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Preparation and characterization of adipic acid dihydrazide derivatives of yeast mannans

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Abstract

Adipic acid dihydrazide (ADH) as one of the most widely used homobifunctional linkers in bioconjugate chemistry was used to prepare active forms of originally neutral mannans from the three pathogenic yeasts (*Candida albicans*, *Candida tropicalis*, *Candida glabrata*). A combination of the simple analytical methods (elemental analysis, colorimetric assays: Park–Johnson assay, trinitrobenzenesulfonic acid assay, and size exclusion chromatography) was applied for exhaustive structural characterization of the products. Significant cross-linking effect of ADH was revealed, despite the high excess of ADH involved in the reaction.

Keywords: Mannan; Adipic acid dihydrazide; Cross-linking

1. Introduction

Mannans are easily accessible neutral polysaccharides, which occur in the outermost layer of yeast-like microorganisms. Their structure consists of a conservative α-(1-6)-linked polymannosyl backbone and side chains of variable length containing α-(1-2)-linked mannoses with traces of α-(1-3)-attached mannose (Kogan, Pavliak, Šandula, & Masler, 1991). Solubility of the mannans in water indicates the possibility of their functionalization and application in the biological systems. First step of their functionalization, e.g. for synthesis of glycoconjugates, consists of their conversion to the chemically reactive form. In principle, mannans are able to bind other substrates by two ways. One is employing hydroxyl groups by direct activation using cyanylation agents (Bystrický, Machová, Bartek, Kolarova, & Kogan, 2000; Kohn & Wilchek, 1983; Lees, Nelson, & Mond, 1996), while the other one involves introducing chemically reactive groups. A widely used simple method is periodate oxidation followed by reductive amination (Jennings & Sood, 1994; Masárová, Mislovičová, & Gemeiner, 2001). The principal problem is to define optimum conditions of oxidation in order to generate a satisfactory amount of carbonyl groups and to preserve polysaccharidic character of the molecules (Masárová et al., 2001). Amination can be performed with compounds containing amino or hydrazine functions. Adipic acid dihydrazide (ADH) is a popular linker for introduction of reactive hydrazide group into polysaccharides (Hermanson, 1996). However, effectiveness of free hydrazide groups introduction can be markedly reduced by their involvement in cross-linking (Bystrický, Machová, Maloví-ková, & Kogan, 1999).

In the present work, a combination of simple analytical methods, which enables also to quantitatively assay the effect of chemical modification of yeast mannans is described. We have used three structurally different mannans from pathogenic yeasts, highly branched mannans from *Candida albicans* and *Candida glabrata*, and a slightly branched mannan from *Candida tropicalis*.

2. Materials and methods

2.1. Chemicals

α-D-Mannans from *C. albicans* CCY 29-3-32, *C. tropicalis* CCY 29-7-6, and *C. glabrata* CCY 26-20-1 from the Culture Collection of Yeasts and Yeast-like Microorganisms (CCY), Institute of Chemistry, Slovak Academy of Sciences, were isolated and purified using precipitation with Fehling's reagent according to the procedure described previously (Kogan, Pavliak, & Masler, 1988).

ADH, 2,4,6-trinitrobenzenesulfonic acid (TNBS) (0.25%

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aqueous solution) were from Sigma. Sodium dodecyl sulfate (SDS) was from Serva, sodium cyanoborohydride ($NaBH_3CN$) was from Merck. Sodium periodate ($NaIO_4$), glycerol, D-glucose, potassium ferricyanide ($K_3[Fe(CN)_6]$), sodium carbonate (Na_2CO_3), potassium cyanide (KCN), ferric ammonium sulfate ($Fe(NH_4)(SO_4)_2$) were from Lachema (Prague, Czech Republic). Set of pullulan standards (Shodex Standard P-82) was from Macherey-Nagel, GmbH (Düren, Germany).

2.2. Periodate oxidation of mannans

Polysaccharide (50 mg) was dissolved in distilled water (15 ml). Sodium periodate (**I**, 7 mg (30-fold molar excess); **II**, 10.8 mg (45-fold molar excess)) in solid form was added and the reaction mixture was stirred for 2 h at room temperature in the dark. The reaction was quenched by adding six drops of glycerol and the solution was stirred for an additional hour. The mixture was exhaustively dialyzed against distilled water and lyophilized. The contents of carbonyl groups were determined by Park–Johnson colorimetric assay (Park & Johnson, 1949).

