

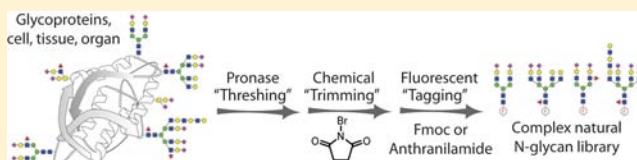
Novel Strategy to Release and Tag N-Glycans for Functional Glycomics

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S Supporting Information

ABSTRACT: Functional glycomics has been impeded by the lack of inexpensive enzymatic and mild chemical methods to acquire natural glycans in significant amounts. In this study, we have developed a new strategy we term “threshing and trimming” (TaT) to quickly obtain N-glycans from glycoproteins and animal tissues. TaT employs low-cost Pronase to degrade peptides and N-bromosuccinimide (NBS) to effect oxidative decarboxylation under very mild reaction conditions to generate homogeneous aglycon moieties as nitriles or aldehydes. These aglycons can be readily conjugated with fluorescent tags for profiling and functional study. TaT is an affordable alternative to expensive specialty enzymes and strong chemical treatment and unpleasant reagents, and should further drive the functional glycomics of N-glycans.



INTRODUCTION

Functional glycomics, as the systematic study of structures and functions of the glycome, has seen great progress in the past decade.^{1–3} This is largely due to increasing interest in glycans related to various biological pathways and diseases. Glycans play important roles in protein folding, cell–cell adhesion, host–pathogen interactions, and cell signaling.⁴ The study of glycans/carbohydrates is inherently more complicated than nucleic acids and proteins, due to their more complicated structure.⁵ Unlike the situation for nucleic acids and proteins, there is neither practical automatic synthesis nor high throughput sequencing techniques available for glycans, despite the great efforts and advances made in synthetic/analytical chemistry of glycans. While the prototype glycan synthesizer has been reported,⁶ the limitation of its applicability to larger glycans and the lack of monosaccharide building blocks prevent it from being employed widely in the near future. The sequencing of glycan structures has been focused on high resolution mass spectrometry (MS), which has produced an enormous amount of information on glycomics.^{7–12} However, due to limited material, the structural information is often partial and detailed analysis requires significant instrumentation and expertise.

In recent years, glycan microarrays have emerged as a powerful tool to study functions of glycans by facilitating studies on protein–glycan interactions in a high throughput fashion. A library of glycans, with either defined or unknown structures, can be printed on a single glass slide and interrogated for protein binding using antibodies and lectins, generally defined as glycan-binding proteins (GBPs). Despite the great success of glycan microarrays using synthetic glycans with defined structures, the bottleneck is the unavailability of more complex glycans. We and others have been seeking alternative ways to prepare glycan libraries from natural glycans

for microarray by developing fluorescent bifunctional linkers, which greatly facilitate the detection, monitoring, quantification, and immobilization of natural glycans.^{13–17} While techniques including multidimensional HPLC separation can be used successfully to isolate individual natural glycans, the large-scale release of glycans from various natural sources is expensive and not easy.

N-Glycans can be released from glycoproteins either enzymatically or chemically.^{18–20} Peptide:N-glycanases (PNGase F) have been widely used to remove N-glycans from glycoproteins and have contributed enormously to the development of the glycosciences. However, these specialty enzymes are often too expensive to be applied at a preparative scale. The use of these enzymes often involves a complex denaturing procedure, including reduction/alkylation and trypsin digestion. The specificity of PNGase F also raises questions about the completeness of the prepared glycome, especially from nonmammalian systems, since unusual modification of the core structures of N-glycans can adversely affect PNGase F recognition. The only validated chemical method to quantitatively release N-glycans is hydrazinolysis at elevated temperature. This is seriously detrimental to many labile groups and the reagent is highly toxic and dangerous to handle, especially at larger scales. Both of these methods release glycans in free reducing form that can be easily labeled with a fluorescent tag by reductive amination, but the reducing end structural features are lost.

