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Oriented immobilization of peptide-N-glycosidase F on a monolithic support for glycosylation analysis

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ABSTRACT

In this paper, we report on a novel oriented peptide-N-glycosidase F (PNGase F) immobilization approach onto methacrylate based monolithic support for rapid, reproducible and efficient release of the N-linked carbohydrate moieties from glycoproteins. The glutathione-S-transferase-fusion PNGase F (PNGase F-GST) was expressed in *Escherichia coli* using regular vector technology. The monolithic pore surface was functionalized with glutathione via a succinimidyl-6-(iodoacetyl-amino)-hexanoate linker and the specific affinity of GST toward glutathione was utilized for the oriented coupling. This novel immobilization procedure was compared with reductive amination technique commonly used for non-oriented enzyme immobilization via primary amine functionalities. Both coupling approaches were compared using enzymatic treatment of several glycoproteins, such as ribonuclease B, fetuin and immunoglobulin G followed by MALDI/MS and CE-LIF analysis of the released glycans. Orientedly immobilized PNGase F via GST-glutathione coupling showed significantly higher activity, remained stable for several months, and allowed rapid release of various types of glycans (high-mannose, core fucosylated, sialylated, etc.) from glycoproteins. Complete protein deglycosylation was obtained as fast as in several seconds when using flow-through immobilized microreactors.

1. Introduction

Microreactors are becoming important tools in many research and development areas ranging from organic synthesis [1] to diesel fuel production [2,3]. Immobilized enzyme reactors are under rapid recent development, especially for bioanalytical purposes related to the omics fields. Besides the most common immobilized trypsin reactors [4,5] other enzymes, including, e.g., the endoproteinase LysC have been also successfully used [6]. While many different forms of solid support can be used for enzyme immobilization, including chromatographic particles [7], self-assembled magnetic beads [8] or open fused silica capillary surfaces [9], porous monoliths represent a promising choice due to their excellent mechanical and chemical properties, which can be easily fine-tuned for a plethora of special applications [10].

Enzymatic release of glycans from glycoproteins represents a key step in analytical glycomics. Peptide-N-glycosidase F (PNGase F) is one of the most frequently used endoglycosidases utilized to release N-linked glycans. The common in-solution deglycosylation method is a relatively time-consuming process requiring several hours up to overnight for complete removal of all N-linked glycans. While it has been shown that the deglycosylation time can be reduced to minutes by microwave irradiation [11,12] or pressure-cycling [13], the simplest way to speed up the reaction is increasing the enzyme to substrate ratio by immobilization of PNGase F on a solid support. Both particulate and monolithic supports were used for PNGase F immobilization via non-specific coupling chemistries including CNBr-activated Sepharose 4B [14], glycidoxypropyltrimethoxysilane modified silica beads [15], carboxylated nanodiamonds [16], or acrylic polymer particles containing amine functionalities [17]. Solid supports with immobilized PNGase F can be suspended in the glycoprotein solution [16], packed into a column [14], capillary [17] or microfluidic chip [15] in order to create flow-through reactors. Monolithic PNGase F reactors prepared in capillary format were reported

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earlier [18–20] as promising alternatives to packed column reactors. Palm and Novotny immobilized PNGase F on the surface of polyacrylamide based monoliths via succinimide functionalities [18]. Immobilization of PNGase F on monolithic support via azlactone chemistry [19] or reductive amination [20] was just recently published.

Currently, all coupling methods employ non-specific reactions between the primary amines of PNGase F with reactive functionalities generated on a solid support surface. Since amino groups are also present at the active site of the enzyme molecule, the resulting non-oriented immobilization may negatively affect accessibility of the active site leading to reduced activity of the immobilized enzyme. It can be expected that enzyme immobilization through a selected functional group instead of randomly reacting amines will result in predictable, and therefore better performing microreactors.

With the use of recombinant DNA technology a wide variety of proteins, including enzymes, can be produced with specific sequences allowing site selective binding. Such fusion systems were developed for purification of recombinant proteins from cell lysates through affinity capture. While the tags are usually removed from the protein molecules before further use, they offer a special advantage as specific moieties for oriented immobilization. In particular, a discrete modification using a highly specific tag attached to the enzyme molecule during its synthesis can be employed for direct coupling reactions away from the active center of the enzyme molecule.

In this work, we report on a novel oriented PNGase F immobilization method onto a methacrylate based monolithic support prepared in a 250 μm ID fused silica capillary for rapid and efficient N-glycan removal from glycoproteins. Our approach is based on the affinity of glutathione (GSH) immobilized on the monolithic surface toward glutathione-S-transferase fusion PNGase F (PNGase F-GST). The developed oriented immobilization was compared with the non-oriented approach in enzymatic treatments of several standard glycoproteins including ribonuclease B, fetuin and immunoglobulin G, followed by off-line MALDI/MS and CE-LIF analysis of released glycans.

