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Endo-β-*N*-acetylglucosaminidase H de-N-glycosylation in a domestic microwave oven: Application to biomarker discovery

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ABSTRACT

Sample preparation is the rate-limiting step in glycan analysis workflows. Among all of the steps, enzymatic digestions, which are usually performed overnight, are the most time-consuming. In the current study, we report an economical and fast preparation of *N*-glycans from serum, including microwave-assisted enzymatic digestion in the absence of denaturing chemicals and solvents during the release. To this end, we used a household microwave oven to accelerate both pronase and endo-β-*N*-acetylglucosaminidase H (Endo H) digestions. Purification was then performed using self-made SP20SS and carbon tips. We were able to prepare samples in 55 min instead of 21 h. Finally, the method was applied in the context of oncological biomarker discovery exemplarily to ovarian and colon cancer. We observed a significant downregulation of sialylated hybrid structures in ovarian cancer samples using capillary electrophoresis–laser-induced fluorescence (CE–LIF). Furthermore, sepsis, a systemic inflammatory response syndrome, was also included in the study to understand whether the changes observed in ovarian cancer patients were due to the cancer itself or to the inflammation that usually accompanies its development. Because sialylated hybrid structures were upregulated in sepsis samples, the downregulation of these structures in ovarian cancer is specific to the cancer itself and, therefore, could be used as a biomarker.

Glycosylation is a frequent posttranslational modification occurring in eukaryotic proteins [1]. Glycans not only are responsible for stability or protection toward proteolytic digestions but also are strategic players in many biological and cellular processes such as signaling [2] and immune reactions [3]. Changes of protein glycosylation have been described in many diseases, including inflammation [4] and cancer [5]. Therefore, glycan modulations are being investigated as potential disease markers in body fluids. Blood is a complex material to analyze and requires the adaptation of standard protocols that are usually established with pure glycoproteins that are free of salts and non-glycoprotein substances.

Efficient de-N-glycosylation is the key to successful and accurate glycan characterization. *N*-Glycan release is usually performed enzymatically because enzymes are easy to handle in any laboratory and enzymatic release of *N*-glycans avoids handling harsh flammable solvents that are used for example during hydrazinolysis. Endo-β-*N*-acetylglucosaminidase H (Endo H,[1] EC 3.2.1.96), cleaves high-mannose and hybrid-type *N*-glycans between the first two GlcNAc residues at the reducing end.

Use of proteases and/or denaturing reagents is classically made prior to enzymatic *N*-glycan release to maximize the accessibility of the active site of the enzyme to the cleavage sites of their target glycoproteins. Over past years, fast and sensitive mass spectrometers, chromatographic and electrophoretic systems have been developed, rendering sample preparation to be the major limiting factor to high throughput. Innovative workflows, therefore, are being developed to prepare maximum samples in minimum time. Online tryptic digestion prior to liquid chromatography–mass spectrometry (LC–MS) as well as on-column peptide-N^4-

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[1] *Abbreviations used:* Endo H, endo-β-*N*-acetylglucosaminidase H; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; MAED, microwave-assisted enzymatic digestion; TFA, trifluoroacetic acid; ACN, acetonitrile; RNase B, ribonuclease B; DTE, dithioerythritol; IAA, iodoacetamide; APTS, 8-aminopyrene-1,3,6-trisulfonic acid; THF, tetrahydrofuran; CE, capillary electrophoresis; LIF, laser-induced fluorescence; BGE, background electrolyte; SD, standard deviation.

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(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F) digestion were reported by several groups [6–10], but these systems were tested only with purified standard glycoproteins and not with more complex samples such as serum and plasma.

Another approach is through accelerating glycoprotein de-N-glycosylation by replacing the heating source by microwave irradiation. Although microwave-assisted enzymatic digestions (MAEDs) are nowadays used for peptide mapping and peptide deglycosylation [10], only few reports have been published on the resulting glycan products. Glycopeptides were generated by pronase digestion using a domestic microwave oven, reducing the time to prepare samples from 2 or 3 days to 2 min [9]. A few groups successfully released and investigated *N*-glycans with PNGase F using microwave reactors, whereby microwave reactors are not adapted to heating several samples of small volumes simultaneously [7]. Therefore, we chose a domestic microwave oven because it is possible to have it in any laboratory and it offered the possibility to prepare several samples at a time. We report for the first time the use of Endo H digestions carried out in a microwave oven in a fast and economical protocol that allows the preparation of *N*-glycans from standard glycoproteins or human serum within a single day. Aiming at oncological biomarker discovery, we used this method to analyze the *N*-glycome of serum from ovarian and colon cancer patients. In addition, we wanted to rule out whether the observed differences stem from the cancer itself or from the inflammation that usually accompanies its progression. Therefore, we also included sepsis, a systemic inflammatory response syndrome, into this study.

Materials and methods

Materials

Endo H, expressed in *Escherichia coli*, was obtained from Roche Applied Science (Indianapolis, IN, USA). Chloroform, sodium hydroxide, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany), and acetonitrile (ACN) was purchased from VWR (Darmstadt, Germany). MAEDs were conducted in a domestic microwave oven (Severin 700). All of the other chemicals were bought from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise.

