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O-linked glycosylation analysis of recombinant human granulocyte colony-stimulating factor produced in glycoengineered *Pichia pastoris* by liquid chromatography and mass spectrometry

Bing Gong*, Irina Burnina, Heather Lynaugh, Huijuan Li

GlycoFi, Biologics Discovery, Merck & Co., Inc., 16 Cavendish Court, Lebanon, NH 03766, USA

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ABSTRACT

Glycosylation is a major biochemical attribute of therapeutic proteins and detailed analyses including the structures and sites of such modifications are often required for product quality control and assurance. Using liquid chromatography and tandem mass spectrometry techniques, we analyzed the *O*-linked glycosylation of recombinant human granulocyte colony-stimulating factor (rhG-CSF) derived from glycoengineered *Pichia pastoris* with regard to its nature, structure, occupancy, and location. Peptide mappings using protease and chemical cleavages were performed to determine the specific *O*-linked glycosylation site used by *Pichia*-derived rhG-CSF. Our results demonstrated that Thr134, the equivalent *O*-linked glycosylation site found on endogenous human G-CSF, is the only site modified with a single mannose, allowing glycoengineered *P. pastoris* to be used as a viable production platform for therapeutic rhG-CSF.

1. Introduction

Granulocyte colony-stimulating factor (G-CSF), a hematopoietic cytokine stimulating proliferation and differentiation of neutrophil progenitor, plays essential functions in maintaining circulating level of active neutrophils [1]. Its recombinant form (rhG-CSF) has been used as an important therapeutic to treat patients who developed neutropenia post chemo-/radio-therapy or had bone marrow transplantation [2]. It has also been used to treat infections due to its capability of attracting and localizing mature neutrophils to the infection sites [3,4].

As a relatively small glycoprotein, endogenous human G-CSF comprises 174 amino acids with a single *O*-linked glycosylation modification at Thr133. Currently, two main therapeutic rhG-CSF proteins are produced in *Escherichia coli* (filgrastim) and Chinese hamster ovary cells (lenograstim). With comparable *in vivo* efficacy and safety profiles [5], filgrastim and lenograstim demonstrated the differences inherited from their expression hosts. Instead of 174 amino acids, filgrastim has an extra Met at its N-terminus that is required to initiate the translation in *E. coli*. Moreover, *E. coli*-derived filgrastim lacks the *O*-linked glycosylation found on lenograstim and endogenous human G-CSF. Even though there is no significant clinical impact because of the *O*-linked glycosylation, *in vitro* experiments demonstrated different biochemical and biological properties between glycosylated and aglycosylated rhG-CSF. Kishita et al. showed that, after incubation with human serum individually, lenograstim retained most of its activity while the activity of filgrastim was substantially diminished [6]. Glycosylated rhG-CSF was also found to be more resistant to human neutrophil elastase degradation in an *in vitro* enzymatic assay [7]. It has also been shown that glycosylation prevents polymerization and improves thermo stability of rhG-CSF [8]. In addition to *E. coli* and CHO cells, yeast cells including *Saccharomyces cerevisiae* and *Pichia pastoris* have also been used to express rhG-CSF [9,10]. Using an Agilent BioAnalyzer gel electrophoresis system and site-specific mutagenesis, Apte-Deshpande et al. analyzed the *O*-linked glycosylation on rhG-CSF from wild-type *P. pastoris*. However, the glycan structures were not determined [9]. While *O*-linked mannosylation has been reported in mammalian cell-produced glycoproteins [12,13], the *O*-linked mannosylation from wild-type yeast cells is undesirable because it contains phosphorylation and β-linked mannose structures, which have not been observed on human proteins and could be immunogenic [11].

Recently, glycoengineered *P. pastoris* capable of synthesizing human *N*-linked glycans and controlling *O*-linked glycans has been reported, allowing production of therapeutic proteins with human glycans from yeast cells [14]. Here we analyzed the *O*-linked glycosylation of rhG-CSF derived from such glycoengineered *P. pastoris*. Compared to *N*-linked glycosylation, *O*-linked glycosylation analysis is more complex and challenging due to the facts that no consensus sequences and effective enzymes are

* Corresponding author. Tel.: +1 603 727 5133; fax: +1 603 643 8194.
E-mail address: bing.gong@merck.com (B. Gong).

