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Short Communication

Picomolar Scale Determination of Carbohydrates Covalently Immobilized on Activated Beads Using Hydroxyl Functionality

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Since carbohydrates are major mediators in cell-to-cell adhesion and communication, the development of specific and strong binders against them could generate promising therapeutics. As the first step towards that goal, sugar molecules have to be immobilized to be used as an affinity matrix. The amino functionality in sugar is the most active nucleophile for the immobilization, if the amino group is available. An alternative and general method is to use the hydroxyl group as a direct nucleophile, but the quantitation of immobilized hydroxyl groups is not easily done. To overcome this limitation, we have developed a method to immobilize various isomers of monosaccharides with *p*-nitrophenyl groups to the beads by using their hydroxyl groups. It was found that the amount of immobilized sugar was independent of the structure of the sugar, but was dependent on the number of hydroxyl groups. We also developed a sensitive method to quantify the amount of immobilized sugar at the picomolar scale by utilizing commercially available glycosidases to release a sensitive reporter molecule, *p*-nitrophenol, and detect it by HPLC. This new technique would allow a facile quantitation method for immobilized sugar molecules, which could be used as the affinity matrix to develop strong binders against biologically important sugars.

Keywords: Carbohydrates, Enzymatic assay, Immobilization.

Introduction

Glycoproteins and glycolipids on the cell surface are important in cell-to-cell communication and cell adhesion (Dwek *et al.*, 1993; Gahmberg and Tolvanene, 1996). Cell surface sugars are composed of heterogeneous glycans which are linked by glycosidic bonds to specific residues of membrane proteins and lipids (Yang *et al.*, 1990; Kim *et al.*, 1994; Park *et al.*, 1994). This complicated structure plays a critical role in communication between cells due to its structural diversity and specificity. Such a specific carbohydrate residue can bind to the specific target molecule on the other cell surface (Weis, 1996). If this interaction between carbohydrate and corresponding mediator is blocked, it is expected that cell adhesion and communication will be perturbed or unregulated. It is reported that excessive or abnormal cell adhesion and cell communication are characteristics of various pathological conditions, such as inflammatory diseases and cancer cell metastasis (MeEver, 1997). Therefore, the development of strong binding molecules to the cell surface sugars can be used as reasonable therapeutics that could block or regulate unwanted carbohydrate interactions in these diseases. Specific antibodies (Bjork *et al.*, 1993; Liu *et al.*, 1995; Kuan & Pastan, 1996) and other neutralizing molecules using combinatorial technologies (Lato *et al.*, 1995) have been attempted to develop such therapeutics, but more work needs to be done.

As a first step to develop such a carbohydrate bound molecule, an affinity matrix with the specific sugar molecules has to be generated and the amount of immobilized sugar has to be quantitated. The sugar molecule is usually immobilized to the activated matrix through a nucleophilic amino group to form an amide bond (Smith, 1980). If there is no amino functionality in the molecule, chemical transformation from a hydroxyl group to an amino group has to be done due to the superior reactivity of the amino group over other nucleophilic groups (Spinola & Jeanloz, 1970). However, the yield of the chemical process is not satisfactory and it usually requires tedious protection/deprotection steps (Dunstan &

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Hough, 1972). Transformation of the hydroxyl group to an aldehyde is widely used for the immobilization, but important functionalities might be ruined by oxidizing agents (Bayer *et al.*, 1988; Hoffman & O'Shannessy, 1988). Instead of such a complex chemical transformation, the hydroxyl group could be used as a direct nucleophile to form an ester bond. However, this method is not widely used, because it is hard to control the reaction due to the similar reactivities of hydroxyl groups in sugar and water. When the structural information on the sugar molecule is limited, it is more difficult to control the reactivity and the amount of immobilized sugar. Even when the sugar is covalently attached to beads, one needs multi-step processes to quantify the amount of immobilized sugars using a variety of glycosidase enzymes (Harvey, 1998a). Furthermore, the conditions of these methods are generally harsh and irreversible. The matrix and the immobilized sugar molecules could be ruined during the quantification. Generally, the preparation of the affinity matrix and the quantification require enormous time and effort. Therefore, a facile and mild method to quantify the amount of ester-linked sugars to the beads in one step has to be developed to save time and effort for the preparation of the affinity matrix. In this report, we present a facile method to obliviate these problems by immobilizing sugars with hydroxyl groups and by quantifying them with a simple enzymatic method.

