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Rapid *N*-glycan release from glycoproteins using immobilized PNGase F microcolumns

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ABSTRACT

N-glycosylation profiling of glycoprotein biotherapeutics is an essential step in each phase of product development in the biopharmaceutical industry. For example, during clone selection, hundreds of clones should be analyzed quickly from limited amounts of samples. On the other hand, identification of disease related glycosylation alterations can serve as early indicators (glycobiomarkers) for various pathological conditions in the biomedical field. Therefore, there is a growing demand for rapid and easy to automate sample preparation methods for *N*-glycosylation analysis. In this paper, we report on the design and implementation of immobilized recombinant glutathione-*S*-transferase (GST) tagged PNGase F enzyme microcolumns for rapid and efficient removal of N-linked carbohydrates from glycoproteins. Digestion speed and efficiency were compared to conventional in-solution based protocols. The use of PNGase F functionalized microcolumns resulted in efficient *N*-glycan removal in 10 min from all major N-linked glycoprotein types of: (i) neutral (IgG), (ii) highly sialylated (fetuin), and (iii) high mannose (ribonuclease B) carbohydrate containing glycoprotein standards. The approach can be readily applied to automated sample preparation systems, such as liquid handling robots.

1. Introduction

There is a growing demand in the biopharmaceutical industry for high throughput and large scale *N*-glycosylation profiling of biotherapeutics in all phases of the product development process. In 2015, monoclonal antibodies represented almost half of the biotherapeutic drugs with an estimated $70 billion dollar business worldwide [1,2], emphasizing the importance of their efficient analysis. On the other hand, glycoproteins play significant roles in cell development, differentiation and interactions [3]. Thus, analysis of the structural changes of the glycan moieties of these highly complex molecules in living systems may give the opportunity to shed light on the courses of diseases leading to new glycosylation based biomarkers [4]. However, comprehensive glycoanalytical methods should be able to handle large number of samples in a high throughput fashion [5] demanding rapid and easily to automate glycan analysis tools.

The most frequently used carbohydrate analysis techniques are nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS), liquid chromatography (HPLC) and capillary electrophoresis (CE) [6–8]. Capillary electrophoresis features an important advantage as capable to differentiate oligosaccharides based on their molecular shape – even if the mass to charge ratios of two analytes are the same – thus readily separates linkage and positional isomers [9]. In addition, CE requires only nanoliter sample amounts to be injected for full structural analysis, compared to other techniques that need microliter quantities for the same [10]. The workflow of CE based *N*-glycan analysis includes the following sample preparation steps: glycan release, fluorophore labeling, purification and pre-concentration prior to separation.

In general, Peptide-*N*4-(*N*-acetyl-beta-glucosaminyl) asparagine-amidase (PNGase F) is the most frequently used enzyme for N-linked carbohydrate release, due its specific cleavage capability under mild conditions between the innermost

Abbreviations: PNGase F, Peptide-*N*4-(*N*-acetyl-beta-glucosaminyl) asparagine-amidase; GST, glutathione-*S*-transferase; RNase B, ribonuclease B; IgG, immunoglobulin G; APTS, 8-aminopyrene-1,3,6-trisulfonic acid; CGE-LIF, capillary gel electrophoresis with laser induced fluorescence detection; SPRI, solid phase reversible immobilization technology; BFS, bare fused silica.

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core *N*-acetylglucosamine of the carbohydrate structures and its holding asparagine residue [11]. The reaction is commonly performed in-solution, overnight at 37 °C. Efforts have been taken to accelerate this enzymatic reaction by microwave irradiation [12], ultrahigh pressure [13] or immobilized PNGase F in microfluidics format [14]. Enzyme immobilization has many advantages compared to in-solution based reactions such as long-term operational stability, rapid reaction speed, no enzyme contamination after the reaction and the option of repetitive usage [15]. Pioneering works in the early 1970s successfully utilized immobilized trypsin for rapid protein digestion [16]. Since then, enzyme immobilization techniques have been rapidly emerging and used in various fields from research to industrial applications [17].

Glutathione-*S*-transferase (GST) tag is widely used in various protein purification and enrichment protocols [18]. GST tagged proteins can be captured at pH 7 by glutathione functionalized microbeads. In this study, PNGase F cloned from *Flavobacterium meningosepticum* and expressed as a Glutathion-*S*-Transferase (GST) fusion protein was employed. The GST tagged PNGase F enzyme was immobilized using glutathione affinity microcolumns to support rapid *N*-glycosylation digestion. The released glycans were labeled via reductive amination using 8-aminopyrene-1,3,6-trisulfonic acid (APTS) and analyzed by capillary gel electrophoresis with laser induced fluorescent detection (CE-LIF).

2. Materials and methods

2.1. Chemicals and reagents

Water (HPLC grade), acetonitrile, immunoglobulin G (IgG), RNase B, fetuin, acetic acid (glacial), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), sodium-cyanoborohydride (1 M in THF), lithium acetate, hydrochloric acid and Nonidet P-40 (NP-40) were obtained from Sigma Aldrich (St. Louis, MO, USA). The 8-aminopyrene-1,3,6-trisulfonic acid (APTS) and the carbohydrate separation buffer (NCHO) were from SCIEX (Brea, CA, USA). The Agencourt CleanSEQ magnetic beads were from Beckman Coulter (Indianapolis, IN, USA). Glutathione-S-transferase (GST) tagged PNGase F enzyme (fusion protein in *Escherichia coli*, EC 3.2.218; 3.5.1.52) was from UD-GenoMed Ltd. (Debrecen, Hungary).