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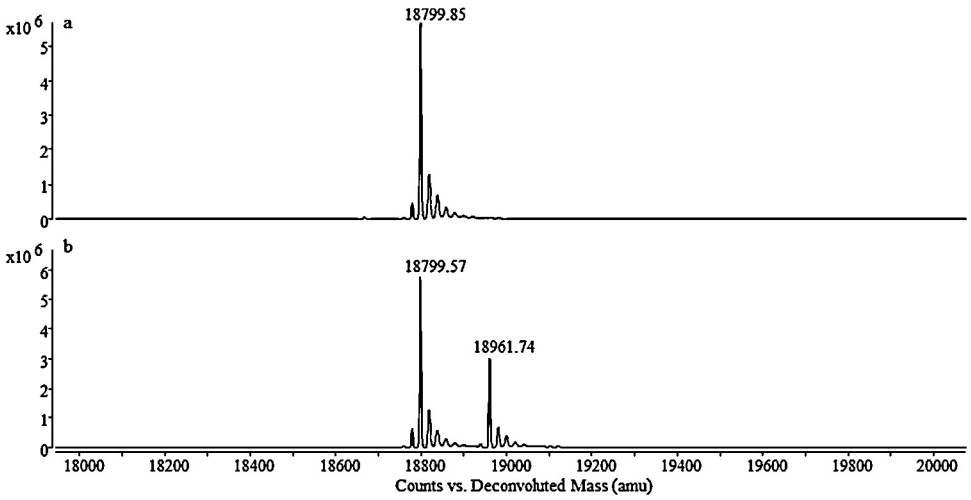

Fig. 1. LC–MS analysis of *E. coli* (a) and *Pichia*-derived rhG-CSF (b).

available to predict the sites and release the glycans. Additionally, glycans tend to fall off before the fragmentation of protein backbone, therefore, the site information is often lost during mass spectrometry analysis. Even though new mass spectrometry techniques such as electron transfer disassociation (ETD) and electron capture dissociation (ECD) have substantially improved the analysis of labile modifications including glycosylation, the results vary among different proteins/peptides [15]. In this work, using liquid chromatography and mass spectrometry with the aid of chemical and enzymatic digestions, we were able to determine the structure, occupancy, and site of *O*-linked glycosylation on rhG-CSF produced in glycoengineered *P. pastoris*.

2. Materials and methods

2.1. Materials

Filgrastim was purchased from Myoderm (Norristown, PA). *Pichia* rhG-CSF was produced in-house. Glu-C protease was from Roche Diagnostics (Indianapolis, IN). Iodoacetamide (IAM), dithiothreitol (DTT), urea, cyanogen bromide, and ammonium bicarbonate were purchased from Sigma Aldrich (St. Louis, MO). HPLC grade water, LC/MS grade water, and acetonitrile with 0.1% (v/v) formic acid or 0.1% TFA were purchased from Fisher Scientific (Pittsburgh, PA). MultiScreen HTS IP Sterile Plate was from Millipore (Billerica, MA). Sepabeads SP20SS were from Supelco (Bellefonte, PA).

2.2. Intact rhG-CSF analysis using LC–MS

Filgrastim and *Pichia* rhG-CSF (2 μg) were analyzed using an Agilent Q-TOF 6520 mass spectrometer coupled with an Agilent 1200 HPLC (Agilent, Santa Barbara, CA). An in-line MassPREP Micro desalt cartridge (Waters, Milford, MA) was used to remove salts from the samples. The protein was eluted using a one-step gradient: 100% buffer A (0.1% formic acid in water) flowed at 2 mL/min for one min, then the flow was switched to 100% buffer B (0.1% formic acid, 10% water, and 90% acetonitrile) at 0.8 mL/min from 1 to 1.5 min. From 1.5 to 4 min, the flow rate was decreased to 0.5 mL/min and the LC–MS data were collected. The MS data were recorded in the profile mode with the range of 500–3200 m/z. The dual ESI ion source was set as follows: gas temp: 350 °C; drying gas: 13 L/min; nebulizer: 45 psig; fragmentor: 150 V; skimmer: 65 V; Oct1 RF VPP: 750 V; Vcap: 3500 V. The MS spectra were analyzed using MassHunter software.

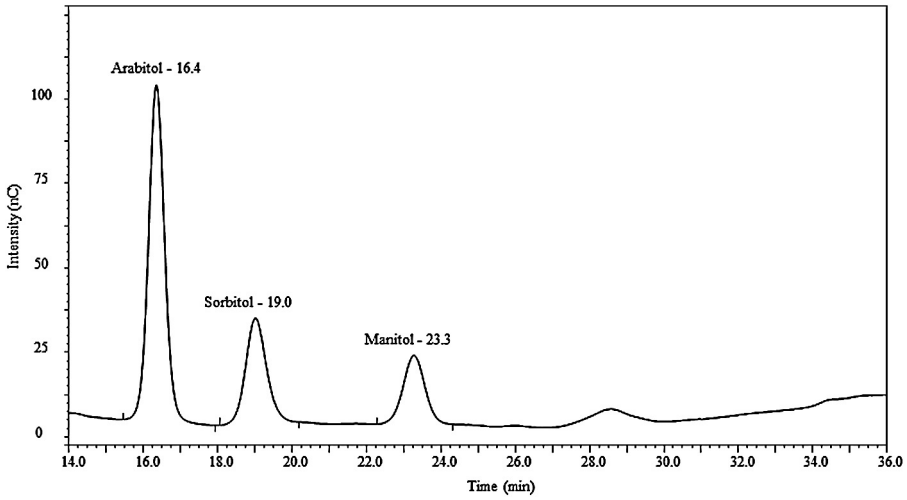

Fig. 2. *O*-linked glycosylation of *Pichia*-derived rhG-CSF analyzed using HPACE-PAD.