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Determination of the cross-linking effect of adipic acid dihydrazide on glycoconjugate preparation

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The cross-linking effect of adipic acid dihydrazide (ADH) on polysaccharide derivatization can be evaluated by applying combination of elemental analysis and colorimetric assay. Elemental analysis is used for estimation of total ADH bound to polysaccharide and a colorimetric trinitrobenzene sulfonic acid assay is used to determine the part of ADH not involved in cross-linking. The difference of values expressed as molar ratios (per repeating unit) provides information on the amount of ADH involved in cross-linking the polysaccharides. Carboxymethylated polysaccharides were derivatized with different amounts of ADH to test the procedure. Analytical results showed that excess of ADH in the reaction only slightly decreased the cross-linking. The number of carboxyl groups remained unmodified even at high excess of ADH and high concentration of carbodiimide (EDC) coupling reagent.

Keywords: **glycoconjugate, adipic acid dihydrazide, β-glucan, cross-linking**

Introduction

Currently, a lot of different glycoconjugates are being synthetically prepared by coupling polysaccharides to proteins [1]. Chemical coupling is easily achieved by using highly reactive homobifunctional linker—adipic acid dihydrazide (ADH). ADH became one of the most widely used linkers since it was first used for the U.S. licensed glycoconjugate vaccine against *Haemophilus influenzae* type b [2]. ADH as the bis-hydrazide linker is extensively used to derivatize, mainly carboxyl, carbonyl and hydroxyl groups in polysaccharides [3–11]. Despite its widespread use, the total content of ADH and the part of ADH which forms cross-link in the polysaccharide were not precisely determined. At a large molar excess of ADH in the coupling reaction it is supposed that one hydrazide group binds to the polysaccharide while the other one remains free, being ready to couple with the protein. The content of these free hydrazide groups can be subsequently determined by colorimetric assay.

However, the reaction of the second hydrazide group with another carboxyl cannot be completely excluded. Cross-linking the polysaccharide in this way may lead to changes in the architecture of the glycoconjugate and alterations in the biological effectiveness of the product. Being aware of these consequences, we describe a method which provides quantitative information on the degree of cross-linking of polysaccharide caused by ADH linker. At present, there is no reliable method for such an estimation. Data provided by size-exclusion chromatography alone assume that macromolecules do not change their shape upon derivatization. Such an assumption, however, is generally not true and may therefore lead to false interpretation of the results. For a quantitative study of ADH cross-linking effect we have used two different types of carboxymethylated (CM) glucans: CM-(1 → 3)-β-D-glucan and CM-(1 → 4)-α-D-glucan (commercial CM-amylose). The behaviour of ADH cross-linked polysaccharides using size-exclusion chromatography is also discussed.

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Materials and Methods

Chemicals

Adipic acid dihydrazide (ADH), 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC), 2-[N-morpholino]ethanesulfonic acid (MES) and its sodium salt, 2,4,6-trinitrobenzenesulfonic acid (TNBS), CM-amylose, $NaNO_3$ and KOH were from Sigma. The carbonate-free KOH solution (0.084 M) was prepared in the laboratory and carbonate–free double-distilled water was used for potentiometric titration. CM-(1 → 3)-β-D-Glucan was an in-house preparation; Sephacryl S-400 was from Pharmacia; Pullulans P-5, P-100, P-200, P-400, and P-800 (Shodex Standard P-82 set) were from Macherey-Nagel GmbH (Düren, Germany).

Preparation of the CM-polysaccharides with lower molecular weight

CM-glucan. High molecular carboxymethylated glucan (subsequently called CM-glucan), with degree of substitution (DS) = 0.92 and M_W = 324 000 was prepared from alkali-insoluble glucan isolated from the cell walls of *Saccharomyces cerevisiae*, according to [12]. It was then treated for 10 min by ultrasonication (20 kHz, 100 W) [13]. After dialysis and gel filtration (Sephacryl S-400), the major fraction of CM-glucan had a M_W of around 146 000.

CM-amylose. The commercial carboxymethylated amylose (subsequently called CM-amylose) was treated by ultrasonication under the above conditions, dialyzed and fractionized (Sephacryl S-400). The M_w of CM-amylose was around 126 000.

The degree of carboxymethylation of both CM-polysaccharides was determined by potentiometric titration.

