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Binding of carbohydrates to solid supports Part 3: Reaction of sugar hydrazones with polystyrene substituted with aromatic aldehydes

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The hydrazones of glucose and *N*-acetylglucosamine, as models for the residues at the reducing termini of glycans, were covalently and reversibly bound in good yield to hydroxybenzaldehydo ligands attached to a polymer support. The binding, by a sugar azine linkage, occurred within two hours at room temperature at neutral pH, and efficient recoveries of sugars from the beads were achieved by displacement with aqueous hydrazine hydrate, ethanolic benzaldehyde, or aqueous acetone. Enzyme modification of glycans was demonstrated by separation of the products of hydrolysis of lactose hydrazone with β-galactosidase, using hydroxybenzaldehyde-derivatized polystyrene beads. Addition of a spacer arm to aminopolystyrene beads, for binding of reducing sugars as Amadori compounds to the aromatic amine function, was also investigated.

***Keywords*: sugar hydrazones, sugar azines, immobilization, aminopolystyrene, glycan modification**

Introduction

The many important and varied biological roles of glycoconjugates [1–3] have lead to increasing interest in methods for glycan analysis. Immobilization of glycans can significantly simplify both their analysis and structural modification. Applications include enzyme sequencing [4], affinity chromatography [5] and immunoassay [6], the removal of terminal residues by exoglycosidases [4], and chain lengthening or glycan synthesis by glycosyltransferases [7, 8], exoglycosidases [9], or by chemical means [10].

Methods of immobilization of glycans generally involve the reducing terminus. Reducing sugars have been bound to amino-derivatized solid supports, as glycosylamines to aliphatic amino ligands [5] or as Amadori compounds to aminopolystyrene [11], or they have been reductively coupled to amino ligands using sodium cyanoborohydride [12]. As the reductive coupling is slow [13], glycans have been activated for binding by conversion to glycosylamines, which are further derivatized to stabilize them before binding [14, 15]. Other methods of derivatization of reducing sugars before binding include reductive amination with 2-(4-aminophenyl)ethylamine [16] or with trifluoroacetamidoaniline [17].

We reported previously a mild and efficient method of immobilization of sugar hydrazones as thiothiosemicarbazones to isothiocyanatopolystyrene beads, from which moderate recoveries of sugars could be achieved [18]. We now present another mild method of higher efficiency for immobilization of sugar hydrazones by coupling them to hydroxybenzaldehyde ligands by means of an azine linkage, from which sugars are recovered in high yield.

Materials and methods

Polystyrene beads with specular finish, 3.2 mm diameter, were from the Precision Plastic Ball Co. (Ilkley, U.K.), D-[U-^{14}C] glucose (3 mCi mmol^{-1} in 3% ethanol) and *N*-acetyl-D-[1-^{14}C] glucosamine (57 mCi mmol^{-1} in 2% ethanol) were from Amersham International plc (Amersham, U.K.) and Ultima Gold scintillant from Packard (Groningen, Holland). β-Galactosidase was from Sigma Chemical Co. (St. Louis, MO), hydrazine hydrate was from Aldrich Chemical Co. (Milwaukee, WI), and all other chemicals were of analytical grade. Phosphate-buffered saline (pH 7.4) contained 0.01 M sodium phosphate and 0.14 M sodium chloride. Chlorhexidine (0.002%) was present in all solutions as a preservative. The aminopolystyrene beads were stored under 0.01 M hydrochloric acid and other

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beads under water at 4°C. Radiochemical counting was carried out on 2 × 100 µl aliquots of supernatants, each pair taken from duplicate tubes. Thin-layer chromatography (TLC) was carried out on aluminium plates precoated with silica gel 60 (E. Merck, Darmstadt, Germany).

Diazotization of aminopolystyrene beads and coupling of hydroxybenzaldehydes

Sodium nitrite (1.2 g, 17 mmol) in chilled water (5 ml) was added slowly to a dispersion of aminopolystyrene beads [11] (10g, *ca.* 600 beads, *ca.* 0.6 mmol of amino groups) in hydrochloric acid (10%, 12 ml, 33 mmol) cooled in an ice bath. After mixing well, the beads were drained, washed with ice-cold water, then added to 2-hydroxybenzaldehyde (630 µl; 6 mmol), or 3- or 4-hydroxybenzaldehyde (735 mg, 6 mmol) in chilled sodium hydroxide (2.5 M, 12 ml). The beads changed color immediately. The mixture was kept on ice for 5 min, then the beads were drained and washed several times first with ethanol and then with water. The hydroxybenzaldehyde-coupled beads retained their activity for at least 12 months in 0.002% chlorhexidine at 4°C.