In this manuscript, we describe the development of a general method to release and tag N-glycans from all natural sources using mild conditions that retain the reducing end linkages and

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common, inexpensive, and easy-to-handle reagents. This new approach, which is directed to glycoproteins, is termed “threshing and trimming” (TaT), and is schematically represented in Figure 1. Glycoproteins in complex samples

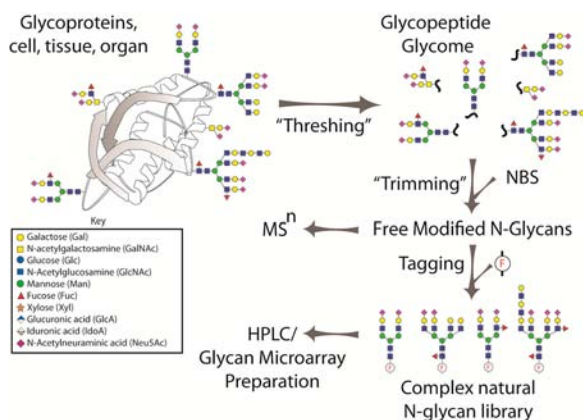


Figure 1. Strategy of “threshing and trimming” (TaT) of glycoconjugates for Functional Glycomics.

including cells, tissues, or organs are digested with Pronase (“threshed”) to remove most of the aglycon components. The threshed glycoconjugates are then “trimmed” by reaction with N-bromosuccinimide (NBS) to form homogeneous aglycons, which can be specifically tagged and separated for functional analysis. This manuscript describes the application of this strategy to N-glycans, perhaps the most studied class of glycans, and demonstrates a simple and inexpensive process to prepare fluorescently tagged N-glycans under mild conditions for functional studies.

RESULTS

Combination of Pronase Digestion and N-Bromosuccinimide (NBS) Treatment Releases Glycans with Homogeneous Aglycon from Glycoproteins. Pronase can nonspecifically digest proteins to small peptides and single amino acids without altering any glycans.²¹ In our previous work, we applied N-fluorenylmethyloxycarbonyl (Fmoc) protection as a reversible fluorescent tag to these glycopeptides to facilitate their separation and use of the glycans for microarray preparations.²² However, the resulting glycopeptides from most glycoproteins are heterogeneous on the peptide components. This heterogeneity coupled with the inherent heterogeneity of the glycans makes it extremely difficult to practically separate and characterize these glycopeptides for preparation of a glycan library. We therefore sought a chemical treatment that can remove the heterogeneous peptide component yet preserve the glycan linkage to Asn. N-Bromosuccinimide (NBS) is a widely used oxidant in synthetic carbohydrate chemistry for protecting group manipulation/activation.^{23,24} It has also been used for decarboxylation of amino acids to prepare corresponding nitriles.^{25,26} We reasoned that NBS could be used to trim the peptide/amino acid components of glycopeptides without affecting glycans. We found that NBS treatment of a Pronase digest of glycoproteins under different conditions gave either the expected nitriles or free reducing glycans, as schematically shown in Figure 2a and Supporting Information Figure S1. We analyzed the MALDI-TOF profiles of the dialyzed Pronase digest from ovalbumin before and after NBS treatment (Figure 2b). Exhaustive

Pronase digestion removes most of the peptide components to generate glycoamino acids (Figure 2b, top panel). Upon NBS treatment at room temperature in phosphate buffer (pH 6–8), glycan nitriles were formed nearly quantitatively (Figure 2b, middle panel). When NBS treatment was carried out in the same buffer at elevated temperature (65 °C), free reducing glycans, including a small component of glycans lacking the reducing terminal GlcNAc, i.e., the one linked to Asn, were observed as the products (Figure 2b, bottom panel). This latter minor component likely arises from a “peeling” reaction by an unknown mechanism that must be different from β -elimination, as it is not pH dependent and no further “peeling” was observed with prolonged reaction time. While free reducing glycans have been commonly used for structural characterization and fluorescent tagging, we focused on the relatively stable glycan nitriles, since they are generated under mild conditions and retain the reducing end linkage to the aglycone. In addition, the nitrile functional group can be specifically derivatized if desired.

Unexpectedly, we found that not only glycoamino acids, but also glycopeptides with short peptides could be trimmed by NBS treatment to nitriles (Figure 2c,d). Bovine immunoglobulin G (IgG) and horseradish peroxidase (HRP) were treated with Pronase and mixtures of glycoamino acids/glycopeptides were obtained (Figure 2c,d, top panels). When these mixtures were treated with NBS, glycan nitriles were obtained as the major products (Figure 2c,d, bottom panels), suggesting that glycopeptides with short peptide chains can also be trimmed down to nitriles. The proposed mechanism is shown in Supporting Information Figure S1, route a. It is also noteworthy that the major HRP N-glycan is resistant to PNGase F digestion due to its unusual core α 3-fucosylation on the innermost GlcNAc, which does not interfere with the trimming by the NBS reaction in this novel approach.