Preparation of the ADH derivatized polysaccharides

ADH-CM-glucan. To CM-glucan (M_w = 146 000) dissolved in MES buffer (0.05 M, pH = 5.1) 3.33, 5.95 or 10.1 mg of ADH/mg CM-glucan was added. After 5 min, EDC was added slowly with stirring to a final concentration of 50 mM and the reaction mixture was stirred for 3 h at pH 5.2–5.5 at room temperature. The pH was maintained with 0.1 M MES (pH = 3.6). The reaction mixtures were exhaustively dialyzed in dialysis tubing (Servapor) against a large amount of distilled water and the dialyzates were freeze-dried.

ADH-CM-amylose. CM-amylose was derivatized according to the above procedure, with 1.88, 3.55 or 6.67 mg ADH/mg CM-amylose. Another two samples of ADH-CM-amylose (1.88 mg ADH/ mg CM-amylose) were prepared using 25 and 100 mM EDC in the reaction mixture.

All reaction products were subjected to the elemental, TNBS, and potentiometric titration analyses as well as to HPLC characterization.

Elemental analysis

Solid samples were analyzed for their carbon, hydrogen and nitrogen content using EA 1108 device (FISONS Instruments, UK). The combustion of samples at very high temperature (1020°C) and intensive oxygen supply in the instrument ensure complete gassification without any pyrolytical side-products.

TNBS analysis

The content of free hydrazide groups in ADH-CM-glucan and ADH-CM-amylose samples was evaluated by the trinitrobenzene sulfonic acid method (with 0.25% TNBS solution) using ADH as a reference [14]. The results were expressed as moles of free hydrazide per repeating unit of CM-polysaccharide.

Determination of carboxyl groups by potentiometric titration

The content of free carboxyl groups in the original CM-polysaccharides and their ADH-derivatized products was determined potentiometrically. Solutions of polysaccharides were passed through a cation exchanger Dowex 50X × 2 (H^+-form) and titrated with a solution of KOH (0.084 M) to the point of equivalence using a combined pH electrode. In the case of original CM-polysaccharides the degree of carboxymethylation was calculated according to the equation suggested by Rinaudo and Hudry-Clergeon [15].

Calculation of amount of ADH involved in cross-linking

The amount of ADH involved in cross-linking represents the difference between the total amount of bound ADH and amount of ADH possessing one free hydrazide group. Combination of at least two independent analytical methods is required to provide unambiguous data. We have tried a combination of elemental analysis and colorimetric TNBS assay. The third method, potentiometric titration was used as a check of accuracy.

First, weight contents of nitrogen and carbon obtained from elemental analysis is transformed to molar ratio of nitrogen and carbon $(N/C)_{exp}$ in the sample. This ratio (obtained from experimental data) should satisfy the expression:

$$(N/C)_{exp} = (N_{PS} + N_{ADH})/(C_{PS} + C_{ADH}) \quad (1)$$

where N_{PS} and C_{PS} represent numbers of nitrogen and carbon atoms, respectively in the repeating unit of polysaccharide and are the constant characteristics of original polysaccharide. Variables $N_{ADH} = 4x$ and $C_{ADH} = 6x$ represent numbers of nitrogen and carbon atoms introduced to polysaccharide by ADH, where x denotes the molar fraction of bound ADH per repeating unit of polysaccharide. The magnitude of x can be obtained from the equation:

$$(N/C)_{exp} = (N_{PS} + 4x)/(C_{PS} + 6x) \quad (2)$$

Since colorimetric (TNBS) assay requires presence of free hydrazide group to produce coloration, it detects the part of ADH bound only with one hydrazide to polysaccharide which represents ADH not involved in cross-linking (y).

The amount of ADH involved in cross-linking of polysaccharide is thus equal to the difference $x - y$.

High-Performance Liquid Chromatography (HPLC)

Size-exclusion HPLC experiments were performed at ambient temperature with a system that included a high-pressure pump (LCP 3001; Laboratorní přístroje, Prague, Czech Republic), an eight-port switching valve equipped with two 100-µl loops (Model PK 1: Vývojové dílny, Czechoslovak Academy of

Sciences, Prague), and two in series connected columns (250 × 8 mm) packed with Biospher GM 300 and Biospher GM 1000 sorbents (mean particle size = 10 μm; Labio, a.s., Prague). The separation process was monitored with a differential refractometric detector. The mobile phase used was 0.1 M aqueous $NaNO_3$ solution. The flow rate was 0.4 ml/min. A set of pullulans was used for the calibration of the HPLC system. The molecular-weight averages of sonicated polysaccharides were calculated using the computer program [16] taking pullulan standards as the reference materials.

