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PNGase F Catalyzed De-N-glycosylation in a Domestic Microwave

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Abstract

Common de-N-glycosylation protocols usually require a lengthy incubation time. Although pressure cycling-technology or scientific microwave reactors can accelerate this enzyme reaction, they may not be easily accessible. In this brief report, we employed an alternative strategy using a standard domestic microwave oven to perform the de-N-glycosylation. Model glycoproteins (bovine RNase B, bovine fetuin, and human IgG) and a complex mixture from human plasma were fully deglycosylated in 20 min, without any apparent adverse effects on the glycans or protein backbones. This new method provides a simple and inexpensive solution to achieve rapid de-N-glycosylation.

Keywords

De-N-glycosylation; domestic microwave; PNGase F; glycoprotein; N-glycan

Due to the complexity and heterogeneity of N-glycosylation, glycans typically need to be released from the glycoproteins to characterize their structure or improve the coverage of the proteins' backbone [1]. Rapid and reproducible release of N-glycosylation would enhance the throughput of glycomics and glycoproteomics based studies [2]. Traditional enzymatic release of N-glycosylation using PNGase F requires an incubation time ranging from a few hours to overnight depending on the nature of the samples. Various approaches can accelerate this digestion including a specialized scientific microwave reactor [3], high-pressure cycling-technology [4], and immobilized enzyme onto solid supports [5]. A specialized scientific microwave can de-N-glycosylate monoclonal antibodies in 10 minutes, and model glycoproteins (e.g. bovine Ribonuclease B) in one hour [3]. Although rapid techniques exist, they are not readily accessible because of the large capital investment required for specialized instruments.

A domestic microwave-assisted protocol yielded rapid trypsin digestion on proteins [6]. Herein we demonstrated a similar strategy using a domestic microwave to assist enzymatical de-N-glycosylation in 20 min. We tested various model glycoproteins, and two complex mixtures derived from human plasma samples. We evaluated the N-glycans from the model

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glycoproteins by MALDI-MS to evaluate the stability of glycans after microwave-assisted digestion.

Bovine pancreas Ribonuclease B (RNase B), bovine fetuin, and human IgG from serum were tested (Sigma-Aldrich, St. Louis, MO). Two human plasma samples were obtained from healthy volunteers under an Institutional Review Board approved protocol. Plasma samples were subjected to MARS-7 depletion (Agilent Co. Santa Clara, CA) using the vendor's protocol. The bound fractions (Top-7 abundant fractions) were collected for PNGase F digestion as our model complex sample. PNGase F and associated buffers (glycoprotein denaturing buffer, NP-40, G7 buffer) for conventional de-N-glycosylation in a water bath was performed according to the vendor's protocol (New England Biolab, Ipswich, MA). In brief, dried glycoproteins were dissolved in 45 μ L of glycoprotein denaturing buffer, and heated at 100 $^{\circ}$ C for 10 min. A final volume of 60 μ L was obtained by adding 6 μ L of NP-40, 6 μ L of G7 buffer, 1–2 μ L PNGase F, and water. The reaction was incubated at a 37 $^{\circ}$ C oven overnight. Acidifying the solution to pH 4 with 1% formic acid stopped the reaction.

The samples for the domestic microwave protocol were prepared as described above, except that the samples were alternatively heated in a domestic microwave oven. A LG household microwave oven (Model #: LRM1230W, China) with a maximum output power of 1200w and a frequency of 2.45 GHz was used for irradiation. To avoid overheating of the samples, the reaction vials (1.5 mL Eppendorf tubes) were placed in a plastic holder floating in a glass beaker with 1.5 liters of room temperature tap water. Samples were irradiated for 20 min at 20% of maximum power. After irradiation, the reaction vials were cooled in an ice bath. To determine whether additional irradiation time beyond 20 min was needed, we repeated 20 min irradiations and replaced the water bath each time. The exact amount of transferred energy by the microwave was not measured, but the final temperature of the water bath was approximately 70 $^{\circ}$ C after irradiation.

Since previous work has demonstrated that low power microwave irradiation does not affect the protein backbone [3; 6], the simplest method to measure the effectiveness of de-N-glycosylation was one-dimensional gel electrophoresis (SDS-PAGE) of samples before and after digestion. The model glycoprotein bovine RNase B is a small glycoprotein (150 amino acids) with a single N-glycosylation site at asparagine-60 that contains a high mannose glycan that accounts for ~2K Da mass per protein [7]. RNase B has a mass of 18K Da when glycosylated (Figure 1A lane 1). Domestic microwave assisted de-N-glycosylation (Figure 1A lanes 2–4) was as effective as the traditional overnight incubation protocol (Figure 1A lane 5). Both protocols demonstrated a shift of RNase B from 18K Da to 16K Da, indicating the loss of a single N-glycan. Irradiation beyond 20 minutes (Figure 1A lanes 3 and 4) did not improve the yield. It is noteworthy that the amount of enzyme used is the minimum suggested by the vendor protocol.

To further test this protocol, bovine fetuin was examined (Figure 1B), because it is an acidic glycoprotein containing three N-glycosylation sites that carry large, complex-type, and multi-antenna glycans with various sialic acid residues [8; 9]. Fetuin has one major band at 56K Da and numerous minor bands, indicating the presence of co-purified proteins (Figure 1B lane 1). Using the 20 min irradiation protocol, we demonstrated effective release (Figure 1B lane 2) as compared to the standard overnight incubation (Figure 1B lane 3). The major fetuin band shifted to 49–50K Da corresponding to a loss of three N-glycans from the fetuin [8]. Interestingly, some of the minor bands (98 and 110 K Da) also shifted after PNGase F treatment implying that these co-purified proteins were also modified by N-glycosylations.