Serum samples

Blood samples from 10 healthy individuals were collected at the Charité Medical University (Berlin, Germany). Serum samples from patients with documented severe sepsis ($n = 10$), ovarian cancer ($n = 10$), and colon cancer ($n = 10$) were obtained from the Institute of Laboratory Medicine and from the Department of Gynecology, in agreement with the ethical rules of the Charité Medical University (approvals EA4/073/06 and EA1/285/09).

Venous blood samples from patients and healthy controls were taken in the morning after 12 h of fasting. Samples were left to sediment for 30 min to 1 h, and serum was isolated by centrifugation at 20 °C for 12 min at 1200*g*, aliquoted, and stored at −80 °C until the time of analysis.

Standard enzymatic digestions and sample cleanup

Ribonuclease B (RNase B, 50 μg) or human serum (10 μl) was dissolved in 25 μl of 50 mM phosphate buffer (pH 5.5). Reduction occurred at 60 °C for 45 min with 2.5 μl of 200 mM dithioerythritol (DTE), and carbamidomethylation was achieved with 10 μl of 200 mM iodoacetamide (IAA) in the dark by room temperature for 1 h. Subsequently, 2.5 μl of 200 mM DTE was added to inhibit the excess IAA. The mixture was diluted with 300 μl of water and 100 μl of phosphate buffer (pH 5.5). Glycans were released overnight at 37 °C using 20 mU of Endo H.

Released glycans were isolated using C18 Sep-Pak cartridges (Alltech, Deerfield, IL, USA). Cartridges were conditioned with 3 × 400 μl of 80% ACN containing 0.1% TFA solution and then equilibrated with 3 × 400 μl of 0.1% aqueous TFA. Samples, dissolved in 0.1% aqueous TFA, were applied to the cartridges. *N*-Glycans, collected in the flow-through, were desalted using Carbograph Extract-Clean columns (Alltech). Columns were preequilibrated with 3 × 400 μl of 80% ACN containing 0.1% TFA, followed by 3 × 400 μl of 0.1% aqueous TFA. After applying the samples, columns were washed with 3 × 400 μl of 0.1% aqueous TFA. Desalted *N*-glycans were subsequently eluted with 3 × 400 μl of 25% ACN containing 0.1% TFA. Finally, samples were evaporated to dryness.

MAED and sample cleanup

RNase B (50 μg) and human serum (10 μl) were dissolved in 50 mM phosphate buffer (pH 5.5) so that all samples had a volume of 50 μl prior to digestions. Then, 1 μl of pronase (1 μg for RNase B, 10 μg for human serum) was added to the samples, which were subjected to 700 W microwave irradiation for 5 min. The rest of the pronase activity was inhibited by incubation at 99 °C for 5 min. Finally, Endo H (20 mU) was added to the samples and exposed to 700 W microwave irradiation for 5 min.

Purification was achieved in self-made microcolumns using a table centrifuge. Briefly, 10-μl pipette tips equipped with a filter at the bottom were filled with either 50 μl of SP20SS hydrophobic resin (Supelco, Bellefonte, PA, USA) or 10 mg of graphite powder (Grace, Deerfield, IL, USA). SP20SS microcolumns were equilibrated with 3 × 30 μl of 0.1% aqueous TFA. Samples were applied and *N*-glycans, which did not bind to the resin, were eluted with 3 × 30 μl of 0.1% aqueous TFA. Proteins remained bound to the column material [8]. After being lyophilized, samples were desalted on self-made graphite microcolumns that were preequilibrated with 3 × 30 μl of 80% ACN/0.1% TFA. After applying the samples, microcolumns were washed with 3 × 30 μl of 0.1% TFA and *N*-glycans were eluted with 3 × 30 μl of 25% ACN/0.1% TFA. Eluates were dried in a vacuum centrifuge.

APTS labeling and CE–LIF

8-Aminopyrene-1,3,6-trisulfonic acid (APTS, 10 mg) was dissolved in 100 μl of 15% acetic acid (buffer A). Labeling buffer consisted of 15 μl buffer A and 15 μl tetrahydrofuran (THF). Subsequently, 5 μl of 1 M $NaCNBH_3$ dissolved in THF and 5 μl of APTS solution were added to the labeling buffer. Dried glycans were resuspended in 4 μl of this labeling solution and incubated overnight at 37 °C in darkness. Samples were finally diluted with 21 μl of Milli-Q water.

APTS-labeled *N*-glycans were separated by capillary electrophoresis–laser-induced fluorescence (CE–LIF) using a P/ACE MDQ device (Beckman Coulter, Fullerton, CA, USA) equipped with LIF detection ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 520 \pm 10$ nm). The separation was achieved with reversed polarity on a polyvinyl alcohol (PVA)-coated capillary (Beckman, 50 μm i.d., 40 cm effective length to window, 50.2 cm total length) using the background electrolyte (BGE) of the ProteomeLab Carbohydrate Labeling and Analysis Kit (Beckman Coulter). Briefly, the BGE consisted of 25 mM acetate buffer (pH 4.75) containing 0.4% polyethylene oxide. The sample, diluted 1:5 in Milli-Q water, was introduced at a pressure of 0.5 psi for 3 s, and the separation was performed at 20 °C at a constant voltage of −30 kV for 20 min. APTS-labeled maltose was used as the internal standard to normalize the detected migration times