Released N-Glycans from Glycoproteins Can Be Labeled with 2-Aminobenzamide (2-AB) by Palladium/Carbon Catalyzed N-Alkylation. Nitriles are usually considered chemically inert, yet there are specific chemical transformations to activate nitriles to a more active functional group, such as aldehydes and amines,^{25,27,28} which are highly useful for further functionalization. In our ongoing efforts to utilize natural glycans for protein–glycan interaction studies, we found that fluorescent tagging is often essential for detection, characterization, and quantification of minor amounts of glycans. Therefore, we developed a simple method to fluorescently tag these glycan nitriles. Recently, nitriles have been used in the catalytic N-alkylation of amine and nitro groups, although often excess nitriles are used to drive the N-alkylation to completion.^{29–31} We reasoned that by using an excess of fluorescent amine, we should be able to directly tag the glycan nitriles (Figure 3a) for subsequent profiling and functional study. This was confirmed by NBS treatment of Pronase-digested bovine fetuin followed by palladium/carbon (Pd/C) catalyzed reaction with 2-aminobenzamide (2-AB) (Figure 3b,c). When Pronase-derived glycopeptides of fetuin were treated with NBS followed by permethylation, we clearly observed N-glycan peaks corresponding to the expected permethylated nitrile (Figure 3b, top panel). Interestingly, O-glycans were observed as permethylated reducing glycans, as shown in the full spectrum in Supporting Information Figure S2. It is known that permethylation of Pronase digest of glycoproteins yield permethylated reducing O-glycans.³² It is possible that O-glycans inhibit Pronase so that only O-