Estimation of the degree of derivatization of hydroxybenzaldehydeazopolystyrene beads

Residual amino groups were measured by reaction with 4-nitrobenzaldehyde [11]. Hydroxybenzaldehyde groups were measured by equilibrating the beads for 1 h in phosphate buffer (0.1 M, pH 6), then drained and added to 4-anisidine (4 mM, 2 ml, 4 beads per tube), and shaken gently at room temperature for 16 h. Aliquots (200 µl) of the supernatants were diluted to 3 ml with ethanol and the change in ultraviolet absorbance at 232 nm measured.

Addition of spacer arms to aminopolystyrene beads

Reductive coupling of glutaraldehyde to aminopolystyrene

Aminopolystyrene beads (24, *ca.* 12 µmol amino groups) were added to a stirred solution of glutaraldehyde (25%, 45 µl, 0.12 mmol) in methanol (5 ml). Sodium cyanoborohydride (15 mg, 0.24 mmol) was added, and the pH was maintained at approximately 6 for 30 min by addition of aliquots of hydrochloric acid (0.01 M). After stirring overnight, the solution had a final pH of 7.7. The beads were drained and washed well with water.

Reductive coupling of 3- or 4-nitroaniline and reduction of the terminal nitro group

Sodium cyanoborohydride (20 mg, 0.32 mmol) in methanol (1 ml) and 3- or 4-nitroaniline (50 mg, 0.36 mmol) in methanol (4 ml) and were added to vials containing glutaraldehyde-derivatized beads (50 each, *ca.* 45 µmol) and stirred overnight. After removal of the supernatant and washing with methanol and water, the terminal nitro groups on the beads were reduced with stannous chloride in concentrated hydrochloric acid [11]. The binding of glucose was carried out under the same conditions as for aminopolystyrene [11].

Binding of sugar hydrazones to 2-, 3- and 4-hydroxybenzaldehydeazopolystyrene beads

The beads were equilibrated in phosphate buffer (pH 6 or 7) for 1 h before binding. The dry hydrazones of [^{14}C] glucose or [^{14}C] *N*-acetylglucosamine, prepared by reacting the sugars with hydrazine hydrate [18], were dissolved in water or 0.2 M phosphate buffer (pH 6 or 7) immediately before use. Duplicate sets of three hydroxybenzaldehydeazopolystyrene beads were added to solutions of [^{14}C] glucose hydrazone (300 µl, 3 nmol) or *N*-acetylglucosamine hydrazone (300 µl, 0.15 nmol) and shaken gently at room temperature. Aliquots (100 µl) of the supernatants were taken for radiochemical counting. The beads were washed rapidly with water (6 × 500 µl).

Stability of binding and recovery of bound sugars

The stability of the azine binding to beads at various pH values was studied by determining loss of radiolabeled sugars in 0.2 M sodium phosphate buffer (300 µl per tube of 3 beads) at room temperature, by counting of the supernatants (Table 1). Recovery of reducing sugars or sugar hydrazones after treatment of the sugar azine-bound beads with ethanolic benzaldehyde, aqueous acetone, or hydrazine hydrate (Table 2) was measured similarly.

Stabilization of binding by reductive coupling

Sodium cyanoborohydride (150 µl, 0.1 M, 15 µmol) in phosphate buffer (1.0 M, pH 6) was added to tubes containing [^{14}C] *N*-acetylglucosamine hydrazone (0.15 nmol) in water (150 µl) and three 2-hydroxybenzaldehydeazopolystyrene beads. Alternatively, sodium borohydride (3 mg, 80 µmol) was added to tubes of three beads containing [^{14}C] *N*-acetylglucosamine hydrazone (0.15 nmol) in water (300 µl). The tubes were gently agitated for 16 h, together with control tubes without reducing agent, and aliquots of the supernatants were radiochemically counted to determine the total bound sugars. The proportion of sugars bound by an unreduced azine link was determined by removal with hydrazine hydrate (100%, 300 µl) at room temperature for 1 h and counting aliquots (100 µl) of the supernatants.