Results and Discussion

The carboxymethylated polysaccharides used in this study differ in the degree of carboxymethyl substitution: CM-glucan DS = 0.92 and CM-amylose DS = 1.33. Uniformity of the molecular size of CM-polysaccharides used in the reactions was achieved by controlled ultrasonication. Chemical derivatization was performed using three different excess amounts of ADH with both polysaccharides and applying three different concentrations of EDC with CM-amylose. Products were characterized by a suggested combination of analytical methods and HPLC. Figure 1 schematically depicts possible saccharide units present in the molecules of CM-amylose (a) and CM-β-glucan (b) upon derivatization with ADH.

First, elemental analysis was used to determine the total amount of ADH bound to the polysaccharide (Table 1, *x*). The molar fraction of ADH per repeating unit obtained from Equation (2) was used for estimation of average molecular weight of polysaccharide repeating unit (Table 1). The accuracy of the calculation can be checked by using the ratio H/C instead of N/C in Equation (2). Second, the TNBS assay for estimation of free hydrazide groups was employed. The molar fraction of bound ADH with one free hydrazide group was obtained in this way (Table 1, *y*).

The cross-linking effect was determined as the arithmetic difference between the total molar fraction of bound ADH and molar fraction of bound ADH with free hydrazide group in

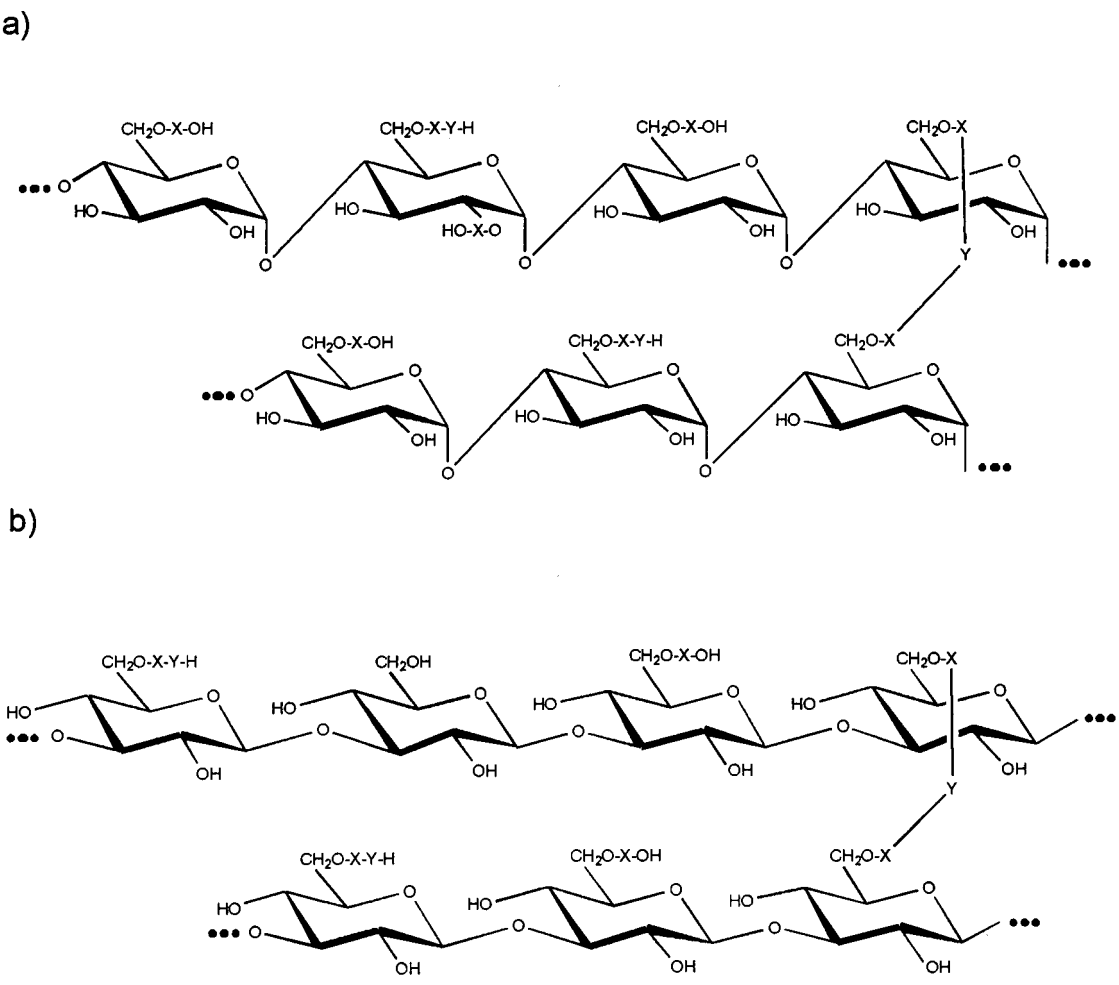

Figure 1. Possible structural segment of (a) ADH-CM-amylose; (b) ADH-CM-glucan
x: $-CH_2-CO-$ (i.e. part of carboxymethyl)
y: $-NH-NH-CO-(CH_2)_4-CO-NH-NH-$ (i.e. ADH)

Table 1. Characterization of ADH-CM-glucan and ADH-CM-amylose derivatives prepared with various amounts of ADH

Sample	*Synthesis*[a] *mol ADH/rep.unit*	*M_w of m.u.*[b]	*x*[c] *ADH_{el}/m.u.*	*y*[d] *ADH_{TNBS}/m.u.*	*x − y* cross/m.u.	*(2x − y)*[e]	*Calculated unoccupied carboxyls*[f]	*Measured free carboxyls*[g]
CM-glucan	4.7	333.8	0.51	0.18	0.33	0.84	0.08	0.14
	8.4	339.7	0.54	0.27	0.27	0.81	0.11	0.15
	14.3	345.0	0.57	0.32	0.25	0.82	0.10	—
CM-amylose	3.0	372.3	0.54	0.11	0.43	0.97	0.36	0.20
	5.7	383.5	0.60	0.15	0.45	1.05	0.28	0.17
	10.7	389.9	0.64	0.17	0.47	1.11	0.22	0.24

[a] molar ratio in chemical reaction.
