

**Research Article**

## **BOILING REDUCES GLYCAN DETECTION ON GLYCOPROTEINS**

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### **ABSTRACT**

Glycobiology is emerging as a primary field of interest for biological research. As such, it is crucial to develop and improve tools for the study of sugar-modified biomolecules. Many research endeavors in the field of protein glycobiology involve boiling of proteins in sample buffer for 5-10 minutes prior to gel electrophoresis and their further characterization. Here we examined the effect of boiling purified glycoproteins, as well as a mixture of protein lysate, in sample buffer on their sugar content and found that prolonged boiling partially reduces detection of certain glycans, such as of the cytoplasmic O-GlcNAc. This should be taken into consideration when characterizing glycans using common assays.

**Keywords:** *Boiling, Glycoproteins, N-Linked Glycans, O-Linked Glycans, Sample Buffer*

### **INTRODUCTION**

The importance of glycobiology is increasingly appreciated (NIH report, 2012). One of the main areas of research in glycobiology is the study of protein glycosylation. Protein glycosylation is a post-translational modification that occurs in the majority of proteins synthesized in the rough ER, i.e. membranal or secreted proteins (Varki, Esko *et al.*, 2009). In the secretory pathway the two main types of protein glycosylation are N- and O- glycosylation (Varki, Esko *et al.*, 2009). N-glycosylation involves a covalent linkage of the sugar chain to an asparagine residue whereas O-glycosylation involves a covalent binding of the sugar chain to a serine or threonine residue. In addition, many proteins within the nucleus, cytoplasm and mitochondria are dynamically modified at the hydroxyl groups of their serine and threonine residues by O-glycosylation, involving the attachment of N-acetylglucosamine monosaccharides, therefore, termed O-GlcNAcylation (Hart and Akimoto, 2009).

Many experimental setups in the protein glycobiology field involve characterization of the glycans on glycoproteins (Yuzwa *et al.*, 2012).

This often involves running the glycoprotein sample on SDS-PAGE electrophoresis in order to separate proteins according to their molecular size, before analyzing their sugar content using various methods. Prior to electrophoresis, the standard method involves boiling of the protein sample in sample buffer for a recommended time of 5-10 minutes. In the present study we examined the effect of boiling purified N-linked and O-linked glycoproteins, as well as a mixture of protein lysate, in sample buffer on their sugar content. We found that prolonged boiling partially reduces detection of certain glycans. We also demonstrated that the detection of O-GlcNAc, a glycan moiety added during protein O-GlcNAcylation, is similarly reduced following boiling.

### **MATERIALS AND METHODS**

**Protein Samples** - Mucin from bovine submaxillary glands (M3895),  $\alpha$ 1-Acid Glycoprotein from human plasma (G9885) and  $\kappa$ -Casein from bovine milk (C0406) were purchased from Sigma–Aldrich (Schnellendorf, Germany). Bovine Serum Albumin (BSA, #0332) was purchased from a MRESCO (Solon, Ohio).  $\alpha$ 1-Acid, Mucin,  $\kappa$ -Casein and BSA were dissolved in ddH<sub>2</sub>O to make a 1mg/ml solution. Protein lysate from mouse brain (Janson *et al.*, 1996) was prepared by homogenization of the tissue using BULLET BLENDER® (Next Advance, Inc., Averill Park, NY) in ice-cold homogenization buffer, containing 50mM Tris-HCl, pH 7.4, 8.5% sucrose, 2.0mM EDTA, 100mM GlcNAc and cOmplete Protease Inhibitor Cocktail (Roche Molecular Biochemicals GmbH, Mannheim, Germany). The

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homogenates were centrifuged at 14,000RPM for 10 min, and the resulting supernatants were used for further analyses.

**SDS-PAGE** – Prior to electrophoresis, protein samples were prepared using a standard Laemmli protein sample buffer (SB, final concentration 60mM Tris-HCl pH 6.8, 10% glycerol, 1% SDS, 0.001% Bromophenol) and were either un-boiled or boiled in SB for various periods of time. SDS-PAGE was performed in a *GeBaRunner* apparatus (GeBa, Interchim, Montluçon, France). The proteins were separated in 4-20% (w/v) polyacrylamide *GeBaGels* under reducing conditions and were either stained with SeeBand staining solution (Gerard Biotech, Oxford, OH) or transferred into PVDF membrane using a dry blot technique (iBlot®, Life Technologies, Grand Island, NY). The membranes were subjected to PAS GelCode® glycoprotein staining kit (Pierce, #24562, Rockford, IL), or to Western or lectin blot analysis.