β-Galactosidase hydrolysis of lactose hydrazone and recovery of glucose

Lactose hydrazone (200 µl, 50 mM, 10 µmol) in phosphate buffer (50 mM, pH 7.0) was incubated with β-galactosidase (25 U) for 4 h at 28°C. An aliquot (100 µl) of the solution was then diluted with water (200 µl) and added to twenty 2- or 3-hydroxybenzaldehyde beads. After standing in the solution overnight, the beads were rinsed

Table 1. Loss of [^{14}C] *N*-acetylglucosamine hydrazone bound as an azine to 2-hydroxybenzaldehydeazopolystyrene beads or as a thiosemicarbazone to isothiocyanatopolystyrene beads [18], on standing in aqueous buffers

Solution	*pH*	*Loss of sugar (%) from azine*[a]	*Loss of sugar (%) from thiosemicarbazone*[b]
0.2 M phosphate buffer	3	48	6
0.2 M phosphate buffer	4	28	4
0.2 M phosphate buffer	5	9	1
0.2 M phosphate buffer	6	7	0.6
0.2 M phosphate buffer	7	10	0.3
0.2 M phosphate buffer	8	11	
0.2 M phosphate buffer	9	13	
0.01M phosphate-buffered saline	7.4	10	0.7

[a]At room temperature for 16 h.
[b]At room temperature for 2 h.

well with water and heated with aqueous acetone (25%) for 2 h in a boiling water bath. The supernatant was concentrated and analyzed by thin layer chromatography, together with the β-galactosidase solutions, before and after treatment with hydroxybenzaldehyde-derivatized beads. The solvent systems used were butanol-acetone-acetic acid-water (35:35:7:23 v/v) [19], acetonitrile-water (85:15 v/v) [20], and butanol-2-propanol-0.5% boric acid (40:50:10 v/v) [21]. The reducing sugars and the sugar hydrazones were detected with lead tetraacetate (1 g) in ethanol (100 ml) or 4-nitrophenylhydrazine (0.1%) in hydrochloric acid (32%)/ethanol (1:9 v/v).

Results

Derivatization of polystyrene

The aminopolystyrene beads were diazotized and coupled to 2-, 3-, or 4-hydroxybenzaldehyde (Figure 1). The extent of azo coupling was evaluated indirectly by determining the decrease in amino functionality, using 4-nitrobenzaldehyde [11]. A reduction from 970 nmol to 100 nmol of amino groups per bead was observed, indicating conversion of 870 nmol of hydroxybenzaldehyde groups per bead. Direct measurement of the hydroxybenzaldehyde groups, by the uptake of 4-anisidine, indicated 650 nmol only of aldehyde groups per bead. This lower estimate is attributed to a less favorable equilibrium for the formation of a Schiff's base in the analysis.

To provide a spacer arm for Amadori binding of reducing sugars to aminopolystyrene [11], glutaraldehyde was first reductively coupled to the amino group with sodium cyanoborohydride, and the aromatic amines, 3-nitro- or 4-nitroaniline, were then reductively coupled to the free aldehyde group, followed by reduction of the terminal nitro group (Figure 2). Measurements of the degree of amino derivatization of the beads showed a reduction from 701

Table 2. Recovery of [^{14}C] *N*-acetylglucosamine by the action of carbonyl compounds or of [^{14}C] *N*-acetylglucosamine hydrazone by the action of hydrazine hydrate, from azine and thiosemicarbazone bindings of sugars to polystyrene beads

Reagent	*Temperature (°C)*	*Sugar (%)*[a] *recovered from azine*	*Sugar (%)*[a] *recovered from thiosemicarbazone*
25% hydrazine hydrate	25	77	27
100% hydrazine hydrate	25	79	46
25% acetone in water	25	16	18[b]
25% benzaldehyde in EtOH	25	16	3
25% hydrazine hydrate	100	90	
100% hydrazine hydrate	100	95	83[c]
25% acetone in water	100	85	45
25% benzaldehyde in EtOH	100	93	67

[a]Treatment for 2 h unless otherwise specified.
[b]Treatment for 2 h at 55°C.
[c]Treatment for 24 h.