[b] average M_w of repeating monomer unit (m.u.) of ADH derivatized CM-polysaccharides.
[c] calculated from elemental analysis.
[d] free hydrazide groups estimated by TNBS method.
[e] occupied carboxyl groups.
[f] initial − (2x − y)/m.u.
[g] free carboxyl groups measured by potentiometric titration/m.u.

Table 1, labelled *x–y*. The next two columns in Table 1 contain data on carboxyl groups and were obtained by calculation. In the column labelled $2x - y$ there are data expressing amount of carboxyl groups occupied with ADH. The number of unoccupied carboxyls per monomer unit was obtained as a difference between the initial number of carboxyl groups and the calculated number of occupied ones. The calculation revealed that there still was a number of unsubstituted carboxyls in both types of polysaccharide. Their amount was roughly twice as high in CM-amylose (DS 1.33) than in CM-glucan (DS 0.92).

We have also used an experimental method to check the presence of free carboxyl groups. The last column in the Table 1 contains data obtained by potentiometric titration. Despite the incomplete solubility at the concentration required for potentiometric titration, we obtained data confirming the presence of free carboxyl groups for all derivatized polysaccharides. The small differences between the values calculated as ADH-unoccupied carboxyls and those experimentally determined can be attributed to errors in experimental assays as well as to irreversible alteration of carboxyl groups by the EDC side reaction.

Table 2 contains analytical data obtained by varying amount of EDC coupling reagent added at the derivatization of CM-amylose with constant amount of ADH (5.7 mol/m.u.) Using EDC in a concentration higher than 100 mM gave compounds that were incompletely soluble. Increased concentration of EDC resulted in enhanced ADH incorporation for both types of derivative, i.e. the one with a free hydrazide and the other with groups involved in cross-linking. Despite this increased incorporation of ADH a certain portion of carboxyls still remained unoccupied. The presence of free carboxyl groups in all the derivatives showed that the chemical reaction did not proceed quantitatively and did not use all the carboxyl groups, even at a high excess of ADH and a high concentration of EDC.