**Western Blot Analysis** - Presence of  $\beta$ -O-linked N-Acetylglucosamine (GlcNAc) on proteins was detected by Western blotting using a pan-GlcNAc mouse monoclonal antibody (CTD 110.6, Covance, Princeton, NJ). Briefly, the membrane was blocked for 1 hr using 3% BSA diluted in TBST (0.1% Tween) while shaking. CTD 110.6, diluted 1:1,000 in 3% BSA diluted in TBST, was added to the membrane overnight, followed by several washes with TBST. Next, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibody for 1 hr at room temperature while shaking. Blots were developed after thorough TBST washes, using an Enhanced Chemiluminescence System (EZ-ECL, Biological Industries, Kibbutz Beit Haemek, Israel) according to the manufacturer's manual. Densitometric analysis of O-GlcNAc levels using CTD 110.6 antibody was performed on entire lanes using GE Healthcare Densitometer (Pharmacia Biotech Inc., Uppsala, Sweden).

**Lectin Blot Analysis** - Presence of GlcNAc and sialic acids, or N-Acetylgalactosamine (GalNAc) on proteins was detected by lectin blotting using biotinylated Wheat Germ agglutinin (WGA) or Soybean agglutinin (SBA) lectins (LK-2000, Vector Laboratories, Burlington, Ontario Canada), respectively. First, the membrane was blocked for 1 hr using 3% BSA diluted in Tris cations (TC) buffer (containing 1mM Tris-HCl, pH=7.5, 1mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, 1mM MnCl<sub>2</sub>, 0.1% Tween) while shaking. Biotinylated lectins, diluted 1:1,000 in 3% BSA in TC buffer, were added to the membrane for 2 hrs, followed by several washes with TBST (0.1% Tween). Next, the membrane was blocked again with BSA 3% in PBS for 1 hr, followed by 1hr incubation with streptavidin-HRP (016-030-084, Jackson ImmunoResearch, West Grove, PA, USA), diluted 1:2000 in PBS under agitation. After thorough washes with TBST, blots were developed using an Enhanced Chemiluminescence System (EZ-ECL, Biological Industries, Kibbutz Beit Haemek, Israel) according to the manufacturer's manual.

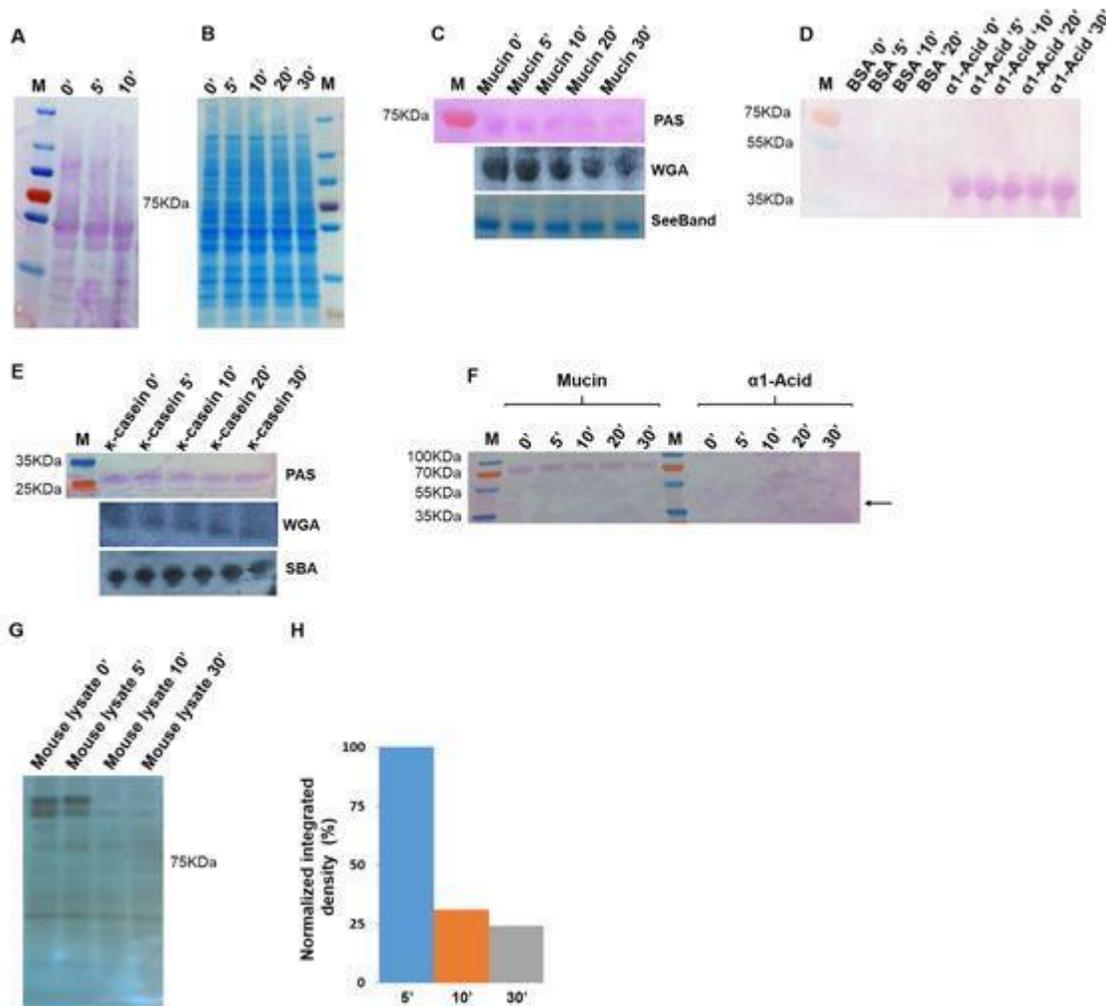
**PNGase-F Treatment**- Mucin or  $\alpha$ 1-Acid were subjected to treatment with the glycosidase PNGase-F according to manufacturer's manual (P7367, Sigma-Aldrich, Schnellendorf, Germany). Briefly, to 45  $\mu$ g of each glycoprotein a denaturation solution was added (final concentration 0.02% SDS with 10 mM 2-mercaptoethanol). Then, solutions were boiled for 10 min to denature the glycoproteins. The solutions were cooled down before adding 3.5 units of PNGase-F and Triton-X (final concentration 1.5% Triton-X). After 3 hrs of incubation at 37°C, reaction was terminated by boiling for 10 min. SB was added and samples were either un-boiled or boiled for various periods of time, before being subjected to SDS-PAGE, transferred onto PVDF membrane and stained using the PAS GelCode® glycoprotein staining kit.