2.3. Preparation of the ADH-derivatized mannans

Oxidized mannan (20 mg) was dissolved in 0.1 M phosphate buffer (pH 8, 25 ml). High molar excess of ADH (15.5 mg) was added and the reaction mixture was stirred for 1.5 h at room temperature. For the reduction of resulting Shiff's base, sodium cyanoborohydride (80 mg) was added and the solution was stirred for an additional hour. The mixture was dialyzed and lyophilized.

All reaction products were subjected to the elemental analysis and TNBS assay as well as to chromatographic characterization.

2.4. Determination of carbonyl groups by Park–Johnson assay

The degree of oxidation of α-D-mannans was determined by Park–Johnson colorimetric assay. The determination of the contents of carbonyl groups is based on the reduction of ferricyanide ions in alkaline solution (Park & Johnson, 1949). D-Glucose was used as the standard. The results were expressed as moles of carbonyl groups per repeating unit of functionalized α-D-mannan.

2.5. TNBS assay

The contents of ADH with free hydrazide groups in ADH-mannan samples were evaluated by the TNBS acid colorimetric assay with 0.25% TNBS solution (Fields, 1972; Habeeb, 1966). ADH was used as the standard. The results were expressed as moles of ADH with free hydrazide group per repeating unit of derivatized α-D-mannan.

2.6. Elemental analysis

EA 1108 device (FISONS Instruments, UK) was used for the measurement of carbon, hydrogen, and nitrogen contents in solid samples. The combustion of samples at a very high temperature (1020 °C) and intensive oxygen supply in the instrument ensured complete gassification without any pyrolytical side products.

2.7. Calculation of the amount of carbonyl groups substituted with ADH

There are two types of the derivatized carbonyl groups, one with ADH possessing free hydrazide groups and the second one occupied by ADH involved in the cross-linking. Calculation of the amount of carbonyl groups cross-linked by ADH is based on a difference between the total amount of ADH and the amount of ADH with one free hydrazide group. The total amount of ADH is obtained from elemental analysis. The experimentally found molar ratio of nitrogen and carbon $(N/C)_{exp}$ is equal to theoretical ratio of nitrogen and carbon in ADH-derivatized polysaccharide

$$(N/C)_{exp} = (N_{PS} + 4x)/(C_{PS} + 6x) \quad (1)$$

where N_{PS} and C_{PS} represent numbers of nitrogen and carbon atoms in repeating unit of the original polysaccharide, respectively. Variables $4x$ and $6x$ represent numbers of nitrogen and carbon atoms introduced to polysaccharide by ADH. The value x denotes the required molar fraction of total ADH bound per repeating unit of polysaccharide. The portion of ADH bound to polysaccharide only with one hydrazide (y) is obtained from TNBS colorimetric assay. The amount of carbonyl groups occupied by ADH is equal to the difference $2x - y$.

2.8. High-performance liquid chromatography

Size exclusion chromatography (SEC) experiments were performed with a system from Laboratorní přístroje (Prague, Czech Republic) containing two in series connected columns (250 × 8 mm) packed with Biospher GM 300 and Biospher GM 1000 sorbents from Labio, a.s. (Prague, Czech Republic). Biospher GM is a co-polymer of glycidylmethacrylate and ethylenedimethacrylate accompanied by special porogens. The separation process was monitored with a differential refractometric detector. The mobile phase used was 0.1 M $NaNO_3$ solution. A set of pullulans was used for molecular weight calibration of SEC system.

3. Results and discussion

Neutral polysaccharides, cell-surface mannans were isolated from *C. albicans*, *C. tropicalis* and *C. glabrata* by a standard procedure using precipitation with Fehling's reagent. Because of their immunogenic and other biological properties, mannans can be with advantage applied in