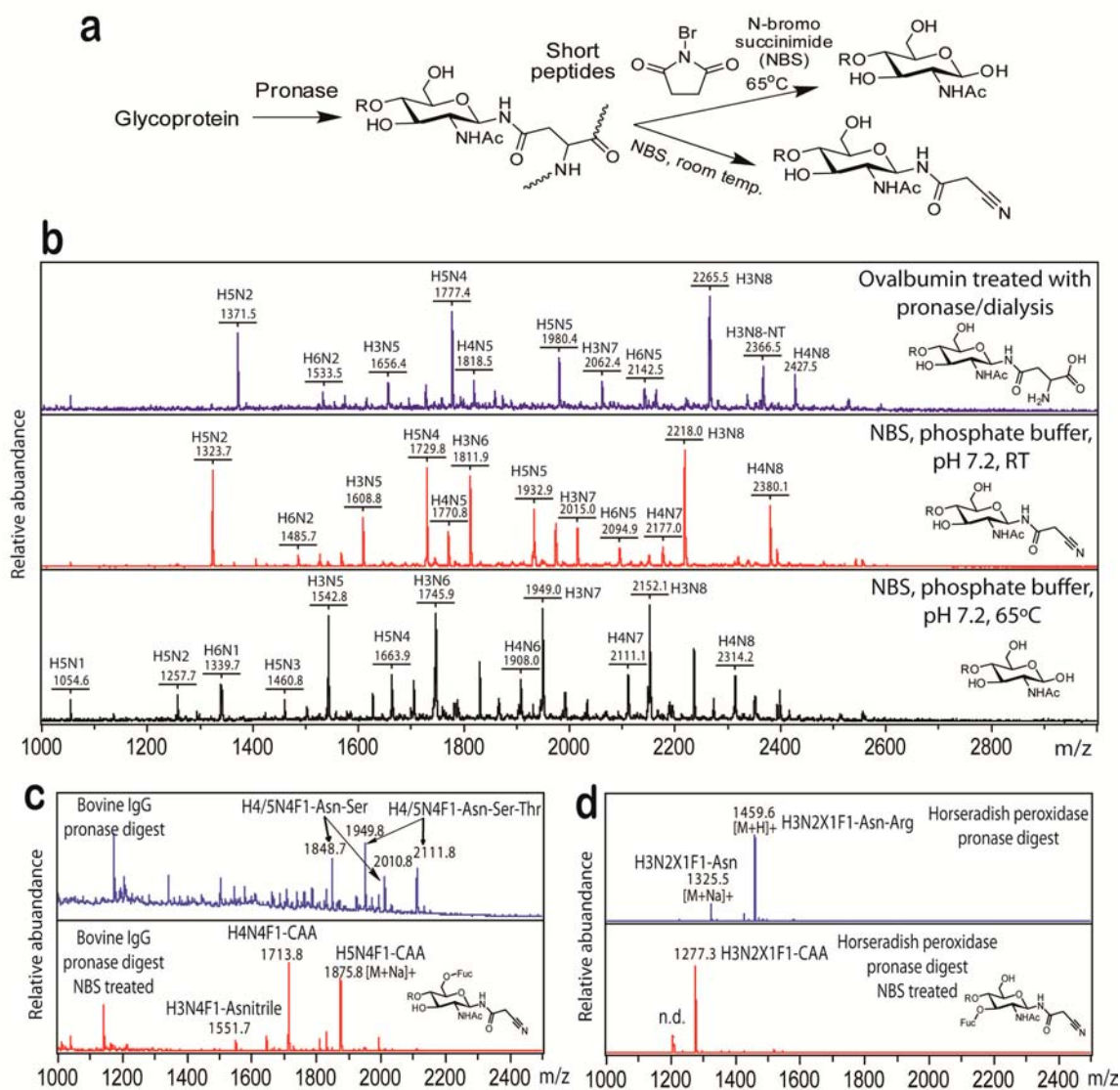


Figure 2. NBS treatment of Pronase digested glycopeptides generates glycan nitriles or reducing sugars. (a) Reaction scheme of Pronase digest and NBS treatment. (b) MALDI-TOF profiles of Pronase digested ovalbumin glycoamino acids (top panel), after NBS treatment under room temperature (middle panel) or 65 °C (bottom panel). (c,d) MALDI-TOF profiles of Pronase glycopeptides from bovine IgG (c) and horseradish peroxidase (d) before (top panels) and after (bottom panels) NBS treatment.

glycopeptides with longer peptide chains are formed, which cannot be trimmed efficiently by NBS, but will release O-glycans during the permethylation. Therefore, our new approach is specific to N-glycan preparation. When the glycan nitriles were treated with 2-AB under catalysis with Pd/C, the nitriles were efficiently conjugated with 2-AB as shown by analysis of the permethylated products (Figure 3b, bottom panel). The 2-AB tagged fetuin N-glycans showed a very similar pattern when compared with the AEAB conjugates of PNGase F digested N-glycans from fetuin, suggesting an efficient and specific derivatization of N-glycans (Figure 3c). Similar to 2-AB, the glycan-nitriles from fetuin can also be conjugated with 2,6-diaminopyridine (DAP)¹⁷ (Supporting Information Figure S3), suggesting that the tagging method is general for fluorescent amines.

Since the reagents used in this new approach are inexpensive, it can possibly be used for functional glycomics analyses on a large scale, which is not practical using enzyme-based methods to release N-glycans. To test the potential of TaT for large-scale

preparations, 700 g (wet weight) of pig liver were homogenized, digested with Pronase, and dialyzed. The Pronase digest and dialysis was repeated once to ensure complete digestion. The glycopeptides were then treated with NBS followed by further dialysis to remove salt and small molecules. After lyophilization, the dialysate yielded 0.9 g crude glycan nitrile derivatives. The MALDI-TOF spectrum of permethylated glycan nitriles (Figure 3d and Supporting Information Figure S4) and fluorescent HPLC profile of 2-AB labeled glycans (Figure 3e) were obtained, which showed successful N-glycan release/tagging. Interestingly, the glycans obtained are mostly neutral and monosialylated glycans, different from profiles that have been observed for mouse and human liver N-glycans previously (<http://www.functionalglycomics.org/>).

Glycan-Aldehyde Can Be Released from Pronase Digested Glycoproteins and Tagged with Bifunctional Fluorescent 2-Amino-N-(2-aminoethyl)benzamide (AEAB). The generation of nitriles from amino acids by NBS