Materials and Methods

Reagents All the sugars used in this research were purchased from Sigma and were used without further purification. The five commercially available beads used were Affi-gel 10 gel (BioRad), Epoxy activated Sepharose 6B (Sigma Cat. # E6754), Cyanogen Bromide Activated Sepharose 4B (Sigma Cat. # C-9142), Epichlorohydrin-Activated Agarose (Sigma Cat. # E-5142), and 6-Aminohexanoic acid (Sigma Cat. # A-6763). The commercially available α-glucosidase (Sigma Cat. # G-6136), amyloglucosidase (Sigma Cat. # A-7420), cellulase (Sigma Cat. # C-0901), α-fucosidase (Sigma Cat. # F-7753), α-mannosidase (Sigma Cat. # M-7257), α-galactosidase (Sigma Cat. # G-8507), and β-N-acetylglucosaminidase (Sigma Cat. # A-2415) were used diluted as indicated in the legends of Tables and Figures.

Attachment of sugar to the matrix bead Twenty five milligrams of beads were washed twice with 500 μl of sodium acetate buffer (10 mM, pH 4.5) to remove stabilizing agents, followed by five times with 500 μl of N-[2-hydroxyethyl] piperazine-N'-2-ethanesulfonic acid] (HEPES) buffer (100 mM, pH 8.0). The resulting beads were then placed in 400 μl of sugar solution in HEPES buffer and stirred for 1 h at room temperature. Then, 40 μl of 1.0 M ethanolamine in the same buffer was added to make 0.1 M ethanolamine solution and stirred for another 1 h at room temperature. The beads were then filtered through micro-centrifuge filters and washed 5 times with 500 μl of HEPES buffer. The resulting beads were stored in the same buffer at 4°C.

Measuring the concentration of immobilized sugar on beads To the sugar-immobilized beads suspended with 200 μl of 2-[N-morpholino]ethanesulfonic acid (MES) buffer (10 mM, 100 mM NaCl, 0.02 % sodium azide), 5 μl of glycosidase solution already dissolved in the same buffer was added and incubated for 30 min at 37 °C. Then, the beads were filtered out and 10 μl of the filtrate was directly injected into a high performance liquid chromatography column (HPLC) without any pre-treatment. *p*-Nitrophenol hydrolyzed by the enzymes was measured at 315 nm. An isocratic run of 50% methanol and 50% water (0.1% trifluoroacetic acid) with 0.8 ml/min flow rate gave a 5.7 min retention time for *p*-nitrophenol using a C18 Microsorb column (4.6 mm × 15 cm with a 4.6 mm × 2.5 cm guard module) as stationary phase. The amount of *p*-nitrophenol in each assay solution was calculated using the standard plots shown in Fig. 1.

Results and Discussion

We focused on two aspects of sugar immobilization in this report: (1) how to control the amount of immobilized hydroxyl groups on the commercially available matrix by changing the concentrations of sugars, and (2) how to develop a sensitive method to measure the amount of sugar immobilized on the matrix. *p*-Nitrophenylated pyranoside sugars were employed as model carbohydrates; not only because they contain the hydroxyl functionality only as a nucleophile, but also because they can be easily cleaved to produce *p*-nitrophenol, a sensitive reporter molecule.