2. Experimental

2.1. Materials and reagents

Glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), 1-dodecanol, cyclohexanol, 2,2′-azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl)propyl methacrylate, sodium periodate, sodium cyanoborohydride, chloroform, iodomethane, dithiotreitol, and iodoacetamide were purchased from Sigma–Aldrich (Prague, Czech Republic). GMA and EDMA were purified by passing them through a column containing basic alumina inhibitor remover (Sigma–Aldrich). Succinimidyl-6-[(iodoacetyl)amino]hexanoate was purchased from Chem-Impex International (Wood Dale, IL). 8-Aminopyrene-1,3,6-trisulphonic acid (APTS) was purchased from Beckman Coulter (Brea, CA).

PNGase F-GST was expressed in *Escherichia coli* using regular vector technology and purified by affinity chromatography [21,22]. The expression of PNGase F-GST was confirmed by SDS-PAGE of cell lysates followed by Coomassie Brilliant Blue R-250 staining.

Polyclonal human immunoglobulin G (hIgG), bovine ribonuclease B (RNase B), bovine fetuin, PNGase F, glutathione, super-dihydroxybenzoic acid (9:1 mixture of dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) and sinapinic acid were purchased from Sigma–Aldrich.

Polyimide-coated fused-silica capillary (250 μm ID, 375 μm OD) was purchased from Polymicro Technologies (Phoenix, AZ).

2.2. Instrumentation

MALDI/MS measurements were carried out using an AB SCIEX TOF/TOF 5800 system (Framingham, MA, USA). CE-LIF separations were performed on a P/ACE MDQ instrument (Beckman Coulter) equipped with an Ar-ion laser (488 nm excitation wavelength) for fluorescent detection.

The elemental composition of monolithic columns was determined by scanning electron microscopy with energy dispersive X-ray analysis (SEM/EDAX) on a JEOL JSM-5500 LV instrument equipped with an analyzer IXRF Systems and a detector Gresham Sirius 10.

2.3. Preparation of monolithic poly(glycidyl methacrylate-co-ethylene dimethacrylate) support

The inner surface of the fused silica capillary was first activated with 3-(trimethoxysilyl)propyl methacrylate [23]. The polymerization mixture consisting of 25.5% GMA, 17.5% EDMA, 40% 1-dodecanol, 17% cyclohexanol, and 1% AIBN (with respect to monomers) (all percentages w/w) was purged with nitrogen for 10 min. The vinylized capillary was filled with this mixture and thermally initiated polymerization was carried out in a water bath at 60 °C for 24 h. After polymerization, the porogenic solvents were removed from the monolith by pumping acetonitrile through the column at a flow rate of 60 μL/h for 1 h.

2.4. Oriented immobilization of PNGase F-GST

The poly(GMA-co-EDMA) monolithic column was filled with a 30% (v/v) solution of ammonium hydroxide, sealed with silicone septa and heated in a column oven to 70 °C for 3 h. The modified column was washed with water (150 μL/h, 1 h) and dimethyl sulfoxide (DMSO) (150 μL/h, 15 min). The resulting primary amine functionalized monolithic column was flushed with a solution of succinimidyl-6-[(iodoacetyl)amino]hexanoate in DMSO (10 mg/mL) at a flow rate of 150 μL/h for 3 h in dark at room temperature. Next, a solution of glutathione (10 mg/mL) in 20 mmol/L Tris–HCl buffer, pH 8.3 was pumped through the iodoacetate functionalized column at a flow rate of 150 μL/h for 3 h in dark at room temperature. The scheme of the activation procedure is in Fig. 1. The glutathione modified column was washed with the 20 mmol/L Tris–HCl buffer, pH 8.3. PNGase F-GST (1 mg/mL) was dissolved in 10 mmol/L phosphate buffered saline (PBS). The enzyme solution was pumped through the glutathione modified monolithic column at room temperature for 2.5 h with a flow rate of 100 μL/h. The PNGase F-GST conjugated monolith was then washed with 10 mmol/L PBS (100 μL/h, 1 h) and stored at 4 °C before further use.

2.5. Non-oriented immobilization of PNGase F-GST

The poly(GMA-co-EDMA) monolithic column was filled with 0.5 mol/L sulfuric acid, sealed with silicone septa and heated in a column oven to 70 °C for 3 h. The monolithic column was washed with water (150 μL/h, 1 h) followed by rinsing with a freshly prepared solution of 0.1 mol/L sodium periodate (150 μL/h, 1.5 h). PNGase F-GST (1 mg/mL) was dissolved in 10 mmol/L PBS, containing 3 mg/mL sodium cyanoborohydride. The enzyme solution was pumped through the aldehyde functionality modified monolith at room temperature for 2.5 h with a flow rate of 100 μL/h. The monolith conjugated with PNGase F-GST was then washed with 10 mmol/L PBS (100 μL/h, 1 h) and stored at 4 °C before further use.