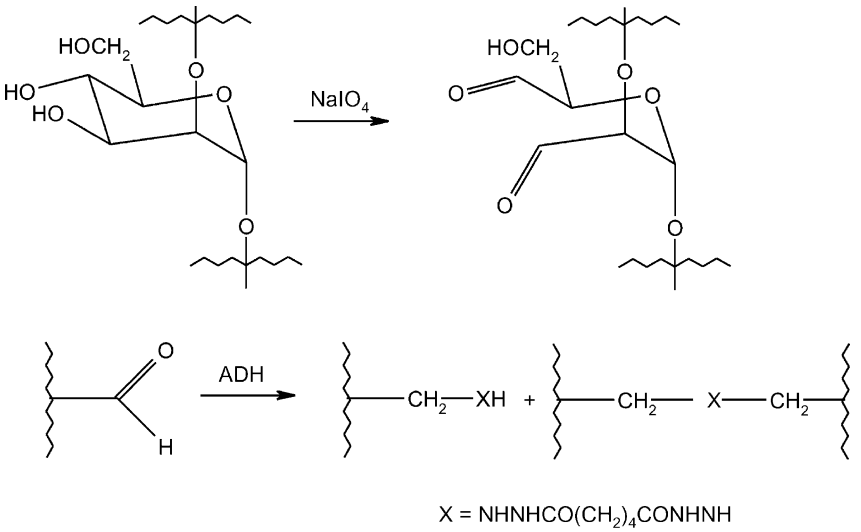

Fig. 1. Chemical reactions of mannan within the branches and possible structures of the products.

various systems upon chemical attachment of other active molecules. For this purpose, a chemically reactive form of the mannan was obtained by controlled periodate oxidation. Mannans containing various amounts of carbonyl groups were rendered using the reaction involving two different amounts of sodium periodate. Optimum reaction conditions preserving the size of macromolecule were found, yielding the maximum of around 12 mol% of carbonyls per repeating unit. Although these mannan carbonyl derivatives are able to directly bind the other effector molecules, it is often recommended to introduce a spacer (such as ADH) for more effective binding in bioconjugate chemistry (Kossaczka & Szu, 2000). Fig. 1 schematically shows possible chemical structures of mannans obtained after oxidation and derivatization with ADH. As shown in Fig. 1, derivatization of polysaccharides with ADH introduces highly reactive hydrazide groups. However, introduced hydrazide groups can react with other carbonyls, and, therefore, possibility of cross-linking cannot be excluded. All reaction products were characterized by a combination of analytical methods and SEC. Results of chemical analyses and calculations are summarized in Table 1. First column shows the effect of two different amounts of sodium periodate in the reaction. As can be seen from the table, increase in the yield of carbonyls was notable with about 50% increase in reagent. Since each oxidized monosaccharide unit contains two carbonyl groups, there are overall approximately 6 mol% of the altered units. Such very moderate modification can be considered to be optimum for functionalization of branched mannan.

Further derivatization was performed with constant amount of ADH. The derivatized products were subject to elemental analysis in order to determine the total amount of ADH bound to the polysaccharide (Table 1, x). The amounts x were calculated from Eq. (1). The mannans with higher contents of carbonyl groups bound generally more ADH. The TNBS assay was employed for estimation of free hydrazide groups content. Found molar fractions of bound ADH with one free hydrazide group (Table 1, y) were considerably lower than the total amount of bound ADH. The difference between the total amount of ADH and that with one free hydrazide group represents the extent of cross-linking ADH (Table 1, $x - y$). Branched structures of polysaccharides play an important role in this derivatization. It was reported (Masárová et al., 2001), that oxidation of mannan takes place mainly within side chains. This statement was confirmed by measurement of interaction of lectin Concanavalin A with oxidized mannan. It is well known, that Concanavalin A interacts with internal α-(1-2)-D-mannopyranosyl units within branches as well as with non-reducing terminal α-D-mannopyranosyl groups. Authors reported a proportional decrease in the level of interaction with lectin with an increase in the oxidation degree of mannan.

We have found that highly branched mannans obtained from *C. albicans* and *C. glabrata* exhibited relatively high degree of the cross-linking, while the degree of the cross-linking observed for mannan from *C. tropicalis* was considerably lower. Generally, ADH binding results in saccharide units which are cross-linked as well as in lesser amount of saccharide units bearing reactive-free hydrazide groups ($2(x - y) > y$, Table 1). We suppose that the explanation for the observed effect could be based on neighboring group effect of proximal carbonyls in connection with potential steric hindrances, since derivatization takes place in side chains. Therefore, even high excess of ADH used in the reaction does not assure complete derivatization yielding ADH derivatives with free hydrazide groups.