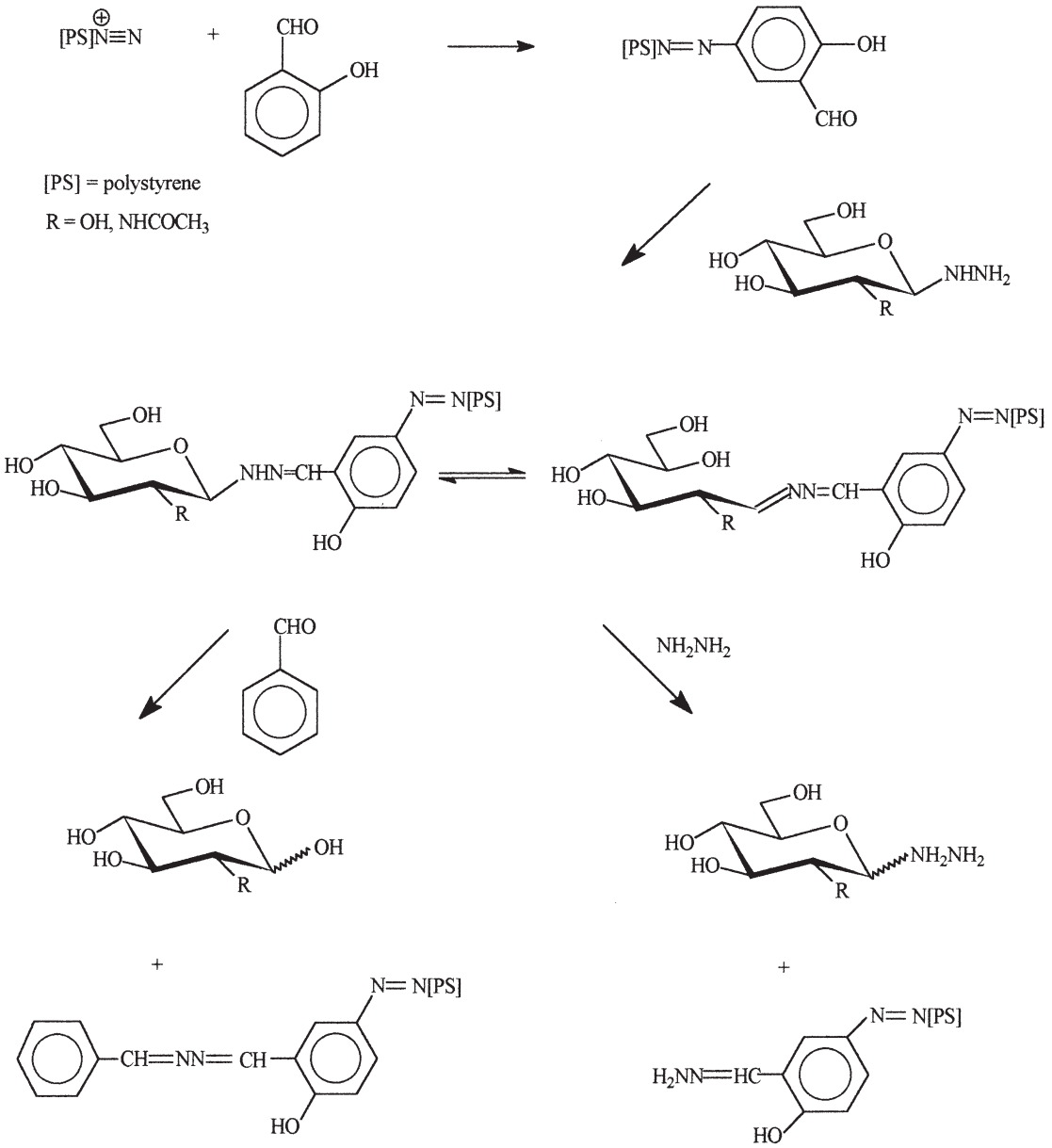

Figure 1. Coupling of hydroxybenzaldehydes to diazotized aminopolystyrene, immobilisation of sugar hydrazones, and recovery of reducing sugars or sugar hydrazones.