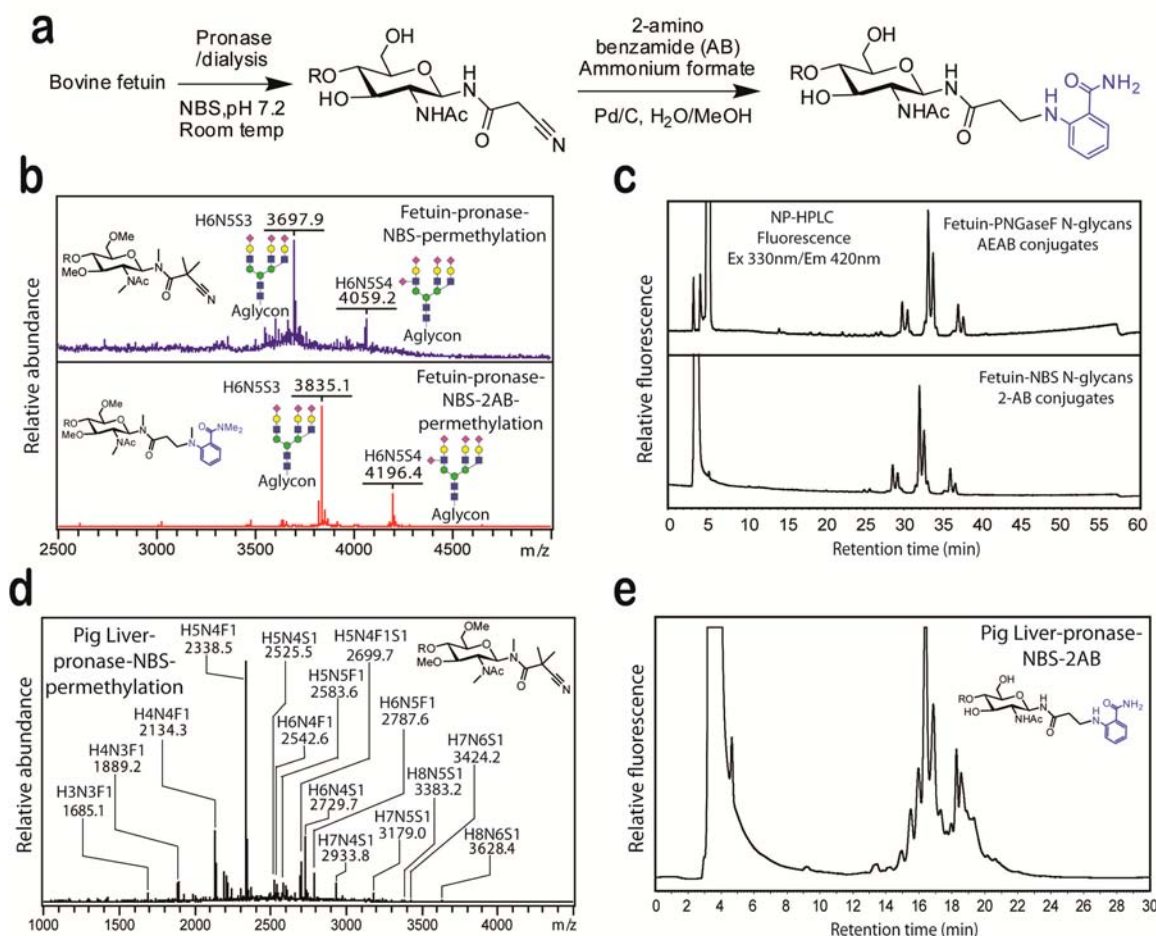


Figure 3. Glycan nitriles from NBS treatment can be fluorescently tagged by Pd/C catalyzed conjugation. (a) Reaction scheme of release and tagging of bovine fetuin N-glycans. (b) MALDI-TOF profiles of permethylated fetuin glycan nitriles (top panel) and Pd/C catalyzed conjugation product (bottom panel). (c) Comparison of HPLC profiles of AEAB conjugated PNGase F digested N-glycans (top panel) and 2-AB conjugated product from glycan nitriles from fetuin (bottom panel). (d) MALDI-TOF MS profile of permethylated pig liver glycans prepared by NBS treatment. (e) HPLC profile of pig liver glycans prepared by NBS treatment and Pd/C catalyzed 2-AB conjugation.

treatment is likely through an intermediate of imine, which is less stable under acidic conditions. Thus, as an alternative route, we explored the NBS treatment of Pronase glycopeptides in 0.1% TFA, which is mild but acidic enough to hydrolyze imines to aldehydes. As expected, the aldehyde product was observed, which can be directly conjugated with AEAB using common reductive amination conditions (Figure 4a). Egg yolk glycopeptide (EYG),³³ ribonuclease B (RNaseB), and HRP were treated with Pronase, followed by NBS under acidic conditions, and directly conjugated with AEAB by reductive amination. The HPLC profiles (Figure 4b) and MALDI-TOF profiles (Figure 4c) indicate that the conjugation occurred smoothly and efficiently. As reported previously,¹⁶ the AEAB conjugated glycans, after separation, characterization, and quantification, can be successfully used in the preparation of glycan microarray.

DISCUSSION

Here we proposed a novel strategy termed “threshing and trimming” (TaT) for the preparation of glycans from glycoproteins. TaT is designed to eliminate the use of specialty enzymes, hazardous chemical reagents, and harsh reaction conditions, and to maintain as much structural integrity of N-glycans as possible. Using a combination of inexpensive

reagents, we successfully developed two routes to prepare fluorescently tagged N-glycans from glycoproteins or from complex mixtures of glycoprotein in large samples of cells, tissues, or organs. These procedures overcome many of the major obstacles limiting large-scale functional glycomics studies.

Pronase, a bacterial product that lacks glycan components, is a broad spectrum protease commonly used to digest most peptide components. Considering the size and hydrophilicity of N-glycans, glycopeptides generated by Pronase digestion can be separated from most amino acids and small peptides. We found that a simple dialysis step using MWCO 500–1000 enriched the desired glycopeptides for subsequent chemical treatment. When Pronase treatment is applied to cells, tissues, or organs, it also facilitates the homogenization, potentially increasing the yield of the glycome preparation.

