Pressure-accelerated Native Protein Deglycosylation by PNGase F Enzyme

Introduction

The positive effect of Pressure Cycling Technology (PCT) on digestion with trypsin is well established [1-4], and has been shown to result in improved sequence coverage, higher peptide intensities and significantly reduced digestion times. Additionally, the enhancing effect of PCT on the activity of several other enzymes, including Lys-C, chymotrypsin, Glu-C, thermolysin, Proteinase K, and lysozyme, has been reported [5-11]. Pressure-accelerated deglycosylation by PNGase F of denatured glycoproteins has previously been shown [12].

Pressure, as well as heat and a variety of chemicals, can be used to denature proteins, but the pressure-perturbed proteins assume conformational forms that are different from those caused by thermal or chemical treatments [13]. Pressure-induced denaturation of substrate proteins leads to better access of enzymes to previously inaccessible, or poorly accessible, target sites. This, in turn, results in improved and accelerated enzyme activity, as long as the level of applied pressure is below the level at which the enzyme itself is denatured and inactivated. In addition, under certain conditions, hydrostatic pressure can have a positive effect on enzyme activity itself, independent of substrate conformation.

Here we report that protein deglycosylation of native proteins by PNGase F enzyme is accelerated when the reaction is carried out under pressure cycling conditions. The goal of this work is to provide the user with the best set of *starting conditions* for pressure-enhanced deglycosylation with PNGase F.

PCT Sample Preparation System (PCT SPS)

Pressure Cycling Technology uses hydrostatic pressure cycles to control biomolecular interactions and to accelerate certain enzymatic reactions. The samples in the pressure chamber are exposed to rapid and repeated pressurization from atmosphere (14.7 psi) to levels as high as 45,000psi (45kpsi), or higher in some applications.

The PCT sample preparation system is comprised of a Barocycler instrument, such as the 2320EXT, and high pressure-resistant sample containers such as PCT MicroTubes with MicroCaps. The specially designed PCT MicroTubes are single-use sample processing containers made of a non-reactive Teflon-like material. These tubes are designed to hold 50-150µl of sample. MicroCaps of different lengths are used to displace excess air in MicroTubes. Other sample containers, that are suitable for larger sample volumes up to 1.4ml, are also available. Up to 16 MicroTubes at a time can be pressure cycled in the 2320EXT, to allow batch-mode processing of multiple samples.

Results and Discussion

PNGase F is a commonly used glycosidase that cleaves N-linked high mannose, hybrid, and complex oligosaccharides from glycoproteins. We tested PNGase F activity under pressure cycling conditions using two model glycoproteins, human alpha1 acid glycoprotein (α1AGP), and bovine fetuin. Alpha1 acid glycoprotein is known to contain five N-linked oligosaccharides; partial deglycosylation results in multiple bands on SDS-PAGE.

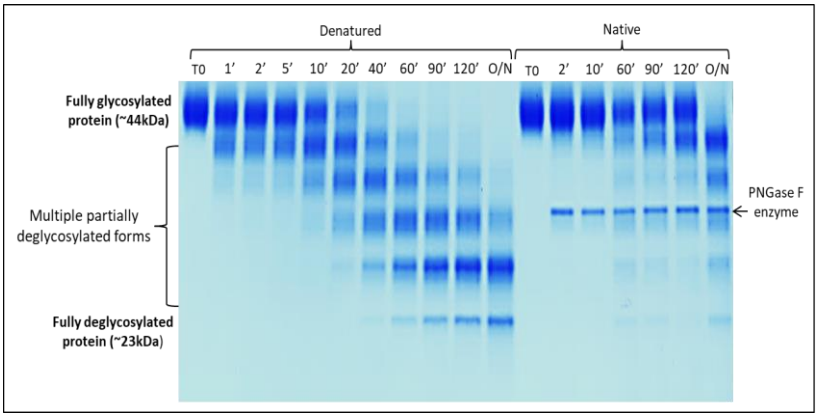

Figure 1. Deglycosylation of native and denatured/reduced α1AGP by PNGase F. Protein was deglycosylated for the indicated time (1 min to overnight) at ambient pressure. Note that enzyme concentration was 170-fold higher in the native protein samples (see strong PNGase F band in those samples) than in denatured/reduced samples. Despite this excess of enzyme, the native protein samples show little of the fully deglycosylated protein, even after overnight incubation, highlighting the difficulty of deglycosylating native protein.

Deglycosylation of native, unreduced protein is a slow process that requires significantly more enzyme compared to deglycosylation of protein that is denatured and reduced [Figure 1]. For this reason, most protocols call for reduction of disulfide bonds and heat denaturation of proteins prior to treatment with PNGase F.