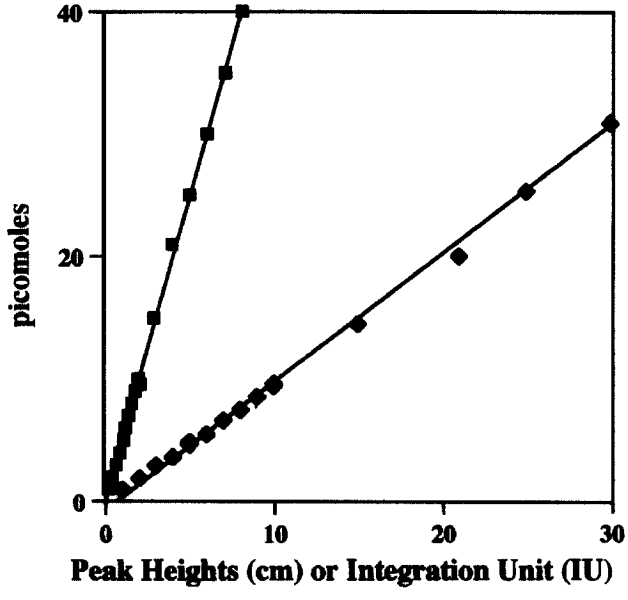

Fig. 1. Standard plots for quantification of *p*-nitrophenol. The concentrations of *p*-nitrophenol in the assay solutions were calculated by the linear relationship between the absolute amount of *p*-nitrophenol (One picomole was quantified by injecting 1.0 μl of 1.0 μM solution in MES buffer into HPLC.) and peak height (cm; –■–) or integration unit (IU; –◆–) of the peak at 5.7 min retention time.

Commercially available glycosidases were used as mild agents to cleave the glycosidic bond of immobilized sugar to release *p*-nitrophenol.

Five commercially available beads were used to attach the *p*-nitrophenylated monosaccharides onto the matrix. The immobilized sugar was quantified by measuring the amount of released *p*-nitrophenol which were previously cleaved by glycosidases. We used glycosidases at conditions near to physiological pH, and the aliquot amount of sample was injected into HPLC without pre-treatment to measure the cleaved reporter molecule. *p*-Nitrophenol was cleaved from the *p*-nitrophenyl-α or β-D-glucopyranoside immobilized on the activated beads within less than 1 h at pH 4.5–7.0 at 37°C and was quantitated using HPLC.

In Table 1, the amount of immobilized sugar is shown using various kinds of sugar and matrices. When different matrices were used, 2–3 fold differences in the amount of immobilized sugar were observed due to the reactivity and concentration differences of the activated groups in the matrices (Entries 1–5). However, when the same matrix was used, almost the same amount of monosaccharide was covalently linked regardless of the structure of the monosaccharide (Entries 5–10). In addition, when α-L-fucopyranoside (Entry 9; absence of a 1° OH) and N-acetyl-β-D-glucosaminide (Entries 10; absence of a 2° OH) were applied, the same amount of sugar was attached. It is suggested that the reactivity difference between primary and secondary hydroxyl groups is negligible. The reactivity of two anomers also showed no differences as in α- and β-glucopyranosides coupling to the matrix (Entries 5–6).

Various concentrations of the sugar solutions were then applied to test whether the amount of immobilized sugar could be controlled by the concentration of the sugar solution. As shown in Fig. 2, the concentrations of sugar solution and the amount of immobilized sugar showed some correlation in all cases. When the concentration of sugar was highly diluted, the amount of immobilized sugar was dependent on the concentration of sugar. However, when the concentration of sugar was similar to or more than the concentration of the activating groups in the bead, the amount of immobilized sugar deviated from the linearity. Our results, however, suggested that the reaction of sugar with the activated group in the bead is faster than that of water. Therefore, the concentration of the immobilized sugar could be controlled by adjusting the concentration of the sugar solution.

