Optimization of Dextran and Mannan Dialdehydes Preparation and Examination of their Biospecific Interaction with Concanavalin A

J. MASÁROVÁ, D. MISLOVIČOVÁ, and P. GEMEINER

Institute of Chemistry, Slovak Academy of Sciences, SK-842 38 Bratislava
e-mail: chemmisl@savba.sk

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The periodate oxidation of dextran and mannan was optimized to prepare activated polysaccharides (containing aldehyde groups) suitable for conjugation with proteins. The mild reaction conditions to achieve available degree of oxidation, while the depolymerization was prevented, were estimated. The products containing up to 16 aldehyde groups per dextran molecule and up to 28 aldehyde groups per mannan molecule were obtained, while a low decrease of M_r of both polysaccharide chains, from 18 400 to 17 000 of dextran and from 55 000 to 49 500 of mannan, respectively, was observed.

Using the precipitation method the interaction of Concanavalin A with both, original as well as modified, subsequently reduced, dialdehyde polysaccharides was investigated. Only the precipitation of mannan and its modified form with Concanavalin A was observed. The oxidation of mannan up to 20 aldehyde groups per mannan molecule caused only a very little decrease of affinity of prepared samples to Concanavalin A.

Methods of protein modification with saccharides have been developed in the last two decades with the purpose to obtain new functional glycoconjugates [1—4]. The attachment of polysaccharides to proteins may change their antimicrobial [5] and emulsifying properties [6] as well as their stability [7—9] and solubility [6]. The neoglycopeptides and neoglycoproteins were prepared also for their use as ligands in order to study lectins or carbohydrate-specific antibodies [10]. Enzymes modified with carbohydrates (neoglycoenzymes) became a powerful tool in cytochemistry and in biochemical detection of lectins in solid-phase assays [10]. The advantages of biospecific immobilization of glycoenzymes on the lectin supports (stability and high remained activity) make the use of neoglycoenzymes extremely attractive for preparation of superactive sorbents and sensitive multilayers [11, 12]. Features such as structure, shape, size, geometry, and valency of carbohydrate ligands proved to be determinant factors in influencing their binding properties to lectins [13].

Dextran and especially mannan are polysaccharides having the affinity [14] to lectin Concanavalin A and, therefore, they are interesting for the synthesis of glycoproteins. Many conjugates of dextran with proteins and enzymes have been prepared and examined for various purposes [5—9]. Several ways were used to form activated dextran polysaccharides available for the binding to proteins. Among the products of various activation procedures (cyanogen bromide [15, 16], 1,1′-carbonyldiimidazole [17], epichlorohydrine [18], and product of periodate oxidation) tested for the dextran, dextran dialdehyde proved to be the most convenient and stable form for the conjugation reaction [8].

Periodate oxidation of polysaccharides leads to cleavage of the diol bonds of monosaccharide units under the formation of dialdehydes [19]. Periodate oxidation of dextran was described as the introduction of aldehyde groups at the C-2 and C-4 position of the D-glucose residue [7]. At mannan in the form of galactomannan the cleavage of C-2—C-3 bond [19] was observed. The prepared dialdehyde of polysaccharides then binds to the amino groups of target protein with covalent bonds. The structural studies, reactions, and applications of oxidation of polysaccharides have been the object of several studies in recent years due to their scientific as well as industrial significance [20—22]. However, the mannan was not yet processed in such a way. Only the synthetic macromolecular mannopyranosylated systems such as glycopolymers and glycodendrimers were prepared and have demonstrated powerful binding properties to model plant lectins [13].

Lectin Concanavalin A interacts with the nonreducing α-D-mannopyranosyl and α-D-glucopyranosyl terminal groups of polysaccharide or glycoprotein chain ends, and with internal (1,2)-α-D-mannopyranosyl units. The extent of interaction of polysaccharides with Concanavalin A is dependent on the degree of branching in the molecule and hence on the large number of chain ends. M_r of polysaccharide also affects Concanavalin A—polysaccharide interaction [14, 23].