2.2. Immobilization of GST tagged PNGase F

For the immobilization process, 1 mL size glutathione affinity resin containing microcolumns were used with 10 μL bed volume (PhyNexus, San Jose, CA, USA). Pipetting was done automatically using *1000+ pipette heads* with the controller software (Capture – Purify – Enrich, Version 2.2.3, PhyNexus). First, the storage solution was removed by applying 0.06 mL min^{-1} flow rate. Then, the bed was washed with 1 mL of 20 mM Tris-HCl buffer (pH 7.3) at room temperature using 1 mL full aspiration and dispense at 0.06 mL min^{-1} flow rate once, and three times at 0.50 mL min^{-1}. After washing the microcolumns, 1.0 μL of GST PNGase F (16 IUB mU/mL) enzyme was mixed with 1 mL of 20 mM Tris-HCl buffer (pH 7.3) and aspirated/dispensed for 3 h using 0.06 mL min^{-1} flow rate at 4 °C for compete binding. The microcolumns were stored at 4 °C filled with fresh 20 mM Tris-HCl buffer (pH 7.3).

2.3. Glycan release and derivatization

Sample preparation was accomplished by using 10 μL of 10 mg/mL standard glycoprotein solution (IgG, fetuin, RNase B) in HPLC grade water, denatured by the addition of 1 μL of 5% SDS and 1 μL of 50 mM of DTT at 65 °C for 10 min. After the denaturation step, 2.5 μL of 10% NP-40 and 85.5 μL of 20 mM Tris-HCl buffer (pH 7.3) were added resulting in 100 μL of total sample volume. The enzymatic digestion was performed using 10 μL bed volume, 1 mL size GST-PNGase F immobilized glutathione microcolumns at 50 °C with 600 μL aspiration/dispense volume at 5 mL min^{-1} flow rate with 15 s pauses between steps. After the enzymatic digestion, 900 μL of acetonitrile (100%) was added to the sample resulting 90.0% final acetonitrile concentration, 200 μL of Agencourt CleanSEQ beads was added (storage solution removed) and vortexed at 2500 rpm for 10 s for carbohydrate partitioning. After 1 min wait, the beads were pulled to the side of the vials by a magnet, and the supernatant was removed. The samples were then labeled on the beads by adding 9 μL of APTS (40 mM in 20% acetic acid) and 1 μL of $NaBH_3CN$ (1 M in THF) followed by incubation at 50 °C for 1 h. After the labeling reaction, the reaction was stopped by the addition of 100 μL of water and the sample was vortexed at 2500 rpm for 10 s. Then, the beads were pulled to the side of the vials by a magnet, and a supernatant was transferred to a new vial. Next, 900 μL of acetonitrile was added and the samples and aspirated/dispensed 25 times at 5 mL min^{-1} speed using PhyNexus Normal Phase microcolumns. The labeled glycans were then eluted by 20 μL of water and analyzed by CGE-LIF. Non-immobilized GST-tagged enzyme was used in the control in-solution enzymatic digestion reaction under identical conditions and reaction volumes.

2.4. Capillary gel electrophoresis

Capillary gel electrophoresis with laser induced fluorescence detection (CGE-LIF) was performed on a PA800 Plus Pharmaceutical Analysis System (SCIEX). For rapid sample analysis, 20 cm effective length NCHO capillary (30 cm total length, 50 μm ID) (SCIEX) was filled with the separation gel buffer of 1% PEO (MW 900 kDa) in 25 mM lithium acetate buffer (pH 4.75). The separation voltage was 15 kV in reversed polarity mode (cathode at the injection side, anode at the detection side) resulting in 500 V/cm applied electric field strength. The samples were pressure injected by 1 psi (6.89 kPa) for 5 s. The 32 Karat (version 9.1) software package (SCIEX) was used for data acquisition and analysis.

3. Results and discussion

In this study, a rapid N-deglycosylation method is introduced using immobilized enzyme microcolumn technology. Carbohydrate release from standard glycoproteins of immunoglobulin G, fetuin and ribonuclease B were accomplished in 10 min by means of recombinant GST tagged PNGase F immobilized on glutathione affinity microcolumns.

First the effect of digestion time was evaluated both for on-column and in-solution reaction between 5 and 20 min in 5 min intervals. Fetuin was used as standard glycoprotein in these experiments. The cumulative RFU values of the four major fetuin glycan peaks (F1–F4) are shown in Fig. 1 as the function of the digestion time for both reaction conditions. The results show somewhat different release characteristics for the two digestion techniques. With the use of on-column digestion method, a steeper increase of deglycosylation was obtained reaching approximately 70% of the maximum *N*-glycan release (overnight digestion result) in 10 min, while the same level was reached only in 20 min by in-solution digestion. Please note that peak distribution (black and gray zones representing F1–F4 peaks) remained the same during the time course.

Next, glycoprotein samples representing the three major *N*-glycosylation subclasses: immunoglobulin G, which contains mostly complex neutral biantennary and a few sialylated glycans; fetuin, with mostly highly sialylated structures; and RNase B possessing high mannose type oligosaccharides, were evaluated to understand possible structural release bias. The released and APTS

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