We then investigated the utility of NBS for trimming the aglycon moiety of N-glycopeptides by simply varying the reaction pH and temperature. Importantly, we were able to isolate N-glycans as nitriles, reducing sugars, or aldehydes, based on the reaction conditions applied. When glycopeptides were treated with NBS in phosphate buffer at room temperature for several hours, the short peptides are transformed to nitriles through oxidative decarboxylation. Due to its triple bond nature, the formation of nitriles demonstrates the

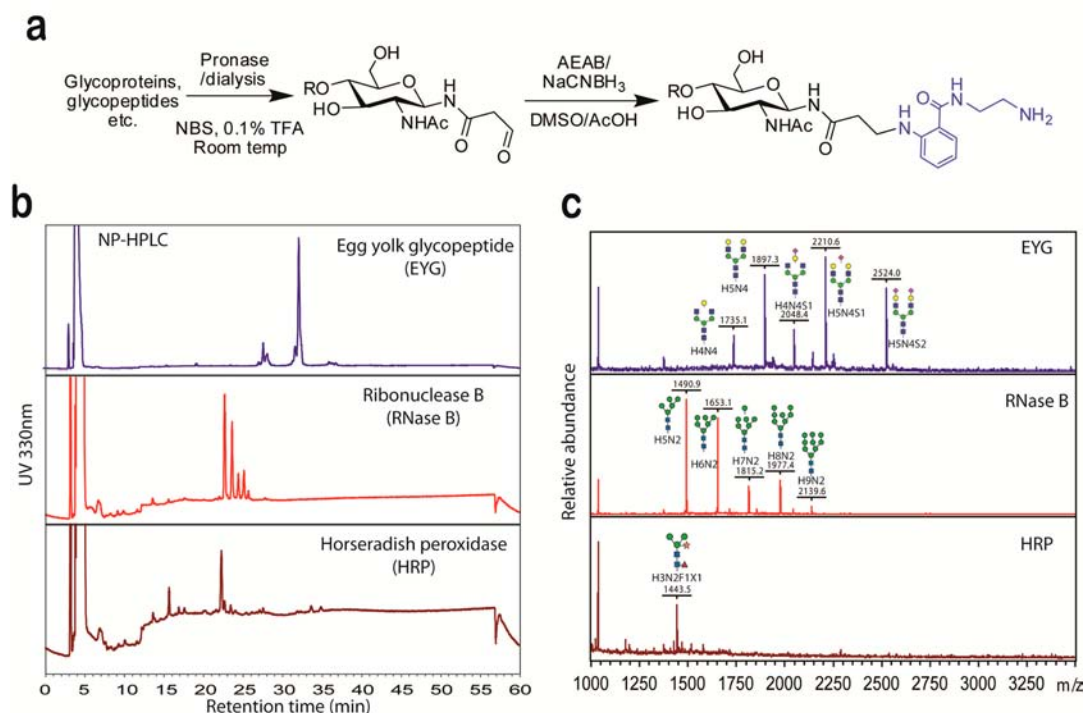


Figure 4. N-Glycan aldehyde can be prepared by NBS treatment of Pronase glycopeptides under acidic conditions and conjugated with AEAB. (a) Reaction scheme. (b) HPLC profiles. (c) MALDI-TOF MS of conjugates of glycan aldehyde with AEAB from egg yolk glycopeptide (top panels), ribonuclease B (middle panels), and horseradish peroxidase (bottom panels).

removal of both the N-terminal and C-terminal aspects of the peptides. Thus, the glycan-nitriles formed in this procedure are homogeneous in the aglycon moiety (Supporting Information Figure S1) and are reasonably stable by themselves. However, with prolonged reaction with NBS at room temperature or 2 h under heating at 65 °C in neutral buffers, the aglycon was removed to form reducing sugars as the only products. Meanwhile, in this later reaction, some reducing N-glycans lost their reducing terminal GlcNAc. Although not fully understood, the mechanism is unlikely β -elimination, as it is not dependent on pH. When we changed the pH of the NBS treatment by using 0.1% TFA instead of phosphate buffer, the products are glycan-aldehydes. This is likely due to the hijacking of the imine intermediates by acidic hydrolysis (Supporting Information Figure S1, route c) to the glycan-aldehydes before further NBS bromination of the glycan-imines and HBr elimination to form glycan-nitriles (Supporting Information Figure S1, route a). The generation of free reducing sugars under heated condition was rather unexpected. It could be through the bromination of the reducing end amide bond, which alters its stability toward hydrolysis (Supporting Information Figure S1, route b).