The data (Tables 1 and 2) revealed another interesting ADH binding characteristic: at higher reagent excess, the products contained higher amount of free hydrazide groups. A slightly lower amount of cross-linking was observed with an excess of ADH for CM-glucan, but this did not occur with CM-amylose. The amount of ADH involved in cross-linking was not proportional to the excess of ADH used in the reaction. This amount depended rather on the number of carboxymethyl groups per monosaccharide unit than on the excess of ADH used. The amount of cross-linked saccharide units i.e., $2(x - y)$ is higher than that of the saccharide units containing no cross-linked ADH. The attempt to change this ratio by

Table 2. Characterization of ADH-CM-amylose derivatives prepared with various amounts of EDC

Synthesis[a] EDC (mM)	*M_w of m.u.*[b]	*x*[c] *ADH_{el}/m.u.*	*y*[d] *ADH_{TNBS}/m.u.*	*x − y* cross/m.u.	*(2x − y)*[e]	*Calculated unoccupied carboxyls*[f]	*Measured free carboxyls*[g]
25	365.5	0.50	0.09	0.41	0.91	0.42	0.33
50	372.3	0.54	0.11	0.43	0.97	0.36	0.20
100	385.4	0.61	0.13	0.48	1.09	0.24	–

[a–g] same as in Table 1.

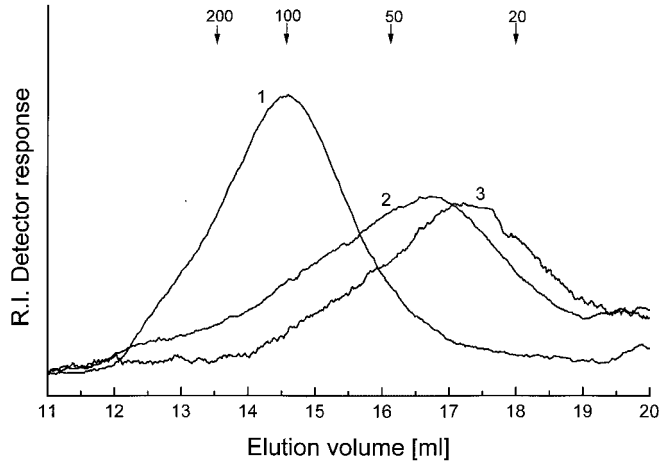

Figure 2. HPLC elution profiles of the original (1) and derivatized CM-glucans: (2)—with 4.7 mol ADH/m.u.; (3)—with 8.4 mol ADH/m.u.

further addition of ADH led to the formation of incompletely soluble compounds. The binding of ADH was limited by the fact that not all carboxyl groups could enter the reaction. The number of the carboxyl groups, which remained free depended only slightly on the amount of ADH used. The total amount of ADH incorporated into polysaccharides was around 50% and was almost independent of CM content and the ADH concentration.

The products were also characterized by HPLC. Binding of ADH changed chromatographic characteristics of both types of CM-polysaccharide. The ADH-CM-polysaccharides were eluted later than original CM-polysaccharide as is illustrated for CM-glucan in Figure 2. This observation implies that ADH binding altered the architecture of the polysaccharide. The reason for such a change is the intramolecular cross-linkages which can bring together remote parts of the chain. Derivatization of carboxyl groups also changes the charge distribution on the polysaccharide. Attraction between free hydrazide groups and negatively charged carboxyl groups within the flexible polysaccharide chain may cause increased folding of the molecule. These two effects can account for unexpected changes in the relative size of the polysaccharides as revealed by HPLC. Our experiment shows that size-exclusion chromatography is not reliable for evaluation of ADH-mediated cross-linking of acidic polysaccharides.

Synthetically prepared glycoconjugates have brought a great improvement to the effectiveness of polysaccharide-based vaccines. As the glycoconjugate chemistry of carbohydrate polymers is not especially clear-cut and substitution reactions may proceed randomly in a non-specific way, almost every synthesized glycoconjugate polymer is unique. Satisfactory reproducibility can be achieved only by accurate monitoring of each step of preparation by suitable analytical procedures. Moreover, analytical methods used for characterization of modern conjugate vaccines should not be expensive, but satisfactorily exhaustive [17]. In this paper we suggest a simple analytical method for the determination of the structural parameters of ADH-polysaccharide glycoconjugate precursor that can be routinely used for the continuous determination of reaction efficiency in the course of preparation of conjugate polysaccharide-protein vaccines.

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