## **RESULTS AND DISCUSSION**

In order to examine the effect of boiling glycoproteins in sample buffer (SB) on their sugar content we boiled mouse brain protein lysate for various periods of time and subjected it to SDS-PAGE and transfer into PVDF membrane, following by Periodic Acid Schiff (PAS) staining (see Materials and Methods). A clear reduction in PAS staining was evident even after 5 minutes of boiling, compared with un-boiled samples, and greater reduction in signal was observed after boiling for 10 minutes (Figure 1A). Similar results were observed when using protein lysates from other organisms (data not shown). In order to verify that the reduction in PAS reactivity was not due to lower amount of protein loaded, we also

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examined the effect of boiling on the heat stability of the proteins in this lysate by subjecting them to SDS-PAGE and SeeBand protein staining (Figure 1B). As opposed to the sugar levels detected, the proteins themselves were found to be very stable through the various boiling periods tested, the longest of which was 30 minutes. A very slight reduction in protein staining was observed after boiling for 30 minutes, whereas a clear reduction in sugar levels detected was observed already after 5 minutes of boiling (Figure 1A).



**Figure 1: Boiling Reduces Detection of Certain Glycans on Glycoproteins but Hardly Degrades the Proteins Themselves**

**A.** PAS staining of mouse protein lysate, un-boiled or boiled in SB for 5’ or 10’. **B.** SeeBand staining of mouse protein lysate, un-boiled or boiled for 5’, 10’, 20’ or 30’. **C.** PAS, WGA and SeeBand staining of purified Mucin, un-boiled or boiled in SB for various periods of time, using different blots. **D.** PAS staining of purified BSA (as a negative control) or  $\alpha$ 1-Acid, un-boiled or boiled in SB for various periods of time. **E.** PAS, WGA and SBA staining of purified  $\kappa$ -Casein, un-boiled or boiled in SB for various periods of time, using different blots. **F.** PAS staining of Mucin and  $\alpha$ 1-Acid after PNGase-F treatment, un-boiled or boiled in SB for various periods of time. Arrow indicates the expected molecular weight of  $\alpha$ 1-Acid. **G.** Western blot analysis of O-GlcNAc moieties using CTD 110.6 antibody on mouse protein lysate, un-boiled or boiled in SB for various periods of time. **H.** Quantification of CTD 110.6 reactivity from (G).

