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Multidimensional system enabling deglycosylation of proteins using a capillary reactor with peptide-N-glycosidase F immobilized on a porous polymer monolith and hydrophilic interaction liquid chromatography–mass spectrometry of glycans

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ABSTRACT

A reactor with immobilized peptide-N-glycosidase F on a monolithic polymer support in a capillary has been developed that allows fast and efficient release of N-linked glycans from immunoglobulin G molecules. Two different monolithic scaffolds based on poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) and poly(butyl methacrylate-*co*-ethylene dimethacrylate) were prepared. A multistep photografting process was used to reduce non-specific adsorption of proteins and to obtain support containing reactive azlactone functionalities enabling the preparation of highly active immobilized peptide-N-glycosidase F. Performance of these reactors was determined through glycan release from several glycoproteins including ribonuclease B, chicken albumin, and human immunoglobulin G and their detection by matrix-assisted laser desorption-ionization/time-of-flight mass spectrometry. The optimized reactor was integrated into a multidimensional system comprising on-line glycan release and their separation via hydrophilic interaction liquid chromatography followed by electrospray ionization/time-of-flight mass spectrometry detection. Using the optimized monolithic reactor with immobilized peptide-N-glycosidase F, human immunoglobulin G was deglycosylated at room temperature in 5.5 min to an extent similar to that achieved with soluble enzyme after 24 h at 37 °C.

1. Introduction

Glycosylation is one of the most common posttranslational modifications of proteins in eukaryotic cells. Glycoproteins are involved in a wide range of biological functions such as receptor binding, cell signaling, immune recognition, inflammation, and pathogenicity [1]. These unique properties of glycoproteins have been recognized by the pharmaceutical industry and have led to production of new recombinant glycoprotein therapeutics used for treatment of various diseases. Currently, more than one-third of all approved biopharmaceuticals such as erythropoietin, blood factors, some interferons and hormones are glycoproteins [2] with their function depending on a specific glycoform. During the last decade, a number of monoclonal antibodies, most often immunoglobulins G (IgG), and their fragments were also developed as potential therapeutic targets [3,4]. Glycosylation of an expressed recombinant protein is affected by factors such as host cell type and culture conditions [5]. It is well known that the changes in glycosylation can affect the physicochemical, pharmacokinetic, and biological properties of therapeutic glycoproteins. Hence, it is important to have analytical tools enabling identification and characterization of glycosylation during the production and purification processes in order to manufacture well-characterized therapeutic targets.

The variable composition, linkage, branching points, configuration of constituent monosaccharides, and the diversity in degree of glycosylation at different sites in glycoproteins are the main reasons resulting in complexity of the current analytical approaches to their characterization. The glycan mapping can be carried out with glycans released from the protein or with glycopeptides generated by enzymatic digestion. Typically, the former approach includes generation of glycans using enzymatic or chemical reaction followed by matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS). Alternatively, released glycans are first derivatized and then separated using reversed-phase liquid chromatography [6] or capillary electrophoresis [7–9] and mass analyzed by mass spectrometry (MS) [10,11]. For example, RPLC involves attachment of a hydrophobic chromophore or fluorophore to the reducing end of the glycan to change its polarity and achieve good separation.

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A modification is also required to increase sensitivity of both UV and fluorescent detection. In addition to RP, hydrophilic interaction liquid chromatography (HILIC) [12] emerged recently as one of the suitable methods used for the separation of glycopeptides [13,14] and neutral glycans [9].

An efficient deglycosylation process is a key requirement for a successful and sensitive glycan analysis. Peptide-N-glycosidase F (PNGase F) is a member of the family of enzymes widely used for the deglycosylation of N-linked glycans. This enzyme releases asparagines (Asn)-linked glycans from glycoproteins and glycopeptides by hydrolyzing the amide bond at the Asn side chain [15]. The released oligosaccharides that can be of high mannose, hybrid, or complex type remain intact and therefore suitable for detailed analysis by mass spectrometry. Although the deglycosylation is usually performed in solution or in gel using soluble PNGase F, immobilization of this glycolytic enzyme can significantly improve the glycan release and identification of protein glycosylation. To our best knowledge, only a single report published by Palm and Novotny described the preparation of immobilized PNGase F in an acrylamide-based monolithic support [16]. Their interesting single-step fabrication process includes polymerization of a mixture comprising acrylamide, N,N′-methylenebisacrylamide, N-acryloxysuccinimide dissolved in a buffer solution together with poly(ethylene glycol), N,N,N′,N′-tetramethylethylenediamine/ammonium peroxodisulfate redox initiator, and PNGase F. This reactor deglycosylated small and mid size proteins. Although the immobilization can proceed via the reaction of nucleophilic functionalities of the enzyme with succinimide moieties, it is likely that some enzyme molecules are also entrapped within the acrylamide matrix. Those molecules are not accessible for large proteins and do not contribute to the desired deglycosylation reaction.