We tried also to evaluate how many unoccupied carbonyl groups were still present after the reaction with ADH. The amount of unoccupied carbonyls is represented by the difference between amounts of originally generated carbonyls

Table 1
ADH derivatization of yeast mannans oxidized with two different amounts of sodium periodate (**I**: 30-fold; **II**: 45-fold molar excess)

Sample	z[a] (carbonyls/m.u.)	x[b] (ADH/m.u.)	y[c] (ADH/m.u.)	$x - y$ cross-linking (ADH/m.u.)	$2x - y$[d] (occupied carbonyls/m.u.)	$z - (2x - y)$[e] (unoccupied carbonyls/m.u.)	Unoccupied carbonyls/m.u.[f]
C. albicans **I**	0.1022	0.0219	0.0072	0.0146	0.0366	0.0656	0.0465
C. albicans **II**	0.1286	0.0409	0.0072	0.0337	0.0746	0.0540	0.0531
C. tropicalis **I**	0.0768	0.0096	0.0052	0.0044	0.0141	0.0627	0.0496
C. tropicalis **II**	0.1217	0.0158	0.0073	0.0086	0.0244	0.0973	0.0543
C. glabrata **I**	0.0682	0.0241	0.0045	0.0195	0.0437	0.0245	0.0385
C. glabrata **II**	0.1220	0.0310	0.0062	0.0248	0.0559	0.0662	0.0533

[a] After oxidation (Park–Johnson assay), before reaction with ADH.
[b] Total ADH (elemental analysis).
[c] ADH with free hydrazide group (TNBS assay).
[d] Calculated, after reaction with ADH.
[e] Calculated.
[f] Measured (Park–Johnson assay).

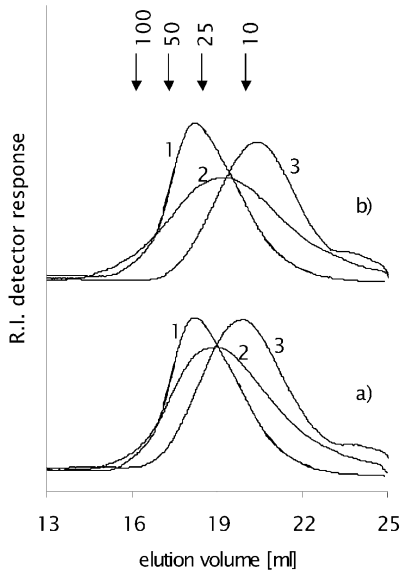

Fig. 2. HPLC profiles of (a) *C. glabrata* (**I**) and (b) *C. glabrata* (**II**). (1) Original mannan; (2) oxidized mannan; (3) ADH-derivatized mannan. For **I** and **II** designation, see Section 2.2. Arrows denote molecular weights (kDa).

(Table 1, z) and the occupied carbonyls (Table 1, $2x - y$). Despite the large excess of the ADH used in the reactions, the products contained significant portions of free carbonyl groups. This fact was confirmed also by measuring free carbonyl groups in the products by Park–Johnson assay (Table 1, right column). Small differences between the experimental values and the calculated ones can be attributed to cumulative errors in experimental assays. The amount of ADH-unoccupied carbonyl groups was higher in the preparations with higher contents of originally generated carbonyls.

Additionally, besides all mentioned analytical methods, all samples were subjected to SEC analysis, in order to specify possible changes in the structure and shape of the macromolecules. The chromatographic characterizations of the original mannans, mannans after the oxidation, and ADH-mannans are shown in Fig. 2. Similar pattern was observed for all types of yeast mannan derivatives. The oxidized mannans were eluted later than original ones. The decrease in molecular sizes can be explained by partial degradation of mannans during the oxidation procedure. The elution positions of ADH derivatives are shifted even further. Two of the possible explanations for the later observed effect could be, e.g.: First, we can speculate, that the decrease in relative size of molecules is based on cross-linking of branches within the mannan molecule. Second possibility could be interactions of free hydrazide groups of ADH derivatives with the chromatographic support. Although the used sorbent was designed to be hydrophilic, non-specific interactions with polysaccharidic material cannot be completely excluded here. ADH-derivatized proteins are also retarded on most HPLC columns (Lees, 2000). Simple SEC analysis revealed not to be plausible enough for evaluation of the chemical changes in this type of derivatives, and will require further, more profound study.

In conclusion, the study demonstrated the effective use of simple analytical methods for exhaustive characterization of yeast mannan derivatives—glycoconjugate precursors. It was also shown that periodate oxidation of mannans is an effective route for introduction of carbonyl groups in neutral-branched mannans, but further activation with homobifunctional hydrazides must be carefully considered due to possible cross-linking.

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