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To further investigate the effect of boiling glycoproteins on sugar detection on them we examined three purified glycoproteins: Mucin from bovine submaxillary glands (Figure 1C),  $\alpha$ 1-Acid from human plasma (Figure 1D) and  $\kappa$ -Casein from bovine milk (Figure 1E). Mucin is highly modified with O-glycans but also with some N-glycans (Perez-Vilar and Hill, 1999),  $\alpha$ 1-Acid is decorated only with N-glycan sugar trees (Fournier *et al.*, 2000), whereas  $\kappa$ -Casein is decorated only with O-glycans (Pisano *et al.*, 1994) (Figure 1C, Figure 1D and Figure 1E, respectively).

BSA, which is not glycosylated at all (Magnelli *et al.*, 2012) served as a negative control and indeed had no PAS reactivity (Figure 1D). Interestingly, while boiling had no effect on PAS staining of N-glycosylated  $\alpha$ 1-Acid (Figure 1D) or of O-glycosylated  $\kappa$ -Casein (Figure 1E), it dramatically reduced PAS staining reactivity of Mucin, which is both O- and N-glycosylated (Figure 1C). As opposed to its reduced PAS reactivity, the Mucin protein itself was stable through boiling in SB for the various periods of time tested up to 30 minutes (Figure 1C).

The decrease in sugar level reactivity of Mucin was verified by staining with the WGA lectin (Figure 1C), which recognizes GlcNAc and sialic acids (see Materials and Methods). We verified that no such decrease in the signal of WGA or SBA lectins is evident for  $\kappa$ -Casein, whose PAS signal remained the same after boiling for various time periods (Figure 1E). To further understand the effect of boiling on sugar detection on glycoproteins we treated Mucin and  $\alpha$ 1-Acid with PNGase-F, a glycosidase that removes all N-glycans.

As expected, no PAS signal was observed for the exclusively N-linked  $\alpha$ 1-Acid after treatment (Figure 1F). In contrast, Mucin still demonstrated PAS signal after treatment (Figure 1F), which is most likely attributed to its O-linked glycans, un-affected by the PNGase-F treatment. However, once N-glycans were removed by the enzyme, no effect of boiling was observed and PAS signal of Mucin remained the same, indicating that loss of PAS reactivity of Mucin following prolonged boiling is probably dependent on existence of N-glycans on it.

Since it appeared that boiling in SB has a marked effect of the ability to detect certain glycans on glycoproteins, we next examined whether a specific type of O-glycosylation, namely O-GlcNAcylation, will similarly be affected by boiling.

O-GlcNAcylation involves the attachment of beta-N-acetylglucosamine (GlcNAc) to Ser/Thr residues and occurs primarily on nucleocytoplasmic proteins. To that end we boiled mouse protein lysate for various periods of time and subjected it to SDS-PAGE and transfer into PVDF membrane, followed by Western blot analysis of O-GlcNAc moieties using CTD 110.6 antibody (Figure 1G). Indeed, a remarkable reduction of O-GlcNAc detected was observed after boiling for various periods of time, although protein level was not affected (Figure 1B).

Strikingly, about 70% of the O-GlcNAcylated glycans were no longer detected after boiling the mouse lysate for 10 minutes (Figure 1H).

Preserving the natural decoration of the glycoprotein of interest is crucial for studying its structure and function as well as for characterization of the glycans it carries. It appears that the detection of some N-linked glycans as well as O-linked glycans, but not all, are affected by prolonged boiling.

One possible explanation for the reduced glycan detection following boiling of the glycoproteins is that boiling removes certain glycans from the glycoproteins. In this regard it is worth noting that the sample buffer used in this study is the most standard one, with a neutral pH of 6.8. We speculate that if indeed boiling removes glycans from glycoproteins, when using a more basic sample buffer,  $\beta$ -elimination of O-glycans will be accelerated. Hence, use of the standard sample buffer is recommended.

Based on our finding, we strongly recommend that when characterizing glycans on glycoproteins, and especially O-GlcNAcylation, the protein sample should be boiled in sample buffer for no more than 5 minutes prior to gel electrophoresis.

The effect of boiling on sugar detection on glycoproteins as reported here should be taken into account when examining glycans via the commonly used assays, such as PAS staining, lectin staining and Western blot using CTD 110.6 antibody or equivalents (such as RL2 antibody).

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### **ABBREVIATIONS**

PAS, Periodic Acid Schiff; SB, Sample Buffer

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