Recently we have developed a multi-step method for immobilization of proteolytic enzymes such as trypsin and endoproteinase LysC using modified poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith as a support [17]. Since the methacrylate-based support itself appeared to be hydrophobic, its surface was first photografted with a highly hydrophilic monomer to reduce the non-specific adsorption followed by activation of the monolith with the photografting of a reactive monomer. This approach enabled rapid and efficient digestion of even large proteins such as immunoglobulins.

This report extends our previous multistep concept and describes the preparation of two monolithic structures differing in chemistry, their activation, and use for fabrication of a monolithic reactor in capillary format with immobilized PNGase F. Performance of these reactors is demonstrated with the release of glycans from model glycoproteins including ribonuclease B (MW ~15 kDa), chicken egg white albumin (MW ~44.3 kDa), and human IgG (MW ~150 kDa) and their detection by MALDI-MS using an integrated multidimensional system comprising on-line release of glycans, their separation in HILIC mode, and electrospray inozation mass spectrometry (ESI-MS) detection.

2. Experimental

2.1. Chemicals and materials

Glycidyl methacrylate (GMA), butyl methacrylate (BMA), lauryl methacrylate (LMA), ethylene dimethacrylate (EDMA), 1-dodecanol, cyclohexanol, 1-propanol, 1,4-butanediol, 2,2′-azobisisobutyronitrile (AIBN), benzophenone, 3-(trimethoxysilyl) propyl methacrylate, ethanolamine, formic acid, acetonitrile (LC–MS grade) and water (LC–MS grade) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethoxylated hydroxyethyl methacrylate (PEGMA, average number-average molecular mass (M_n) 570 g/mol, 11 ethylene glycol units) was purchased from Sartomer (Exton, PA, USA) and 2-vinyl-4,4-dimethylazlactone (vinyl azlactone) was a generous gift from the 3 M Co. (St. Paul, MN, USA). GMA and EDMA were purified by distillation under reduced pressure to remove inhibitors. PEGMA, LMA and BMA were purified by passing them through a column containing basic alumina inhibitor remover (Sigma–Aldrich). Super-dihydroxybenzoic acid (sDHB) and sinapinic acid (SA) were purchased from Fluka (Buchs, Switzerland).

PNGase F from *Elizabethkingia meningoseptica*, ribonuclease B from bovine pancreas (RNase B), albumin from chicken egg white (ovalbumin), and polyclonal human immunoglobulin G (hIgG) were obtained from Sigma–Aldrich.

Both polyimide-coated and PTFE-coated UV-transparent fused-silica 100 μm I.D. capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). The PolyGlycoplex 100 mm × 150 μm I.D. HILIC capillary column was purchased from PolyLC (Columbia, MD, USA).

2.2. Instrumentation

A nanoAcquity UPLC system (Waters, Milford, MA, USA) was used for the separations of released glycans. A Micromass LCT time-of-flight mass spectrometer (Manchester, UK) equipped with a PicoView nanospray source (New Objective, Woburn, MA, USA) was used in all experiments. All ESI time-of-flight (TOF) MS measurements were carried out in positive ion mode.

MALDI–TOF–MS measurements were carried out using a 4800 MALDI–TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). Samples were applied using the "dried-droplet" technique. Super-dihydroxybenzoic acid and sinapinic acid were used as matrices. A 10 mg/mL sDHB solution was prepared in water and used for analysis of released glycans in the positive reflectron ion mode. A 10 mg/mL SA solution was prepared in 0.1% trifluoroacetic acid solution in 1:1 acetonitrile and protein and glycoprotein were analyzed in the positive linear ion mode.

Scanning electron micrographs were obtained using the Zeiss Gemini Ultra-55 analytical scanning electron microscopy (SEM) instrument (Carl Zeiss, Oberkochen, Germany). The samples were sputtered with gold using the SCD 050 sputter coater (BAL-TEC AG, Balzers, Liechtenstein).

2.3. Vinylization of capillaries

The inner surface of UV-transparent fused-silica capillaries was first activated with 3-(trimethoxysilyl)propyl methacrylate. The capillary was quickly rinsed with acetone and water, then flushed with 0.2 mol/L sodium hydroxide solution for 30 min at a flow rate of 0.25 μL/min using a syringe pump (KdScientific, Holliston, MA, USA), and quickly rinsed with water. Next, 0.2 mol/L hydrochloric acid was pumped through the capillary for 30 min at a flow rate of 0.25 μL/min, followed by water and ethanol. A 20% w/w solution of 3-(trimethoxysilyl)propyl methacrylate prepared in 95% ethanol with an apparent pH adjusted to 5 using acetic acid was pumped through the capillary at a flow rate of 0.25 μL/min for 90 min. The capillary was then washed with acetone, dried in a stream of nitrogen, and left at room temperature for 24 h.

2.4. Preparation of monolithic poly(butyl methacrylate-co-ethylene dimethacrylate) support

The vinylized capillary was filled with a mixture containing 24% BMA, 16% EDMA, 34% 1-propanol, 26% 1,4-butanediol and 1% AIBN (with respect to monomers) (all percentages w/w) previously purged with nitrogen for 10 min. The thermally initiated