The quantitative precipitation assay is one of many various methods used for estimation of extent as well as specificity of carbohydrate—lectin interaction [24, 25].

This paper reports on our study of the mild periodate oxidation of dextran (*Leuconostoc* ssp.) and mannan from *Saccharomyces cerevisiae* – the polysaccharides with assumed affinity to Concanavalin A. The aim of this work was to prepare dextran and mannan dialdehydes available (from the point of view of their M_r and molar content of reactive aldehyde groups) for synthesis of a new type of glycoproteins having the affinity to lectin Concanavalin A. The evaluation of such glycoconjugate is perspective in immobilization techniques of enzymes in biotechnology.

EXPERIMENTAL

Two polysaccharides were used in this work: dextran, an α-D-(1→6)-linked polysaccharide of glucose, $M_r \approx 15\,000$—$20\,000$ ($M_r = 18\,400$ determined by HPLC), purchased from Fluka, Buchs, Switzerland and mannan, an α-D-(1→6)-linked mannose with mainly α-D-(1→2) as well as few α-D-(1→3) bonded short (2—3 units) but frequent branches ($M_r \approx 55\,000$) isolated from *Saccharomyces cerevisiae* by *Šandula* [26]. Sodium periodate (Lachema, Brno, Czech Republic) and sodium borohydride (Metallgesellschaft, Frankfurt a.M., Germany), lectin Concanavalin A (Con A) (Lectinola, Prague, Czech Republic) were used. Methyl α-D-glucopyranoside (α-MGP) and methyl α-D-mannopyranoside (α-MMP) were purchased from Fluka, Buchs, Switzerland. Other reagents used were of anal. grade.

The amounts of aldehyde groups in prepared samples were determined by two methods: 1. Somogyi method [27], where a copper tartrate oxidant and chromogenic reagent were used and absorbance at $\lambda = 530$ nm was measured; 2. Park—Johnson method [28] based on the reduction of ferricyanide ions in alkaline solution to Prussian blue (ferric ferrocyanide) and its colorimetric determination at $\lambda = 690$ nm. The content of aldehyde groups in sample is expressed as the mole ratio n(aldehyde)/n(polysaccharide).

The saccharides and the proteins, in the precipitation experiments, were determined by the phenol—sulfuric method [29] (Con A) and by the Lowry method [30], respectively.

M_r of the original as well as oxidized polysaccharide samples (after their reduction) were determined by the HPSEC method. The HPLC system (Shimadzu, Wien, Austria) was composed of a high-pressure pump LC-10AD, a membrane degaser GT-104, an injector Rheodyne 77251, and differential refractometer RID-6A. The system was calibrated with a calibration set of dextran within the M_r 1000—70000 (Fluka, Buchs, Switzerland). The solutions of calibrating dextrans as well as of tested polysaccharides ($\rho = 1$ mg cm^{-3} of water) were applied on an HEMA BIO 100 column (8 mm × 250 mm, 10 μm sorbent of particle size) obtained from Tessek, Prague, Czech Republic and eluted with 0.02 M-phosphate buffer at the flow rate 0.4 cm^3 min^{-1}. The volume of injected sample was 0.02 cm^3. The differential refractometer served as the polysaccharide concentration detector. The elution profile was recorded by Class-VP-chromatography software.

Polysaccharide Dialdehydes

Polysaccharides (100 mg) were dissolved in aqueous solution of sodium periodate of various amounts and concentrations (Table 1). The amounts of the periodate are expressed as the mole ratio $n(NaIO_4)/n$(monosaccharide unit). The reaction mixture was stirred in the dark at 4 °C during 1 h. Then the reaction was stopped by addition of ethylene glycol. The resulted solution was dialyzed (Spectra Por® dialysis tubing MWCO 6000—8000) against water at 4 °C in the dark. Aqueous solutions of oxidized polysaccharides were lyophilized.