nmol per bead to 54 nmol per bead after the first reductive coupling (92% efficiency) and finally an increase after addition of 3- and 4-nitroaniline and subsequent reduction, to 613 nmol per bead and 575 nmol per bead, corresponding to overall efficiencies of spacer arm addition of 88% and 82%, respectively. Similar figures were obtained when comparing binding of [^{14}C] glucose to aminopolystyrene beads (71% binding) and with beads to spacer arms prepared with 3-nitro- or 4-nitroaniline (61% and 62% binding, respectively), corresponding to 86% to 87% of glucose bound to the beads with spacer arms compared with aminopolystyrene itself.

Binding of sugar hydrazones to hydroxybenzaldehyde ligands

The extent of azine binding of the hydrazones of [^{14}C] glucose or [^{14}C] *N*-acetylglucosamine, the stability of the binding (Figure 1), and the recovery of bound sugars were measured radiochemically. Bindings of 85% to 90% of sugar hydrazones to 2- or 3-hydroxybenzaldehyde ligands were obtained within 1 to 2 h at room temperature at pH 7 (Figure 3). The binding of *N*-acetylglucosamine hydrazone to immobilized 2- and 3-hydroxybenzaldehyde ligands was found to be about 50% higher than to 4-hydroxybenzaldehyde ligands.

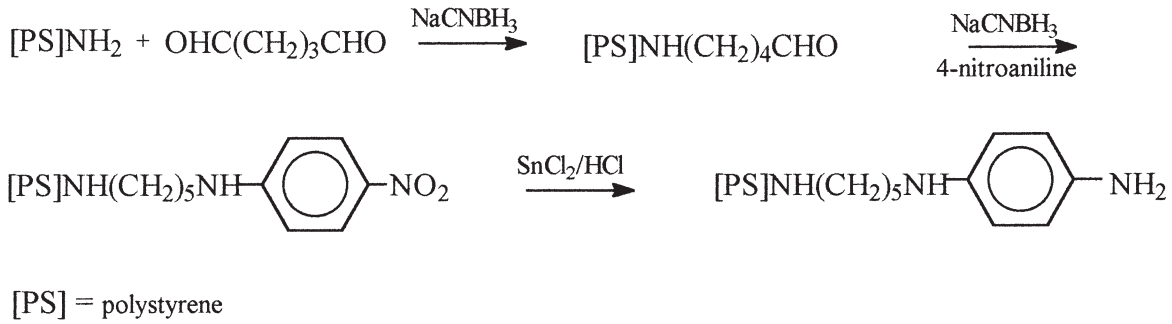

Figure 2. Addition of a spacer arm to aminopolystyrene by reductively binding an excess of glutaraldehyde, then binding 4-nitroaniline and reducing the terminal nitro group.

Stability of the sugar azine binding and recovery of immobilised sugars

Losses of azine-bound sugar hydrazones were 7% to 10% after incubation for 16 h in 0.2 M phosphate buffer (pH 5 to 7.4) (Table 1). Similar losses in this pH range were observed after 1.5 to 2 hrs. *N*-acetylglucosamine hydrazone was recovered from the beads in 75% to 80% yield using hydrazine hydrate at room temperature, and in above 90% yield at 100°C. Reducing sugars were recovered in over 90% yield with benzaldehyde in ethanol and in 85% yield with aqueous acetone at 100°C (Table 2).

Stabilization of the sugar azine binding

Binding of sugar hydrazones to the hydroxybenzaldehyde ligands in the presence of sodium cyanoborohydride in pH 6 phosphate buffer resulted in 64% to 70% uptake of the sugars, compared with 84% in the absence of reducing agent (Table 3). Subsequent treatment of the reductively bound beads with hydrazine hydrate resulted in the loss of 21% of the bound sugar, indicating that reduction of the azine linkage was incomplete. Less than 10% binding was observed in the presence of alkaline sodium borohydride.