The ability to fully control the outcome of products generated by NBS treatment under different reaction conditions provides versatility in this reaction scheme that can be used to great advantage. For example, the free reducing sugars have been commonly used in permethylation analysis and fluorescent tagging, while the glycan-nitriles are rather chemically inert under normal physiological conditions, so they can be directly used in some biological assays if desired. The glycan-nitriles can also be specifically tagged through robust Pd/C mediated N-alkylation of fluorescent amines, which will facilitate separation and characterization. The glycan-aldehydes, on the other hand, are chemically active, and besides the

reductive amination with AEAB to prepare natural glycans for glycan microarray, these glycan-aldehydes are expected to directly conjugate with hydrazide and hydroxyamines easily for bioconjugation with other biomolecules.

We focused on using only mild reaction conditions, which were not likely to affect any known labile groups on glycans, such as sialic acids, phosphoesters, sulfate, etc. NBS was selected because it is a widely used chemical in glycan synthesis with mild and specific oxidation power that we felt might be utilized for a novel approach to preparing glycans from complex mixtures of glycoconjugates. Since the NBS oxidation only occurs on the peptide moiety, it does not interfere with the glycan structure and shows no specificity for different classes of N-glycans. For example, the strategy works not only on glycans with a core α 1–6-linked fucose, but also on N-glycans with a core α 1–3-linked fucose, which are resistant to PNGase F digestion. This approach will presumably also work on other special classes of N-glycans from other natural sources making it useful for the functional glycomics analyses of bacteria and other microorganisms.

To utilize the N-glycans prepared from a natural source, fluorescent and functional tagging is often essential. The glycan nitriles and aldehydes prepared using NBS treatment can be easily tagged with fluorescent compounds such as 2-AB and AEAB for functional study. Pd/C catalyzed N-alkylation of 2-AB has been applied to the nitriles successfully under very mild conditions. The resulting 2-AB conjugates can be used either simply for profiling or for printing of glycan microarrays as reported before. Alternatively, glycan aldehydes prepared by NBS treatment under acidic condition can be directly conjugated with AEAB by reductive amination. The resulting AEAB conjugates provide better immobilization efficiency compared to 2-AB conjugates on NHS-activated glass slides for preparation of glycan microarrays.

TaT has the potential to be the method of choice for generation of glycans in preparative scale from large amounts of cells and tissues or organs and may replace the use of PNGase F and hydrazinolysis in many analytical applications. Of course, PNGase F will remain an important reagent for applications where peptide sequences are of interest. By avoiding specialty enzymes, hazardous chemical reagents, and harsh reaction conditions and by preserving glycan structural integrity, TaT will significantly facilitate the study of functional glycomics, especially when the glycome is studied on a large scale. On an analytical scale, the TaT process is about 50% as efficient as PNGase F for glycan release. However, unlike PNGase F digestion, TaT, which is a chemical method, can be applied to large quantities of tissues. For example, our preliminary study on pig liver has demonstrated the ability to efficiently generate N-glycans from kilograms of tissue. We anticipate that when applied on a large scale TaT will be used to mine milligram to gram quantities of glycans from abundant natural sources and complement synthetic approaches to greatly facilitate the structural and functional analysis of glycome. While only N-glycans are targeted in this study, efforts to extend this new strategy to other classes of glycoconjugates are underway.

■ EXPERIMENTAL PROCEDURES

Materials. All chemicals and HPLC solvents were purchased from Sigma-Aldrich, St. Louis, MO and Fisher Scientific, Pittsburgh, PA. Milli-Q water was used to prepare all aqueous buffers. Pronase was purchased from Calbiochem.

High Performance Liquid Chromatography (HPLC) Analyses. A Shimadzu HPLC CBM-20A system with UV detector SPD-20A and fluorescence detector RF-10Axl was used for HPLC analysis. UV absorption at 330 nm or fluorescence at 330 nm excitation (Ex) and 420 nm emission (Em) was used for detection of both 2-aminobenzamide (2-AB) and 2-amino-N-(2-aminoethyl)benzamide (AEAB) tag. A Restek Pinnacle II Amino Column (5 μ m, 250 mm \times 4.6 mm) was used for normal phase HPLC. The mobile phases were acetonitrile, water, and aqueous ammonium acetate buffer at pH 4.5. A linear gradient from 20 mM ammonium acetate in 80% acetonitrile to 200 mM ammonium acetate in 10% acetonitrile in either 25 or 50 min was used.