Reduction of Polysaccharide Dialdehyde

Aldehyde groups were reduced by treating of dialdehyde samples (10 mg) with 0.05 M solution of sodium borohydride in 0.05 M borate buffer of pH 9.5 ($n(NaBH_4)/n$(aldehyde groups) ≈ 50) for 6 h at room temperature. Then the solution was adjusted with 4 M-HCl to pH 6 and the obtained reduced sample was dialyzed against H_2O and lyophilized.

Quantitative Precipitation Assay

Precipitation of polysaccharides (original as well as treated) with Con A was assayed in phosphate buffer of pH 7 containing 0.05 mol dm^{-3} sodium phosphate, 0.1 mol dm^{-3} NaCl, 0.1 mmol dm^{-3} $CaCl_2$, and 0.1 mmol dm^{-3} $MnCl_2 \cdot 4H_2O$. The total volume of precipitation tests was 1 cm^3, containing 230 μg cm^{-3} of Con A and various concentration of polysaccharide samples within the range 10—150 μg cm^{-3}. The samples were shaken at 25 °C for 2 h. The precipitates were separated in a microcentrifuge MPW-310 (Mechanika Precyzna, Warsaw, Poland) at 10 000 min^{-1} (8400 g) during 10 min, washed twice with 1 M-NaCl, centrifuged and subsequently dissolved in 0.05 M phosphate buffer adjusted to pH 10.5 with 1 M-NaOH.

RESULTS AND DISCUSSION

The reaction of sodium periodate with polysaccharides results in cleavage of the diols bonds and the production of reactive aldehyde groups. According to the amount of the sodium periodate used, oxidized dextrans D-1—D-5 and mannans M-1—M-7 with var-

Table 1. Periodate Oxidation of the Dextran and the Mannan at Various Concentrations and Mole Ratios $n(NaIO_4)/n$(Monosaccharide Units)

Sample	Oxidation		Content of aldehyde groups n(aldehyde)/n(polysac)		M_r
	Mole ratio $n(NaIO)_4/n$(monosac)	$c(NaIO_4)$ / mmol dm^{-3}	Somogyi	Park—Johnson	
Dextran	–	–	1	1	18 400
D-1	0.088	50	4	6	17 400
D-2	0.440	71	16	16	16 200
D-3	0.880	15	14	14	17 300
D-4	0.880	50	19	18	16 500
D-5	1.320	214	20	20	13 000
Mannan	–	–	1	1	55 000
M-1	0.054	50	5	11	54 500
M-2	0.135	50	7	20	51 200
M-3	0.270	15	9	21	52 800
M-4	0.270	50	9	28	49 500
M-5	0.270	100	7	21	45 200
M-6	0.540	50	nd	22*	46 100
M-7	0.710	50	nd	13*	39 600

* Determined in a soluble part; nd – not determined.

ious aldehyde group content and molecular mass (Table 1) were prepared. The two colorimetric methods were used to determine their quantities – the Somogyi's and Park—Johnson's. As shown in Table 1, for dextran the methods provide very similar results. The Somogyi's method is less sensitive for the mannan dialdehydes, which is probably connected with the steric hindrances. Whereas the dextran is an α-D-(1→6)-linked polysaccharide of glucose, the mannan is comprised of many side α-D-(1→2)- and few α-D-(1→3)-bonded branches on the main α-D-(1→6)-linked mannose backbone [31] which may prevent from penetrating of copper salt of the tartaric acid into the clew of polysaccharide. The Park—Johnson's method is, in general, more sensitive (*ca.* 25 times) than Somogyi's method and the calibrations data of various monosaccharides obtained by this method were identical.

During periodate oxidation M_r of prepared dialdehydes is reduced. The decrease is partially due to glycol cleavage of monosaccharide units, however, it is in greater extent due to the degradation of polysaccharide chain.

The influence of various sodium periodate concentration was particularly investigated at a mole ratio $n(NaIO_4)/n(Glc) = 0.880$ (obtained with various volume of periodate solutions). At the concentration of 15 mmol dm^{-3} and 50 mmol dm^{-3} of $NaIO_4$, the oxidation was still effective, but the higher concentrations of the periodate (100 mmol dm^{-3} and 150 mmol dm^{-3}) caused, above all, the extensive degradation of polysaccharide chains (results not shown).