Immobilization of modified glycan hydrazone

Thin layer chromatography was used to demonstrate the cleavage of lactose hydrazone by β-galactosidase to produce galactose and glucose hydrazone, the removal of glucose hydrazone from solution using hydroxybenzaldehyde-substituted beads, and the subsequent recovery of glucose from the beads by aqueous acetone (Figure 4). Butanol-acetone-acetic acid-water (35:35:7:23 v/v) was used to distinguish galactose (R_f 0.54) and glucose hydra-

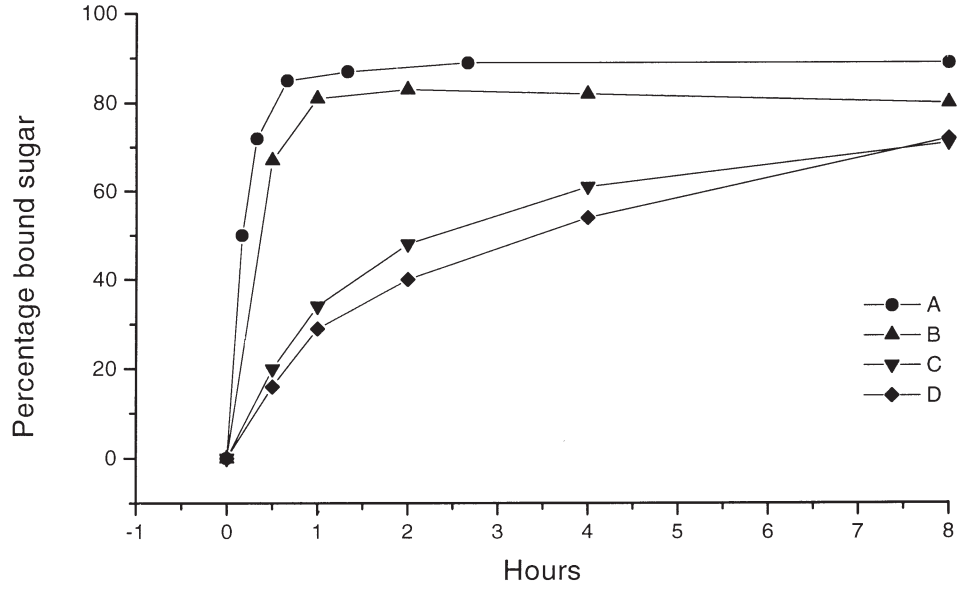

Figure 3. Time courses of the binding at room temperature of **A**. [^{14}C] *N*-acetylglucosamine hydrazone and **B**. [^{14}C] glucose hydrazone to 2-hydroxybenzaldehydeazopolystyrene beads in water, and of **C**. [^{14}C] *N*-acetylglucosamine hydrazone and **D**. [^{14}C] glucose hydrazone to isothiocyanotopolystyrene beads in 0.2 M phosphate buffer at pH 8 [18].

Table 3. Binding of [^{14}C] *N*-acetylglucosamine hydrazone to 2-hydroxybenzaldehyde-derivatized polystyrene beads

Method of binding	*Binding (%)*	*Hydrolysis[b] of bound sugar (%)*	*Recovery[c] of bound sugar (%)*
nonreductive	84	8	63
reductive[a]	64	3	21

[a]Binding in phosphate buffer (1.0 M, pH 6) with sodium cyanoborohydride.
[b]Hydrolysis in 0.1 M phosphate buffered saline at pH 7.4 at room temperature for 16 h.
[c]Treatment with 100% hydrazine hydrate for 1 h.

zone (R_f 0.56) from lactose hydrazone (R_f 0.44) after the β-galactosidase treatment. Since the galactose and glucose hydrazone spots overlapped, the reaction mixture was also run in acetonitrile water (85:15 v/v), in which lactose hydrazone stayed at the origin, while glucose hydrazone ran at R_f 0.04 and galactose at R_f 0.25. The absence of glucose, which would arise from hydrolysis of lactose hydrazone to lactose, followed by its cleavage, was demonstrated in butanol-2-propanol-0.5% boric acid (40:50:10 v/v), where glucose (R_f 0.48) and galactose (R_f 0.41) could be differentiated, and the absence of lactose (R_f 0.31) was also shown. Removal of glucose hydrazone by the hydroxybenzaldehyde-substituted beads and subsequent recovery of glucose when the beads were treated with 25% aqueous acetone for 2 h at 100°C, was demonstrated by TLC in acetonitrile water (85:15 v/v).