To test if shorter conventional heating times would yield similar results to the microwave, the same aliquots of fetuin were digested using the conventional method for 20 min, 1, 3, and 14 hours (overnight). Incomplete digestion, as evidenced by two bands, was present up to 3 hours. The heavier band (~ 51K Da) represented an incomplete deglycosylation product, which decreased over time. Digestion was complete after overnight heating or domestic microwave protocol (Supplemental Figure).

We also tested human IgG. Each IgG molecule (Figure 1C) contains two identical heavy chains (~50K Da mass) and two light chains (~25K Da mass), which are linked by cystine bridges [10]. Each IgG heavy chain contains a conserved N-glycosylation site at Asn-297 in its Fc domain that is essential for receptor binding and function [10]. To better observe the shift of deglycosylation, IgG was separated into heavy and light chains by reduction and alkylation (Figure 1C lane 1). Using the 20 min irradiation protocol we observed a significant shift of the band representing the IgG heavy chain (Figure 1C lane 2), which corresponds to the loss of a single N-glycan (~ 2K Da). No significant shift was observed on the light chain.

Finally, to evaluate the new protocol on complex samples, we tested the top-7 abundant proteins fractions from two human plasma samples. We did not test crude plasma because the over-abundance of proteins would make it difficult to interpret the effectiveness of the protocol by SDS-PAGE. Of the top-7 proteins, six are known to be glycoproteins (α -1-antitrypsin, haptoglobin, serotransferrin, IgG, IgA and fibrinogen- α -chain). Even though the top-7 proteins make up the majority of the sample, there are a large number of co-eluting proteins (more than 20 reported [11]) making this a complex mixture (Figure 1D lane 1 and 3). Both control lanes showed a similar pattern with three regions of high intensity: 20–30K, 50–60K, and 160K Da. Three of the glycoproteins (α -1-antitrypsin, haptoglobin and serotransferrin) have a mass range of 50–60K Da. After 20 min irradiation, there was a visible shift in the 50–60K Da region (Figure 1D lanes 2 and 4). Furthermore, using a glycoprotein specific stain (Pro-Q Emerald 300 glycoprotein stain, Invitrogen, Grand Island, NY) we noted a dramatic decrease in total glycan signal intensity after deglycosylation (Figure 1E, lanes 1 vs. 2, and 3 vs. 4), particularly at the 20–30K Da and 50–60K Da areas (Figure 1E arrows). Because PNGase F specifically removes N-glycosylation, the residual absorption after de-N-glycosylation may be from O-glycosylations that were not removed by PNGase F. Overall, these experiments demonstrated that a domestic microwave could be used to achieve rapid de-N-glycosylation either from purified glycoprotein, or complex mixtures.

To determine the stability of N-glycans during the microwave irradiation, the glycans, from RNase B, fetuin, and IgG, were collected, separated by Sep-Pak C18 SPE (Waters, Milford, MA) and permethylated using solution phase method [12]. Five high mannose-type N-glycans were observed from RNase B with Man5 the most abundant, and Man7 and Man9 less abundant (Figure 2A). Their peak patterns were similar to those previously reported [13], suggesting that domestic microwave protocol did not bias the analysis.

Because of the labile nature of the sialic acid, we were concerned that microwave irradiation would potentially result in a loss of terminal sialic acid residues. Bovine fetuin has multiple acidic glycans (Figure 2B). In the analysis of the released fetuin glycans, the three most abundant peaks (m/z 3602.7, 3963.8, and 2792.2) were all fully terminated by sialic acids. The two minor peaks observed (m/z 2431.1 and 3227.4) were partially sialylated and had been previously reported to occur naturally [9]. The MALDI profile of fetuin N-glycans indicated that even fragile sialic acid linkages are compatible to microwave irradiation. As for IgG glycan analysis, we observed all the major peaks corresponding to complex-type biantennary N-glycans (Figure 2C). The three most abundant peaks (m/z : 1836.02, 2040.13,

and 2244.23) were the well-known G0, G1, and G2 molecules with a core fucose modification [14]. The rest of the peaks were further modified by sialic acid, or bisecting GlcNAc based on these molecules. Thus, it appeared that the domestic microwave protocol had no adverse effect on the released N-glycans.

In summary, we describe a simple protocol to perform rapid N-glycosylation release from either purified or complex samples using a domestic microwave oven. The benefits of this protocol include: a significantly shorter reaction time (20 min), easy manipulation, decreased capital expense, no negative effects on glycans (e.g. desialylation), and its ability to be readily coupled to other up- or down-stream protocols. We expect that this method can be easily adopted in both academic and industrial laboratories, and may help advance overall glycomics and glycoproteomics research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SDS-PAGE of bovine RNase B (A); bovine fetuin (B); human IgG (C); and MARS-7 bound fractions from two human plasma samples (D & E); before and after PNGase F digestion by domestic microwave irradiation (DMW) or standard method (* indicates overnight incubation in a 37 °C oven). (A) to (D) were stained by coomassie blue stains, and (E) was stained by Pro-Q glycoprotein stain. The band of PNGase F (~36K Da) was visible in figures (A to D).

Figure 2. MALDI-MS spectra of permethylated N-glycans from bovine RNase B (A); bovine fetuin (B); and human IgG (C) after the domestic microwave release protocol. Each peak was assigned a representative composition cartoon based on their m/z value and previous reports.