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Protecting group-free immobilization of glycans for affinity chromatography using glycosylsulfonohydrazide donors

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ABSTRACT

A variety of applications in glycobiology exploit affinity chromatography through the immobilization of glycans to a solid support. Although several strategies are known, they may provide certain advantages or disadvantages in how the sugar is attached to the affinity matrix. Additionally, the products of some methods may be hard to characterize chemically due to non-specific reactions. The lack of specificity in standard immobilization reactions makes affinity chromatography with expensive oligosaccharides challenging. As a result, methods for specific and efficient immobilization of oligosaccharides remain of interest. Herein, we present a method for the immobilization of saccharides using *N*′-glycosylsulfonohydrazide (GSH) carbohydrate donors. We have compared GSH immobilization to known strategies, including the use of divinyl sulfone (DVS) and cyanuric chloride (CC), for the generation of affinity matrices. We compared immobilization methods by determining their immobilization efficiency, based on a comparison of the mass of immobilized carbohydrate and the concentration of active binding sites (determined using lectins). Our results indicate that immobilization using GSH donors can provide comparable amounts of carbohydrate epitopes on solid support while consuming almost half of the material required for DVS immobilization. The lectin binding capacity observed for these two methods suggests that GSH immobilization is more efficient. We propose that this method of oligosaccharide immobilization will be an important tool for glycobiologists working with precious glycan samples purified from biological sources.

1. Introduction

Affinity chromatography is a method for the purification of biomolecules based on specific and reversible binding of a receptor and its ligand.[1] We define the ligand as the binding partner that is immobilized onto an insoluble support and the target as the binding partner that is dissolved in a mobile phase. This methodology, developed almost 45 years ago, has revolutionized the fields of modern biology, chemistry, molecular biology, and biotechnology.[2] Affinity chromatography has often been employed for the purification of lectins using immobilized carbohydrates.[3,4] In contrast to protein-based ligands, there are specific issues for affinity chromatography related to the use of carbohydrate-based ligands. Carbohydrate ligands do not typically contain free amine groups, and chemistries that can form covalent linkages between the hydroxyl groups of the ligand and the solid support are required. The selection of the affinity matrix also contributes to the ligand immobilization strategy. The chosen matrix must have minimal non-specific interactions with the target, be macroporous to allow entry of large biomolecules, be physically and chemically stable, and have uniform characteristics.[1] Commercially available matrices used for affinity purification include agarose, cellulose, silica, polyacrylamide, polystyrene, and dextrose. Sepharose, an agarose-based (Gal-β1,4-[3,6]-anhydro-L-Gal) support, is often used for affinity chromatography and is tolerant of a large pH range and organic solvents.[1,5,6]

Linker chemistry for affinity chromatography should ideally be non-destructive to the binding epitope of the ligand and feature a defined point of attachment. For example, reductive amination can be used to immobilize glycans, but the attached product results in a ring-opened reducing end.[7] A variety of coupling methods for the immobilization of carbohydrate ligands to solid support have been developed,[6,8] but few feature defined linker chemistry for carbohydrates. Limited tools exist for affinity chromatography of complex oligosaccharides obtained from biological sources as free reducing sugars. We chose to compare some established methods for immobilization of carbohydrates to Sepharose with newly available protecting-group free glycosidation chemistries.[9]

Divinyl sulfone (DVS) is an electrophilic homobifunctional reagent capable of crosslinking hydroxyl nucleophiles. In an affinity chromatography experiment, DVS can be used to activate a hydroxyl-containing solid support through a Michael addition to one of the vinyl groups of the sulfone. Crosslinking can be minimized through control of stoichiometry.[10] A range of functional groups can react

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with DVS as nucleophiles, including amine and hydroxyl groups. For hydroxyl nucleophiles the reaction must be carried out at a pH greater than 10 to allow efficient formation of the ether linkage. DVS-coupled affinity ligands should not be exposed to conditions exceeding pH 8.5 to avoid potential retro-Michael degradation.[11] DVS activation has been used for immobilization of carbohydrates onto surfaces and solid supports for affinity chromatography.[3,12] Cyanuric chloride (CC) is a trifunctional, heterocyclic reagent which has been used for the immobilization of carbohydrates for affinity chromatography and binding assays.[13] The reactivity of CC is dependent upon substitution of the ring. The resulting linkage between a carbohydrate ligand and a solid support generated by CC coupling has increased stability at high pH relative to DVS-immobilized ligands.[14]

Recently, Nitz and coworkers have reported a method for protecting group-free glycosidation reactions based on the use of *N*′-glycosyltoluenesulfonohydrazide (GSH) donors.[9,15] This method results in selective reaction at the anomeric position of the donor in the presence of a slight excess of the acceptor. Additionally, in the case of 2-acetimido sugars, such as *N*-acetylglucosamine (GlcNAc), the reaction preferentially forms the β-anomer.[9] The conditions for the reaction are mild for the formation of the activated donor, as well as for the glycosidation reaction. We considered that this chemistry would be ideal for the immobilization of reducing oligosaccharides onto solid support. In this configuration, the hydroxy groups of a solid support would act as the glycosyl acceptor and the glycosyl hydrazide would act as the donor. This method should have several advantages over standard immobilization chemistries for carbohydrates. First, the method would not require an activation of the resin and could be performed under very mild conditions. Moreover, no capping or inactivation step would be required at the end of the reaction, reducing the likelihood of non-specific interactions. Most importantly, this strategy would offer the advantage of providing a regioselective and non-destructive immobilization chemistry for complex glycans obtained from biological sources.