No difference between the rates of β-galactosidase hydrolysis of lactose itself and that of lactose hydrazone was observed, and when hydrazine hydrate (0.1% final concentration) was added to a solution of lactose to simulate the presence of the occluded hydrazine often present in sugar hydrazones, the β-galactosidase-catalyzed hydrolysis still proceeded efficiently.

Discussion

The ideal method for the immobilization of reducing sugars has the following attributes: rapid and efficient uptake of the sugar, uptake under mild conditions, and reversible binding to permit subsequent recovery of the sugar. In previous studies, we demonstrated irreversible immobilization of reducing sugars to aminopolystyrene on heating [11] and reversible binding of sugar hydrazones to isothiocyanatopolystyrene [18] under milder conditions. The present method involves coupling of a sugar hydrazone to immobilized aldehyde groups by means of azine linkages. It represents a further advance toward the ideal in that binding is achieved and in higher yield under the same mild conditions (Figure 3), and sugar recovery, also under mild conditions, is appreciably higher (Table 2).

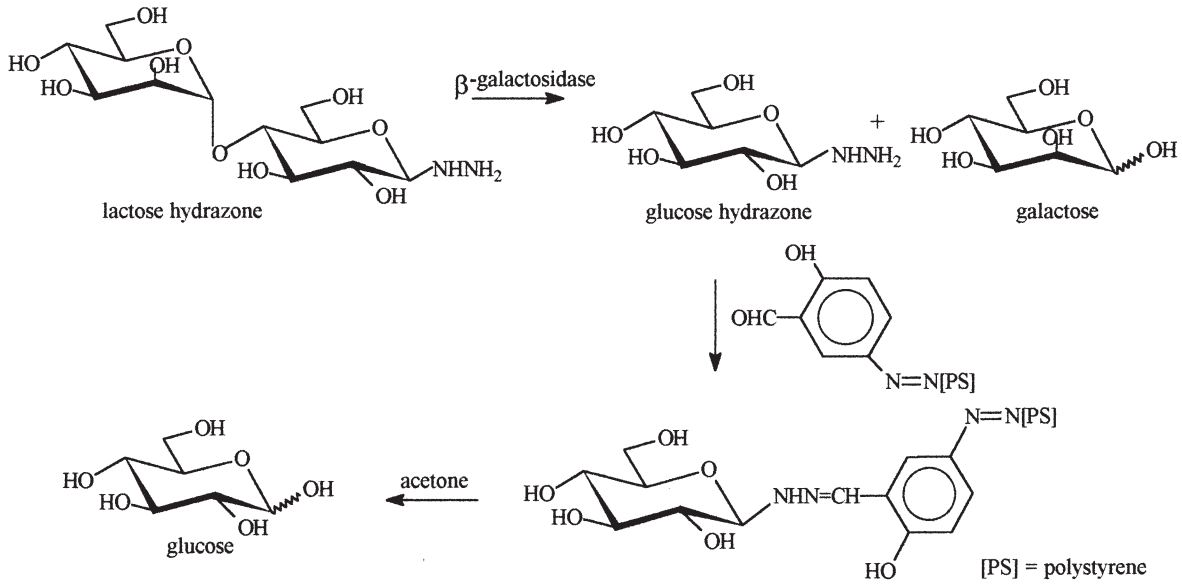

Figure 4. β-Galactosidase treatment of lactose hydrazone, separation of glucose hydrazone from solution by binding to 2-hydroxybenzaldehyde-polystyrene beads, and recovery of glucose from the beads.

The formation of an azine linkage (Figure 1) between a hydrazone and a carbonyl group is an equilibrium process, and is least favored in aqueous solution. The efficiency of binding of a sugar hydrazone to a polymer by means of an azine linkage is therefore determined by the position of equilibrium, which is expected to be optimal for an aromatic aldehyde. The rate of binding of the sugar hydrazones is first order with respect to the sugar hydrazone, and since the active ligands in these studies were in 800-fold excess in the 10 μM glucose hydrazone solution, and in 5000-fold excess in the 0.5 μM *N*-acetylglucosamine hydrazone solution, the uptakes reported here are considered representative of typical bindings in analytical studies.

