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Improved nonreductive *O*-glycan release by hydrazinolysis with ethylenediaminetetraacetic acid addition

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ABSTRACT

The study of protein O-glycosylation is receiving increasing attention in biological, medical, and biopharmaceutical research. Improved techniques are required to allow reproducible and quantitative analysis of *O*-glycans. An established approach for *O*-glycan analysis relies on their chemical release in high yield by hydrazinolysis, followed by fluorescent labeling at the reducing terminus and high-performance liquid chromatography (HPLC) profiling. However, an unwanted degradation known as "peeling" often compromises hydrazinolysis for *O*-glycan analysis. Here we addressed this problem using low-molarity solutions of ethylenediaminetetraacetic acid (EDTA) in hydrazine for *O*-glycan release. O-linked glycans from a range of different glycoproteins were analyzed, including bovine fetuin, bovine submaxillary gland mucin, and serum immunoglobulin A (IgA). The data for the *O*-glycans released by hydrazine with anhydrous EDTA or disodium salt dihydrate EDTA show high yields of the native *O*-glycans compared with the peeled product, resulting in a markedly increased robustness of the *O*-glycan profiling method. The presented method for *O*-glycan release demonstrates significant reduction in peeling and reduces the number of sample handling steps prior to release.

Protein glycosylation is a common posttranslational modification that influences many protein functions [1,2]. Detailed knowledge of O-linked glycan (serine- or threonine-linked oligosaccharides) structures is invaluable for better understanding their biological functions. *O*-Glycans have a number of different functions, including (but not limited to) protecting the underlying protein as well as epithelial cell surfaces, maintaining protein conformations, and controlling epitopes and antigenicity [3]. They have been reported to determine the cell surface expression and function of cell surface receptors and may be involved in growth regulation [3]. They are also known to be involved in blood clotting, embryogenesis, development, and cell death [3–5], and they are associated with cancer [6,7] and other human diseases and disorders [8–10].

Monitoring and controlling O-glycosylation is important in biopharmaceutical development and quality control in the context of existing and potential drugs that are O-glycosylated (e.g., human erythropoietin) [11,12]. The monitoring of any changes in drug glycosylation during manufacturing processes is tightly regulated by the health authorities around the world (e.g., U.S. Food and Drug Administration, European Medicines Agency). Biopharmaceutical manufacturers must demonstrate that any process changes do not alter the clinical safety or efficacy of the biopharmaceutical [13,14].

A number of techniques for the release, recovery, and analysis of *O*-glycans are available [15]. Nearly all of these techniques use chemical release methods because there is no enzyme available for universal *O*-glycan release from proteins. Two endo-α-*N*-acetylgalactosaminidases have been described that have a high specificity but are limited to the release of the neutral core 1 disaccharide, Galβ1-3GalNAcα1 [16–18].

The most common method for the chemical liberation of *O*-glycans is reductive β-elimination [19–21]. This method of release leads to *O*-glycans that are present in their reduced forms (alditols), which means that they are not amenable to

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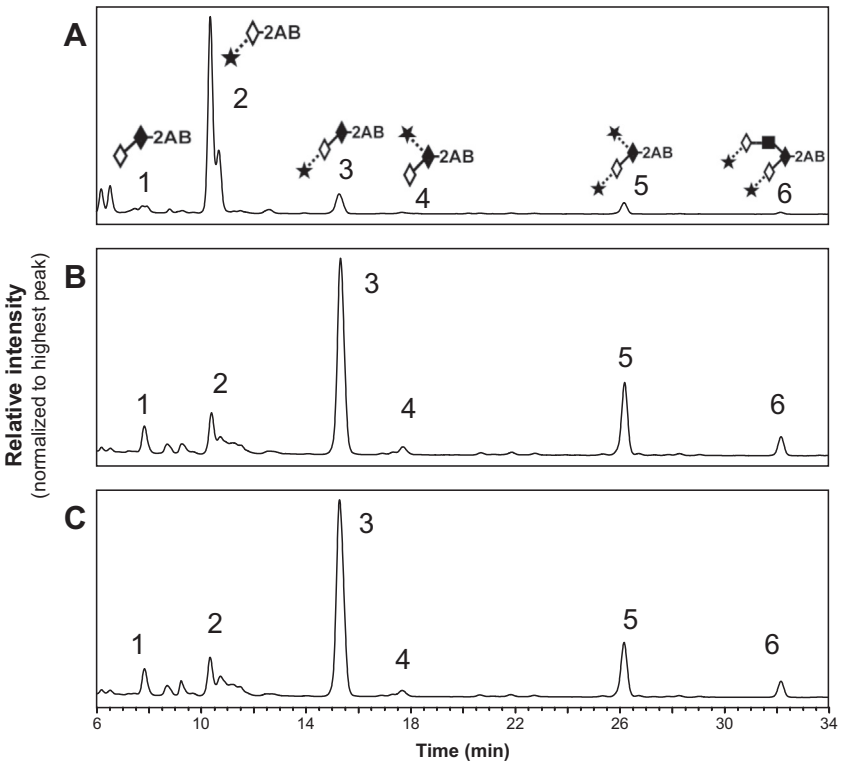