As shown in Table 1, the amount of immobilized sugar was mainly dependent on the number of hydroxyl groups in the sugar. This result was confirmed by using di-, tri-, and longer oligosaccharides as immobilizing sugar sources, as shown in Fig. 3. The amount of immobilized sugar increases as the number of pyranoside rings in the saccharides increases. Approximately 2 pmol of β-monosaccharide per mg of CNBr-activated beads were attached, while when β-di, tri-, tetra-, and penta-saccharides were applied the amounts of immobilized sugar were doubled

Table 1. Amount of various sugar substrates immobilized to five different activated bead matrices.

Entry	Matrix	Sugar Substrate	Enzyme[c]	Amount of Sugar Immobilized (pmol/mg)
1	Epoxy-activated sepharose 6B 25 mg[a]	*p*-Nitrophenyl-α-D-glucopyranoside	α-glucosidase	0.56
2	Cyanogen-activated sepharose 4B 25 mg[a]	*p*-Nitrophenyl-α-D-glucopyranoside	α-glucosidase	2.1
3	Epichlorohydrin-activated agarose 25 mg[a]	*p*-Nitrophenyl-α-D-glucopyranoside	α-glucosidase	0.53
4	6-Aminohexanoic acid 36 mg[b]	*p*-Nitrophenyl-α-D-glucopyranoside	α-glucosidase	0.45
5	Affi-gel 10 gel 25 mg[b]	*p*-Nitrophenyl-α-D-glucopyranoside	α-glucosidase	0.88
6	Affi-gel 10 gel 22 mg[b]	*p*-Nitrophenyl-β-D-glucopyranoside	β-glucosidase	0.91
7	Affi-gel 10 gel 21 mg[b]	*p*-Nitrophenyl-α-D-galactopyranoside	α-galactosidase	1.1
8	Affi-gel 10 gel 30 mg[b]	*p*-Nitrophenyl-α-D-mannopyranoside	α-mannosidase	0.80
9	Affi-gel 10 gel 21 mg[b]	*p*-Nitrophenyl-α-L-fucopyranoside	α-fucosidase	1.2
10	Affi-gel 10 gel 56 mg[b]	*p*-Nitrophenyl-N-acetyl β-D-glucosaminide	β-N-acetylgluco-saminidase	1.1

[a] The matrices were weighed before coupling.

[b] The matrices were weighed after removal of the solvent and dried out.

[c] The units of α-glucosidase, β-glucosidase, α-galactosidase, α-mannosidase, α-fucosidase, and β-N-acetylglucosaminidase used were 1.2, 0.21, 0.24, 0.55, 0.017, and 0.060, respectively.

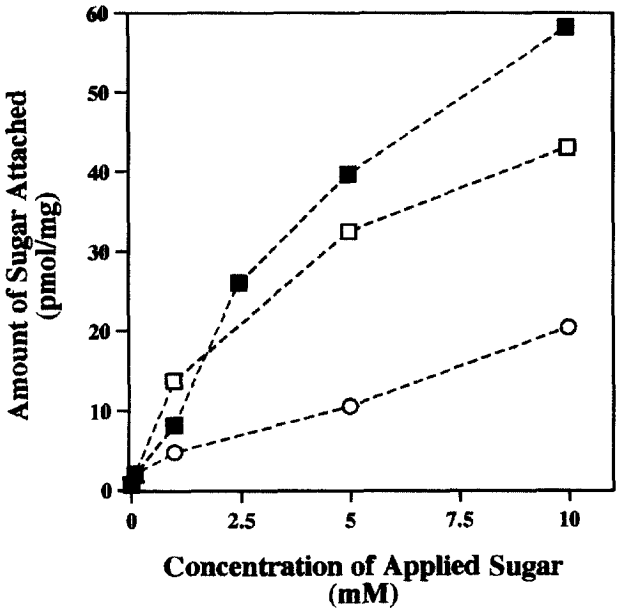

Fig. 2. Effect of concentration of applied sugar on amount of immobilized sugar. Series of *p*-Nitrophenyl-α-D-glucopyranoside/CNBr-activated beads (–■–), *p*-nitrophenyl-β-D-glucopyranoside/CNBr-activated beads (–□–), and *p*-nitrophenyl-α-D-glucopyranoside/6-Amino beads (–○–) were performed. The units of α-glucosidase and β-glucosidase used were 1.2 and 0.3, respectively.

