless to a lesser extent than afucosylated complex glycans (Kanda et al., 2007).

Recently, many companies have increased the number of biosimilars in development due to patent expiry of biologics (Chugh and Roy, 2014). In that context, the identification of levers affecting recombinant protein quality has become a main focus. Glycoheterogeneity occurs naturally in the Golgi apparatus (Wong et al., 2010). Gene and expression levels, as well as spatial localization of the enzymes and nucleotide-sugar substrate influence the level of antennarity and the degree of glycan transformation (Hossler et al., 2009). In biosimilar development, the aim is the consistent expression of a highly similar glycan fingerprint compared with the originator molecule (Kantardjieff and Zhou, 2014). The literature describes three approaches to modulate protein quality: cell line engineering (Butler and Meneses-Acosta, 2012; Kremkow and Lee, 2013; Zhu, 2012), change of process parameters (Gramer, 2014; Hossler et al., 2009; Pacis et al., 2011), and cell culture media design (Brühlmann et al., 2015). Researchers reported that in supplemented medium the cell transformed fluorinated peracetylated fucose and sialic acid into the corresponding fluorinated nucleotide sugars by means of its salvage pathway (Rillahan et al., 2012). They observed a specific and efficient inhibition of the fucosyl- and sialyl-transferases, respectively, when adding one of these compounds to the media at the micromolar level. Instead of inhibiting one of the glycosylation transformation enzymes in the endoplasmic reticulum (ER) or the Golgi apparatus, the substrate generation may be targeted. Cultures supplemented with N-acetylglucosamine (GlcNAc) at the millimolar level resulted in reduced complexity of glycan profiles, hence favoring the G0 glycoform (Blondeel et al., 2015). Media supplementation with kifunensine, a potent α -mannosidase I inhibitor entailed an increase of oligomannose containing monoclonal antibodies (mAb) (Zhou et al., 2008) profile toward HM species (Hossler et al., 2014). In a perfusion process, a novel approach utilized mannose as a carbon source and the ratio of mannose to the total hexose in the feed media correlated with the abundance of HM glycan species (Huang et al., 2015).

In this study, we present a robust approach to increase the abundance of high mannose type monoclonal antibodies, using raffinose, a naturally occurring trisaccharide composed of galactose, glucose, and fructose. Cell culture media supplementation was assessed in high-throughput systems including 96-deepwell plates (DWP) and shake tubes (ST). Confirmation of the findings was conducted in lab-scale 3.5-L bioreactors. Although HM should be minimized due to immunogenic reactions (Kilgore et al., 2008), we developed a method, which may be utilized in the frame of biosimilar development to match the quality profile of the reference medicinal product (RMP). Raffinose, a water soluble carbohydrate, was first found in the Australian Eucalyptus manna, in cotton seed, in sugar beet molasses and both barley and wheat (Stone and Baird, 1897). Nowadays, it is known that raffinose can be found in all plants (Elsayed et al., 2014). Raffinose family oligosaccharides have miscellaneous functions in plants including transport and storage of carbon and are involved in the protection against abiotic stress in plants (Hannah et al., 2006). Several studies described the effect of raffinose on various metabolic pathways of other cell types. Prebiotic treatment of fertile eggs injecting raffinose into the amniotic fluid significantly increased the relative expression of aminopeptidase, sucrase isomaltase, ATPase, and sodium glucose co-transporter 1. As a result, the iron bioavailability was altered (Pacifci et al., 2016). In humans raffinose intake was correlated with leukotoxic effects and oxidative stress (Nieman et al., 2014). The results presented in this work demonstrate that in fed-batch processes raffinose supplementation reproducibly increases the amount of HM glycans.

2. Materials and methods

2.1. Inoculum preparation

Two recombinant cell lines were used in the frame of this study. A CHO-K1 derived clonal cell line expressing a humanized monoclonal IgG1 antibody (cell line 1) and a CHO-S derived clonal cell line expressing a human monoclonal IgG1 antibody (cell line 2). Cells were first expanded in multiple passages in shake tubes or shake bottles in Merck proprietary medium containing methionine sulfoximine (MSX) for at least 14 days in a shaker incubator at 36.5 °C, 5% CO₂, 80% humidity and 320 rpm agitation (ISF1-X, Adolf Kühner, Birsfelden, Switzerland or Multitron Cell, Infors HT, Bottmingen, Switzerland).

2.2. Cell culture in 96-deepwell plates

The fed-batch cell culture was performed on a robotic liquid handling platform (Biomek FX, Beckman Coulter, Brea, CA). CHO-K1 cells were seeded into a shaking 96-DWP filled with Merck proprietary medium enriched with 0–50 mM raffinose (Sigma, Darmstadt, Germany) in the absence of MSX at a viable cell density of 0.20×10^6 viable cells/mL and CHO-S cells at 0.30×10^6 viable cells/mL. A second round of experiments was carried out, using a constant medium osmolality approach. The medium was enriched with 0–128 mM raffinose, and subsequently, distinct amounts of NaCl added to reach a final osmolality of 315 mOsm/kg in all experimental conditions. The plates were incubated with vented lids to minimize evaporation in a shaker incubator at 36.5 °C, 5% CO₂, 90% humidity and 320 rpm agitation (ISF1-X, Adolf Kühner, Birsfelden, Switzerland) for 14 days. 400 g/L glucose solution, chemically defined feed containing over 30 components and alkaline amino acid solution were added on day 3, 5, 7, 10 and 12. Prior to each feeding and at the end of the culture (day 14), samples ($\leq 40 \mu\text{L}$) were drawn for growth and viability assessment and product titer quantification.

2.3. Cell culture in shake tubes

CHO-K1 cells were seeded into a TPP® TubeSpin bioreactor tubes (referred to *shake tubes* or *ST*) filled with either proprietary medium enriched with 0–100 mM raffinose adjusted with NaCl to 315 mOsm/kg in the absence of MSX, or with Cellvento CHO200 (Merck Life Science, Darmstadt, Germany) enriched with 0–50 mM raffinose at a viable cell density of 0.20×10^6 viable cells/mL and CHO-S cells at 0.30×10^6 viable cells/mL in proprietary medium. Two different media were used in order to evaluate whether the increase of high mannose species was reproducible in an off-the-shelf medium (if not specifically highlighted hereafter, the experiments were performed in proprietary medium). The ST were incubated in a shaker incubator at 36.5 °C, 5% CO₂, 80% humidity and 320 rpm agitation (ISF1-X, Adolf Kühner, Birsfelden, Switzerland) for 14 days. Into the ST filled with Merck proprietary medium, chemically defined feed (CDF) containing over 30 components and an alkaline amino acid solution were added on days 3, 5, 7 and 10, while the 400 g/L glucose solution (GlcS) was added on these days and day 12 as well. The Cellvento CHO200 containing tubes were fed with Cellvento Feed-200 (Merck Life Science, Darmstadt, Germany) on days 3, 5, 7 and 9 and with cysteine/tyrosine stock solution on the same days according to supplier recommendations. The 400 g/L GlcS was added on days 3, 5, 7, 9 and 12. Prior to each feeding and at harvest (day 14), aliquots ($\leq 2.5 \text{ mL}$) were taken for viable cell counting, extracellular metabolite profiling (not shown) and product titer determination (not shown).

2.4. Cell culture in 3.5-L bioreactors

The passaged CHO-K1 cells were seeded in 3.5-L bioreactors, (Biostat B, Sartorius, Göttingen, Germany; final volume: 3.0 L) filled

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