Analytical Biochemistry 546 (2018) 23–27

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Reactivity-driven cleanup of 2-Aminobenzamide derivatized oligosaccharides

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ARTICLE INFO

Keywords:
Oligosaccharides
N-Glycan
N-Glycan profiling
Glycoprotein
2-Aminobenzamide

ABSTRACT

N-glycan profiling is commonly accomplished by the derivatization of the enzymatically released oligosaccharides with a fluorophore, thereby facilitating their analysis by hydrophilic-interaction liquid chromatography (HILIC). These fluorescent dyes are often present in large excess during derivatization reactions, and their removal is typically required to minimize chromatographic interference. Herein, we report a reactivity-driven 2-phase extraction protocol with the aldehyde reagent octanal, which demonstrated efficient 2-aminobenzamide cleanup as well as high derivatized *N*-glycan recovery. This cleanup method can be performed within minutes, and therefore provides an alternative sample preparation route for *N*-glycan profiling with improved time efficiency and operational simplicity.

Recombinant glycoproteins including monoclonal antibodies (mAb) as a class of therapeutic agents have drawn increasing interest over the past several decades. The inherent structural diversity of the *N*-linked oligosaccharide moieties on these biotherapeutics represents a major source of their heterogeneity, and it is now well-recognized that *N*-glycosylation can significantly impact biological activity (including effector functionality), safety, immunogenicity and clearance [1–5]. As a result, routine characterization of *N*-linked oligosaccharides has become essential to the assessment of product quality and manufacturing consistency [6].

A variety of methods for *N*-glycan profiling of glycoproteins have been reported to date. While direct characterization of either intact [7,8] or digested glycopeptides [9] has been demonstrated with mass spectrometric detection modes, most of the current methods focus specifically on the *N*-glycan moieties that are released from their protein backbone by PNGase F digestion [10], with multiple liquid chromatographic [11–13] and capillary electrophoretic [14,15] methods used for analysis. To enhance the detection sensitivity, the released glycans are typically first derivatized on their reducing end by chemical conjugation with a fluorophore [16,17]. After the derivatization step, the fluorophore, often present in significant excess, is removed by a cleanup procedure to minimize interference in the subsequent analysis [17].

2-Aminobenzamide (2-AB) is one of the most widely adopted labels in HPLC *N*-glycan analysis, with an extensive database of the elution profiles established [18]. Efficient liquid-liquid extraction protocols to remove 2-AB label from the aqueous reaction have not been reported, likely due to the label's inherent hydrophilicity. Instead, its cleanup typically relies on solid-phase extraction (SPE) [13,18] or paper filtration approaches [19]. However, these methods are often laborious and expensive, highlighting the need for the development of alternative cleanup protocols for this hydrophilic label [20].

We envisioned that the primary amine functionality presented on the excess 2-AB label can be employed as a nucleophilic handle that, by reacting with a matching electrophile, can be selectively scavenged and removed from the derivatized glycan sample. In this regard, aldehyde-based reagents appear to be promising candidates due to their well-studied reactivity with nucleophilic amines [21]. Indeed, a wide array of aldehyde-functionalized polymeric resins has been reported as scavengers of primary amines in solution-phase synthesis [22–26]. A preliminary attempt at performing a reaction-filtration sequence with Wang's aldehyde resin, however, revealed it to be ineffective at removing residual 2-AB label from the derivatized glycan sample (data not shown). Alternatively, liquid-form aldehydes can also be considered as the electrophilic species for this application. Simple short-chain aliphatic aldehydes, such as formaldehydes and acetaldehydes, are not desirable due to their high aqueous miscibility, odor and well-documented toxicity [27–30]. Higher chain-length aliphatic aldehydes are significantly less hazardous and are, unlike short-chain aldehydes, mostly immiscible with water with minimal cross-layer solubility. This

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https://doi.org/10.1016/j.ab.2018.01.012
Received 7 December 2017; Received in revised form 11 January 2018; Accepted 15 January 2018