Mass Spectrometry (MS). A Bruker Daltonics Ultraflex-II MALDI-TOF/TOF system and an anchorchip target plate were used for MS analysis. Reflective positive mode was used for glycans before and after permethylation. 2,5-Dihydroxybenzoic acid (DHB) (5 mg/mL in 50% acetonitrile with 0.1% trifluoroacetic acid) was used as matrix.

Release of N-Glycan Nitriles and Aldehydes from Glycoproteins or Tissues. Glycoproteins were dissolved in water (or 0.1 M Tris buffer, pH 8.0, 0.1% sodium azide) to 10 mg/mL and Pronase was added to 0.2 mg/mL. The mixture was digested at 55 °C in a dialysis bag with MWCO 1000 for 24 h. Another batch of Pronase (0.1 mg/mL by calculation based on the volume) was added and the digestion/dialysis was repeated for 24 h at 55 °C. The dialysate was buffered with 0.25–0.5 M phosphate to pH 6–8 or processed under acidic conditions using 0.1% trifluoroacetic acid (TFA) depending on the outcome desired as discussed below. NBS was added to 15 mg/mL and the solution was stirred at room temperature for 4 h to release glycan nitriles or aldehydes. The reaction mixture was quenched by addition of sodium sulfite to 15 mg/mL, dialyzed, and the glycan nitriles or aldehydes were directly lyophilized for MS analysis or fluorescent tagging. As an

example, bovine fetuin (120 mg) was treated with Pronase digestion/dialysis for 2 \times 24 h. The dialysate was lyophilized to give 19.1 mg glycoamino acids/glycopeptides. Twelve milligrams of this mixture was treated with NBS in phosphate buffer (0.5 M, pH 7.2) and dialyzed against water after quenching with sodium sulfite. After lyophilization, 10.3 mg crude glycan nitrile product was yielded (14% by weight from glycoprotein), which can be directly labeled with 2-AB under Pd/C catalysis to obtain the fluorescent derivatives. In another example, bovine IgG (310 mg) was treated with Pronase digestion/dialysis and yielded 15.1 mg glycoamino acids/glycopeptides. 9.2 mg of this product was treated with NBS and yielded 6.7 mg crude glycan nitrile product (3.5% by weight from glycoprotein).

The release of glycan nitriles and aldehydes from N-glycans contained in more complex samples such as cells, tissues, and organs follows essentially the same procedure except that Pronase digestion was repeated once. Tissue was homogenized by blending with water (3 mL/g) and digested with Pronase for 48 h and centrifuged. The supernatant was concentrated using a rotary evaporator, dialyzed, and digested with Pronase again. The digest was passed through a C18 Sep-Pak to remove lipid-linked glycans, and the flow-through solution was processed as described above for glycoproteins.

Permethylation Analysis. Permethylation of glycan samples was carried out according to reported procedures³⁴ to increase the sensitivity of MS analysis. Briefly, a lyophilized sample was treated with DMSO/NaOH slurry (100–200 μ L) and methyl iodide (25–50 μ L) for 10–30 min. The supernatant was then partitioned between water (500 μ L) and chloroform (500 μ L). The organic layer was washed with 500 μ L water, dried, and redissolved in 50% methanol for MS analysis.

Fluorescent Tagging of Glycan-Nitriles with 2-Aminobenzamide (2-AB) by Pd/C Mediated N-Alkylation. In a typical procedure, desalted glycan nitriles were mixed with 2-AB (50–100 mM), ammonium formate (0.5–1 M) in 75% methanol. Then, palladium 10% on carbon (Pd/C) was added (2–5 mg/mL). The mixture was rotated at room temperature for 4 h, then filtered and dried in a Speed-vac to remove methanol. The resulting tagged glycans can be dialyzed or passed over Carbograph to remove byproducts and desalt the sample prior to MS and HPLC analysis.

Fluorescent Tagging of Reducing Glycans and Glycan Aldehydes by Reductive Amination. Free reducing glycans from PNGase F digestion and glycan aldehyde prepared by NBS treatment of glycopeptides were conjugated with 2-AB or AEAB using essentially the same reductive amination procedure.¹⁶ Briefly, to a dried sample, equal volumes (10–50 μ L) of 2-AB or AEAB (0.35 M) and sodium cyanoborohydride (1 M) in DMSO/AcOH (7/3 v/v) was added. The mixture was heated at 65 °C for 2 h and the glycan derivatives were precipitated by addition of 10 volumes of acetonitrile. The precipitates were collected by centrifugation for MS and HPLC analysis.

■ ASSOCIATED CONTENT

Supporting Information

Four supplementary figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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