The aromatic aldehyde functionality was introduced into aminopolystyrene beads by diazotization of the amino groups, followed by azo coupling to 2-, 3-, and 4-hydroxybenzaldehyde (Figure 1). These modified polymers were incubated with sugar hydrazones at room temperature. In the optimal pH range of 5–8, there was an uptake of up to 90% in 2 h. In view of the equilibrium nature of the binding, for more complete removal of sugar hydrazones from solution, it would be necessary to use two or more sets of beads in sequence.

It was anticipated that the presence of a hydroxyl substituent, adjacent to the aldehyde function (Figure 1), would stabilize the azine linkage by internal hydrogen bonding, as in pyridoxal aroylhydrazones [22]. However, binding of sugar hydrazones to 3-hydroxybenzaldehyde beads, where no hydrogen bonding is possible, is just as effective as for the 2-hydroxy isomer, possibly because the electronic advantages of binding to the 2-hydroxy isomer are offset by the increased steric hindrance. As anticipated, binding to the 4-hydroxybenzaldehyde beads is less efficient as its *p*-quinonoid structure is likely to be susceptible to oxidation, while internal hydrogen bonding [22] would stabilize the *o*-quinonoid structure of the 2-hydroxybenzaldehyde binding.

N-Acetylglucosamine hydrazone was used as a model for complex glycans in most studies since it is the residue at the reducing terminus of *N*-linked glycoproteins and since *N*-acetylgalactosamine is frequently at the reducing terminus of *O*-linked glycoproteins. Glucose was also included in the binding studies and lactose in the enzyme modification study. Binding of sugar hydrazones was best when the beads, recovered from strong base after azo-coupling, were washed with ethanol and water without acid treatment, then equilibrated in buffer at pH 6–7. The binding of the sugar hydrazones could then be carried out either in buffer or in water. It was most important to ensure that traces of hydrazine, occluded in the sugar hydrazones, are removed by reevaporation from water before binding [8], since hydrazine competes with the sugar hydrazones for the aldehyde ligands.

Equilibrium binding means that there will always be a leakage of bound sugars into the medium, particularly at low pH, so that azine binding may not be suitable for some applications, such as immunochemical studies, which require incubations in several solutions or continuous washing, which would result in a small proportional loss at each step. However, successive brief washings of the sugar-bound beads did not cause serious losses, as shown by high recoveries of sugars by chemical treatment of the beads (Table 2). The azine binding is well suited to applications such as solid-phase modification methods, requiring the recovery of bound sugars [8–10]. Alternatively, it is possible to prepare a glycan hydrazone, subject it to enzymatic modification, and then use azine binding to beads to recover the reducing terminal domain of the modified glycan from salt and byproducts of the enzyme treatment. This approach is illustrated by the treatment of lactose hydrazone with β-galactosidase to form galactose and glucose hydrazone. Using the solid-phase binding of the glucose hydrazone, glucose was recovered free from the other solutes (Figure 4). No free lactose or glucose was detected in the medium, indicating that the stability of the sugar hydrazones in the neutral aqueous medium was excellent over the timescale of the experiment. Because sugar hydrazones are readily prepared from reducing sugars [23, 24], this method is a practical alternative to chromatographic techniques for the recovery of glycans after enzymatic modification.

Two additional bead modification studies were also carried out. The potential for increased stabilization of the azine linkage by reductive binding was studied. Reductive binding of *N*-acetylglucosamine hydrazone with sodium cyanoborohydride to the hydroxybenzaldehyde ligands was moderately successful (Table 3), but reductive coupling with alkaline borohydride was unsatisfactory. Other investigators have reported similar difficulties with borohydride reduction of hydrazones with a proton on the outer nitrogen atom [25]. The addition of a spacer arm to aminopolystyrene was carried out to improve the accessibility of the amino groups to reducing glycans in Amadori immobilization [11] and that of the immobilized glycans to antibodies or enzymes in subsequent immunoassays or enzyme modifications [11].

These studies complement two previously reported methods of binding glycans to solid supports [11, 18]. Immobilization of sugar hydrazones to hydroxybenzaldehyde ligands has the advantage of faster binding of sugar hydrazones in high yield under conditions that are appropriate for the acid-labile neuraminic acid-containing glycans [18], and the higher recoveries of bound sugars [18] make the method particularly suitable for enzyme modification.

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