Fig.1. Comparison of HPLC profiles of 2AB-labeled fetuin *O*-glycans. The *O*-glycans were released with hydrazine (A), 50 mM EDTA salt in hydrazine (B), and 20 mM anhydrous EDTA in hydrazine (C). The following symbols are used to depict glycan structures [42]: ◇, galactose; ♦, *N*-acetylgalactosamine; ◈, fucose; ■, *N*-acetylglucosamine; ★, *N*-acetylneuraminic acid; ✯, *N*-glycolylneuraminic acid; dashed line, α-linkage; solid line, β-linkage.

labeling with a fluorescent or ultraviolet (UV)[1] tag. Therefore, the range of analytical methods available for the characterization of *O*-glycans released by reductive β-elimination is restricted, with high-pH anion-exchange chromatography with pulsed amperometric detection [22], mass spectrometry, and nuclear magnetic resonance being the predominantly applied methods [23–26].

Several chemical methods for obtaining *O*-glycans in their nonreduced form have been described, using ethylamine [27], ammonia [28], or lithium hydroxide in an automatic setup [29], but the yields of released *O*-glycans were low and variable. It has been reported that the best method for universal removal of *O*-glycans in their nonreduced form in high yields is hydrazinolysis [15,30–32]. Release of glycans by nonreductive methods is compatible with reducing-end labeling using reagents such as 2-aminobenzamide (2AB) and 2-aminobenzoic acid (2AA) allowing high-performance liquid chromatography (HPLC) with fluorescent or colorimetric (UV) detection [33].

Analysis of O-glycosylation by HPLC-based methods is preferred for a number of reasons. For example, the HPLC or ultra-performance liquid chromatography (UPLC) profiling of 2AB-labeled *O*-glycans is quantitative and highly sensitive, has a reasonably high throughput, and has the ability to provide detailed information about both monosaccharide sequence and the types of glycan linkage [34,35].

Unfortunately, all of the chemical *O*-glycan release methods (reductive and nonreductive) show stepwise degradation of the polysaccharides. The degradation is observed as the removal of one sugar residue at a time from the reducing end. This unwanted side reaction is known as "peeling" [15,31].

Peeling is a general problem when performing the release of *O*-glycans and often results in poor repeatability with variable amounts of the small peeled glycans. This is a major problem for comparability studies or quality control. Many researchers have investigated this peeling phenomenon, and it has been reported that the degree of peeling can be minimized by removal of water prior to hydrazinolysis [31]. We recently showed that the degree of peeling can be greatly reduced by the removal of calcium or other cations prior to hydrazinolysis by employing several washes of the glycoprotein samples with a solution of 0.1% trifluoroacetic acid (TFA) or low-molarity solutions of ethylenediaminetetraacetic acid (EDTA) [35].

Although these "washing" steps are effective, they are also time-consuming and can lead to loss of sample. Here we present an improved hydrazinolysis protocol that allows efficient suppression of peeling but does not require the series of washing steps prior to the release reaction [35]. The method relies on including EDTA—either as its disodium dihydrate salt or in its anhydrous form—as part of the hydrazinolysis reaction mixture. Analysis of the *O*-glycans by both HPLC with fluorescence detection and liquid chromatography–mass spectrometry (LC–MS) demonstrates the efficient suppression of peeling reactions, allowing the acquisition of *O*-glycan profiles in a reproducible manner. The method is shown to work on commercially available standards but also proves to be successful when analyzing immunoglobulin A (IgA) purified by affinity enrichment from human plasma.

Materials and methods

Materials

Anhydrous hydrazine (99.9%) and all other reagents for hydrazinolysis were obtained from Ludger (Oxford, UK). EDTA disodium

[1] *Abbreviations used:* UV, ultraviolet; 2AB, 2-aminobenzamide; 2AA, 2-aminobenzoic acid; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; LC–MS, liquid chromatography–mass spectrometry; IgA, immunoglobulin A; BSM, bovine submaxillary gland mucin (type I-S); HILIC, hydrophilic interaction liquid chromatography; GU, glucose unit; ESI, electrospray ionization.