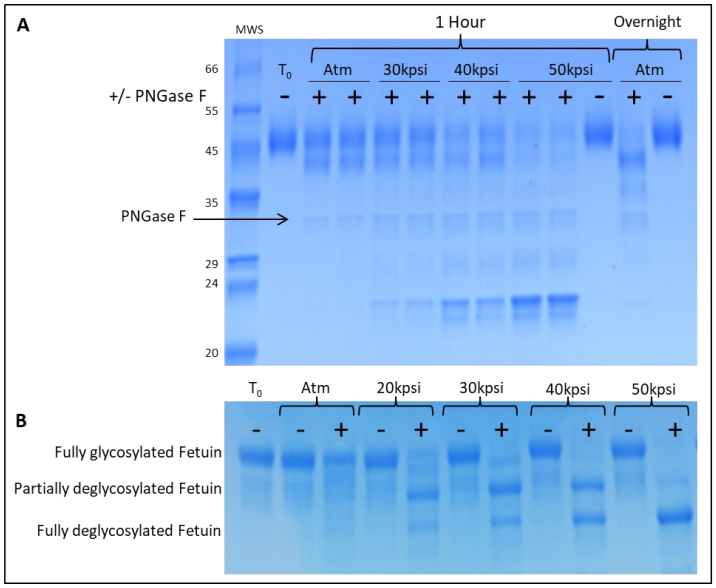

Figure 2. Pressure cycling significantly accelerates deglycosylation of proteins by PNGase F.
A. One hour deglycosylation of α1AGP at ambient pressure (atm) or with pressure cycling at the indicated pressure. Pressure cycling at 40-50kpsi allows almost complete deglycosylation of native (S=S intact) human α1AGP in 1 hour, in contrast to the control reaction at ambient pressure, which is not complete even after overnight incubation. Control reactions (-) were incubated without enzyme. **B**. Thirty minute deglycosylation of native bovine fetuin at the indicated pressures. Pressure cycling-accelerated deglycosylation of fetuin shows a very similar pattern to above. The reaction is accelerated at higher pressures and is almost complete within 30min at 50kpsi. As seen with α1AGP, pressure cycling in the absence of enzyme (-) has no effect on the protein.

However, for many applications, rapid and efficient deglycosylation of native proteins is desirable. Pressure cycling significantly accelerates protein deglycosylation by PNGase F, in a pressure-level dependent manner [Figures 2 and 3]. This enzyme is quite pressure-stable and shows enhanced activity even at pressures above 50,000psi (50kpsi). As seen in Figures 2 and 3, pressure cycling in the absence of enzyme (-) has no effect on protein size on SDS-PAGE, confirming that the size shift of the protein bands is due to accelerated deglycosylation by the enzyme.

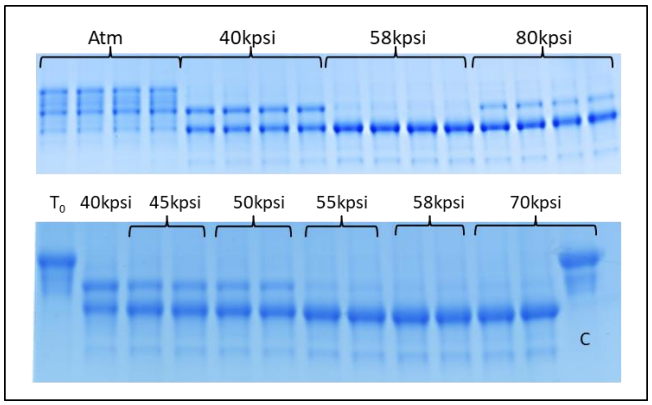

Figure 3. Pressure cycling above 50kpsi further accelerates deglycosylation of proteins by PNGase F.
Deglycosylation of native fetuin at ambient pressure (0kpsi) or with pressure cycling at the indicated pressure (30 cycles). Maximal deglycosylation is observed at 55-70kpsi. Further increasing the pressure to 80kpsi results in less effective deglycosylation, likely as a result of enzyme denaturation. Control (C) fetuin incubated at 70kpsi without enzyme.