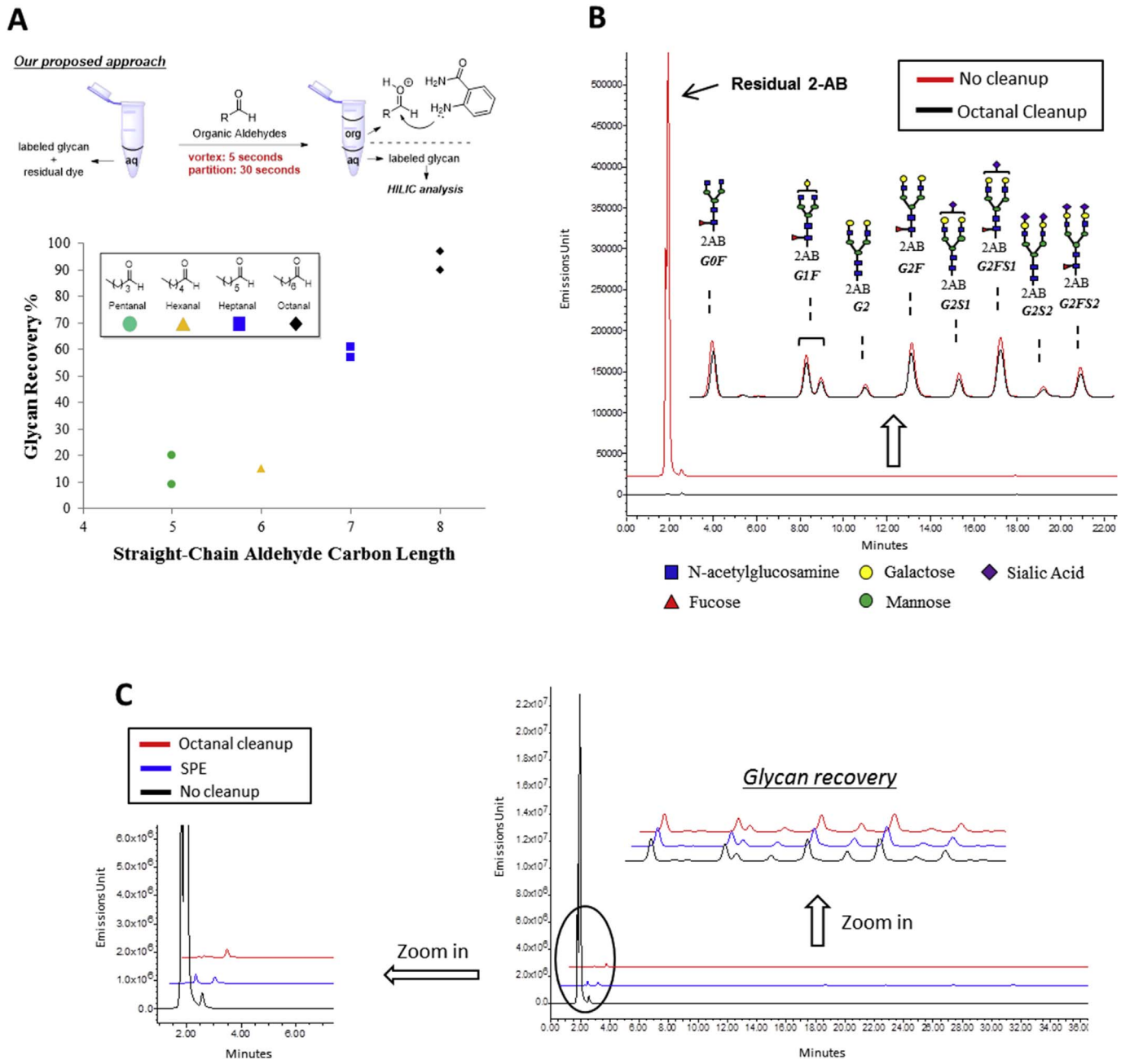

Fig. 1. Demonstration of an alternative 2-aminobenzamide (2-AB) cleanup method using aldehyde derivatives. **(A)**: The derivatized *N*-glycan sample was allowed to mix and partition with a series of varying length aliphatic aldehydes. This resulted in the formation of a two-phase solution, and the bottom aqueous layer was subsequently analyzed by HILIC. Recovery was assessed by normalizing the total peak counts of the derivatized *N*-glycan against the no-cleanup control. **(B):** Representative *N*-glycan HILIC chromatogram post octanal-mediated cleanup, showing the relative amount of residual dyes as well as recovered *N*-glycan profile compared to the no-cleanup control. **(C)**: Comparison of the efficiency of 2-AB removal between our procedure (octanal) and the use of a commercially available SPE *N*-glycan cleanup cartridge.

is important as it allowed us to achieve a one-pot reaction-extraction cleanup scheme (Fig. 1A), in which the glycan labeling mixture can simply be mixed with a chosen aldehyde reagent, thus forcing the amine-aldehyde reaction to take place and ultimately allowing the aqueous/organic partitioning to extract residual 2-AB label from the glycan-containing aqueous layer. Following this rationale, we investigated the use of several higher chain-length aliphatic aldehydes, ranging from 5 to 8 carbons, in carrying out this proposed scheme, using a glycosylated Fc-fusion protein as our model substrate. This fusion protein contains charged and neutral biantennary *N*-glycans. As shown in Fig. 1A and Fig S1, we discovered that the recovery of labeled glycan was inversely correlated with the chain-length of the aldehyde reagents that were examined. Although the exact reason for this observation is unclear, we reasoned that the significant glycan sample loss in the cases of lower chain-length aldehydes can be attributed to either their relatively enhanced electrophilicity that led to competing side-reactions with the derivatized glycan species, or the higher overall miscibility between the organic/aqueous layers. Regardless, octanal cleanup provided not only excellent derivatized sample recovery (Figs. 1A and 2B and Fig S1), but also significant reduction of residual 2-AB label peak in the resulting HILIC chromatogram (Fig. 1B). Importantly, octanal extraction did not lead to any noticeable speciation of the derivatized glycans when the resulting aqueous layer was analyzed by HILIC (Table S1), confirming the reliability of this cleanup method in *N*-glycan sample preparation workflows.

This alternative cleanup method was also compared with a