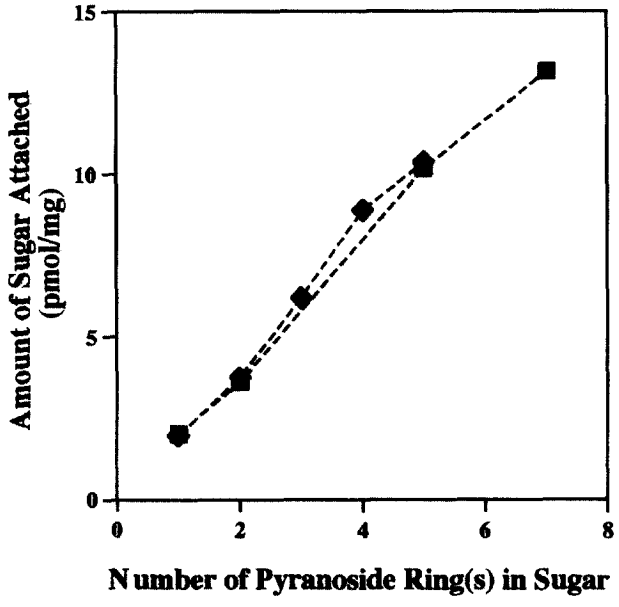

Fig. 3. Amount of immobilized sugar with different number of pyranoside rings in applied sugar. Series of *p*-nitrophenyl-α-anomeric saccharides (–■–). and *p*-nitrophenyl-β-anomeric saccharides (–◆–) were performed. A mixture of α-glucosidase and amyloglucosidase and a mixture of β-glucosidase and cellulase were used for the cleavage of α- and β-glucosidic bonds, respectively. The units of α-glucosidase, amyloglucosidase, β-glucosidase, and cellulase used were 1.2, 3.7, 0.3, and 2.8, respectively.

(3.8 pmol/mg), tripled (6.2 pmol/mg), quadrupled (8.9 pmol/mg), and quintupled (10.3 pmol/mg), respectively. This trend was also observed in α-anomers of oligosaccharides up to the heptasaccharide. In conclusion, the amount of sugar immobilized on activated beads depends only on the number of pyranoside rings in the sugar, irrespective of the structure of the sugar.

The glycosidases are known to specifically distinguish a variety of sugar isomers. However, all of the immobilized sugars in our results were promptly cleaved by these enzymes within 30 min near the physiological pH, affording that glycosidase specificity is not changed by a loss of hydroxyl functionality in the immobilization, regardless of the position in the pyranoside. This result might be due to the broad specificities (mM order) or high k_{cat} values which are characteristic of the general glycosidase enzymes. Considering the structural and conformational complexities of cell surface carbohydrates, it is difficult to fully elucidate the detailed structure. However, it is relatively easy to deduce the number of pyranoside rings in the carbohydrate antigen before any information on the structure is available (Harvey, 1996a; 1996b). In this respect, our results could be useful for estimating how much sugar could be immobilized if we know the number of pyranoside rings in the target sugar molecule.

In summary, we have demonstrated that picomolar quantities of sugars could be immobilized via hydroxyl groups to the activated beads and it could be easily determined by the amount of *p*-nitrophenol released from the immobilized sugar by natural glycosidases near physiological pH. It could also be used as a general method to estimate the exact amount of activated groups in the matrix beads. If a more sensitive reporter molecule such as a fluorescent tagging molecule is used, sub-picomolar quantities of immobilized carbohydrates could be detected where such sensitivity is necessary (Rye, 1992).

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