Further studies were conducted to determine whether the deglycosylation reaction was more effective when high pressure was held steady (static pressure), or with pressure cycling. During pressure cycling, protein conformation is disrupted by rapid changes in pressure. It has been demonstrated with trypsin that pressure cycling can be more effective than static high pressure for acceleration of the digest reaction, presumably because the rapid pressure changes keep the substrate proteins in a more unfolded and accessible conformation. To test whether this holds true for PNGase F reactions, the effect of static and cycled pressure on deglycosylation of model proteins was examined. The results, shown in Figure 4 indicate that at both 45kpsi and 58kpsi, static pressure is more effective than cycled pressure for acceleration of the PNGase F reaction. Interestingly, at 80kpsi the reverse is observed (not shown). This is likely due to the negative impact of the 80kpsi pressure level on the enzyme (see Figure 3). During static pressure treatment at 80kpsi, the enzyme is held at this pressure and is only poorly active. However, during pressure cycling, part of the time the reaction is at lower pressures, presumably allowing the enzyme to recover some activity.

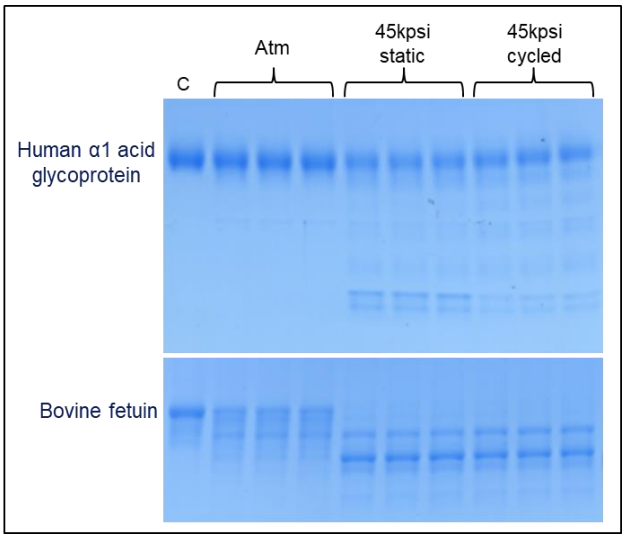

Figure 4. Static pressure is slightly more effective than pressure cycling for deglycosylation of model proteins by PNGase F.
Deglycosylation of disulfide-intact α1 acid glycoprotein (top) and fetuin (bottom) at 45kpsi with steady (static) pressure or pressure cycling (cycled). Both pressure conditions significantly accelerate the reaction relative to incubation at ambient pressure (Atm), but the static pressure condition shows slightly faster generation of the fully deglycosylated form.

Conclusion

Here we examined the benefit of hydrostatic pressure for accelerated deglycosylation with PNGase F. Data generated with two different model proteins (human alpha1 acid glycoprotein and bovine fetuin) agree well, and suggest that the enzyme reaction can be greatly accelerated by performing the reactions under pressure cycling conditions. Pressure cycling for an hour or less, resulted in significantly more complete deglycosylation than when the same reaction was incubated overnight at ambient pressure.

Based on our data, the best starting conditions for PCT-accelerated deglycosylation with this enzyme are summarized in Table 1. These suggested conditions can be used “as-is” or as a starting point for further optimization by individual users to generate optimized pressure-enhanced protocols for specific applications and sample types.

Table1. Suggested Starting Conditions for Pressure-Accelerated PNGase F Deglycosylation

Pressure	45-58kpsi (higher pressures may be beneficial in some applications)
Cycle profile	Static pressure
Temperature	37°C. At higher or lower temperatures, additional optimization of PCT conditions and/or buffer components may be required.

Materials and Methods

PNGase F enzyme was purchased from New England Biolabs (cat # P0704S). Reactions were carried out essentially as described by the manufacturer at 37^0 C using reagents provided with the enzyme. For denatured/reduced protein reactions, the protein was first boiled with NEB glycoprotein denaturing buffer (containing DTT and SDS), per manufacturer instructions. Reactions were then carried out in NEB GlycoBuffer 2 supplemented with NP-40 to counteract the effect of SDS. For native protein reactions, protein was diluted directly in GlycoBuffer 2 and reactions were carried out without NP-40, per manufacturer’s instructions for deglycosylation of native proteins. Model proteins (human alpha1 glycoprotein and bovine fetuin) were purchased from Sigma (Cat # G9885 and F2379). Deglycosylated and control samples were denatured and reduced with DTT in laemmli buffer, prior to gel electrophoresis. Deglycosylation was assayed by changes in apparent molecular weight on SDS-PAGE. Reactions shown in Figures 2 and 4 were carried out at 37^0 C in PCT MicroTubes in a Barocycler 2320EXT using one minute cycling parameters (50 seconds at high pressure and 10 seconds at ambient pressure, per cycle). Reactions shown in Figure 3 were carried out at 37^0 C in PCT MicroTubes in a HUB880 Explorer with pressure cycling in Pump Mode using one minute cycling parameters (50 second hold at high pressure and 10 seconds at ambient pressure, per cycle).

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