Herein we develop a mild and protecting group-free protocol for the immobilization of carbohydrate ligands to a cross-linked agarose support (Sepharose CL-6B) using GSH donors. We envisioned that this method could provide similar levels of immobilization to existing chemistries but would be complimentary due to its regio- and stereo-selectivity. We compared our method with DVS and CC chemistries as a benchmark for existing methods used in lectin affinity chromatography. Each method was compared based on its immobilization efficiency and carbohydrate binding capacity of the resulting matrix. The immobilized ligands prepared by GSH were also characterized by high-resolution magic angle spinning (HR-MAS) ^{1}H NMR.

2. Results

2.1. Functionalization of monosaccharides with DVS in solution

We first set out to compare the selected immobilization chemistries in solution to help establish reaction conditions and characterization data.[16,17] We took as a starting point the method reported by Fornstedt and Porath for the immobilization of D-mannose onto a solid support with DVS as a linker.[3] Initially we carried out the reaction using five equivalents of DVS to one equivalent of Gal (Fig. 1a). These conditions afforded a mixture of products that included mono, di, tri, and tetra-DVS-functionalized glycosides of Gal (**1**) in 60% yield. Using a similar methodology with reduced equivalents of DVS, we were able to isolate a mixture of modified glycosides of GlcNAc (**2**) in 51% crude yield (Fig. 1b). Lower ratios of DVS to monosaccharide (0.2:1) simplified the product mixture, likely by limiting the number of multiply-functionalized saccharides.[10] The ^{1}H NMR spectra of the mixture **2** revealed that H-1 shifted upfield by 0.15–0.20 ppm for both anomers (from 5.09 to 4.89 ppm for H-1α and from 4.57 to 4.42 ppm for H-1β). Due to overlap with the residual HOD signal, an accurate α/β ratio could not be obtained. These data suggest that substitution occurred primarily at O-1 of the monosaccharide, in addition to minor products resulting from reaction at other sites on the ring. This finding is consistent with observations from Cheng, et al.[12,18] and confirms that the promiscuity of the DVS reaction with glycans can result in complex mixtures.

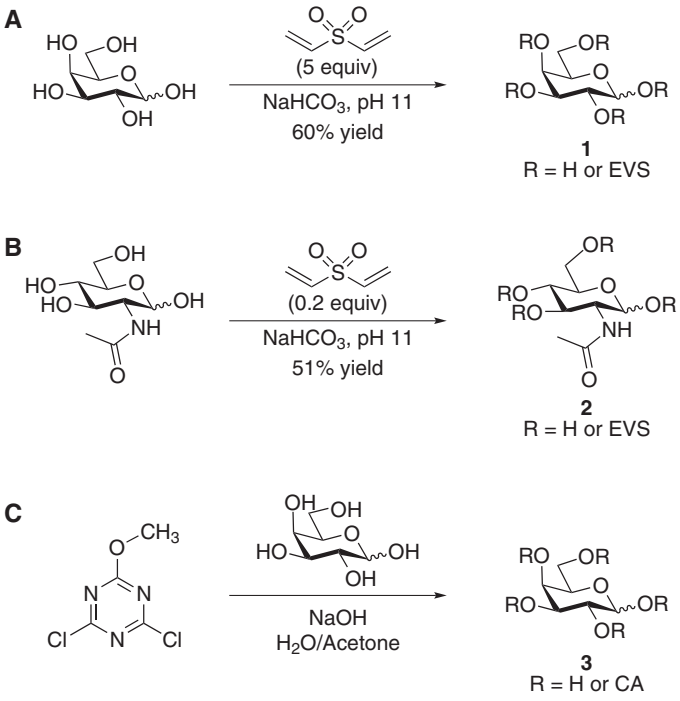

Fig. 1. DVS-functionalization of monosaccharides in solution. Monosaccharides (a) Gal and (b) GlcNAc were reacted with DVS in alkaline conditions to provide mixtures of ethyl vinyl sulfone (EVS) products (1 and 2). Using conditions previously reported,[13] we attempted derivitization of Gal with CC chemistry (c). The reaction afforded a mixture of mono- and di-substituted products (3).

2.2. Functionalization of monosaccharides with cyanuric chloride in solution

Cyanuric chloride (CC) has previously been used for immobilization of carbohydrates to solid support. We attempted to follow reported protocols;[19] however, in our hands these conditions were low yielding and resulted in hydrolysis of the cyanuric chloride to afford the corresponding cyanuric acid (CA). To investigate the site of attachment and the efficiency of the immobilization of carbohydrates using CC, we tested modified conditions (Fig. 1c).[13] The reaction afforded a mixture of products including the hydrolyzed, monosubstituted, and disubstituted triazine (**3**). The products were isolated using preparative TLC as described in Section 4. The ^{1}H NMR spectrum of the material shows a complex set of peaks between 5.5 and 4.4 ppm indicating substitution at more than one of the hydroxyl groups on the monosaccharide and consistent with previous reports.[13]

2.3. Glycosidations of N′-glycosyltoluenesulfonohydrazide donors in solution

Previous reports have used GSH donors for protecting group-free glycosidations in solution.[9,15] Using reported conditions, we generated glycosides using octanol as an acceptor. We generated the octyl glycosides of Gal, lactose (Lac), and GlcNAc (Fig. 2; compounds **5**, **7**, and **9** respectively).[9,15] The glycosidation reactions were