Fig. 1 shows the influence of various mole ratio $n(NaIO_4)/n(Glc)$ on the degree of oxidation and on the degradation of the dextran at the constant concentration of periodate (50 mmol dm^{-3}). The highest increase of reactive aldehyde groups in dextran molecules at their low degradation was registered by elevation of periodate/glucose mole ratio from 0.088 to 0.440. The application of two- and three-fold amount of periodate, respectively, with regard to the last ratio mentioned above brought finally neither the more evident influence on the oxidation course, nor the decrease of DP. The mole ratio $n(NaIO_4)/n(Glc) = 1.76$ led to the great cleavage of polysaccharide backbone followed by reduction of the degree of oxidation.

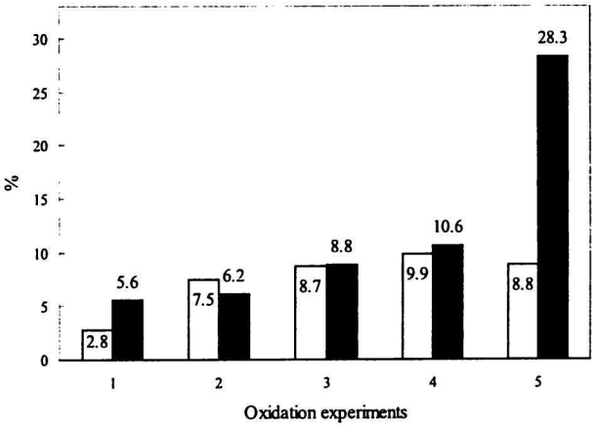

Fig. 1. Effect of the mole ratio $n(NaIO_4)/n$(glucose) on the degree of dextran oxidation (□) and degradation (■) (at $c(NaIO_4) = 50$ mmol dm^{-3}). Mole ratio: *1.* 0.088, *2.* 0.440, *3.* 0.880, *4.* 1.320, *5.* 1.760.

Unlike oxidation of the dextran, oxidation of the mannan progressed at the mildest conditions without degradation. The start of degradation course was

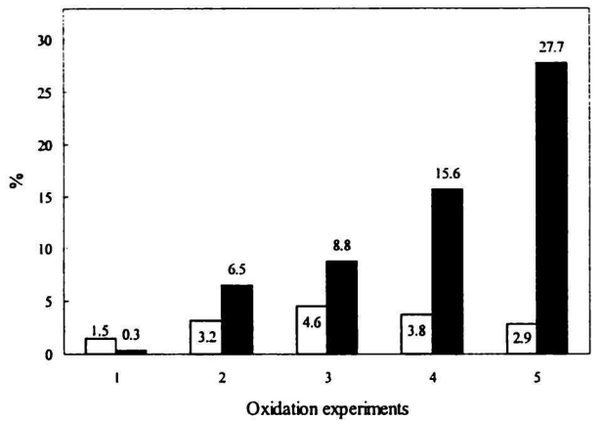

Fig. 2. Effect of the mole ratio $n(NaIO_4)/n(mannose)$ on the degree of mannan oxidation (□) and degradation (■) (at $c(NaIO_4) = 50$ mmol dm^{-3}). Mole ratio: *1.* 0.054, *2.* 0.135, *3.* 0.270, *4.* 0.540, *5.* 0.710.

eventually due to the enhancement of the amount of oxidation reagent used. The influence of a various concentration of $NaIO_4$ at a constant mole ratio $n(NaIO_4)/n(Man) = 0.270$ was investigated. By the change of the concentration from 15 mmol dm^{-3} to 50 mmol dm^{-3} of the periodate, the amount of the aldehyde groups was somehow increased. However, the reaction of a 100 mM solution of periodate resulted in the two-fold enhancement of the degradation against 50 mM solution, whereas the degree of oxidation dropped (Table 1).

The course of periodate oxidation of the mannan by using a constant concentration of $NaIO_4 =$ 50 mmol dm^{-3}, but at a different mole ratio of $n(NaIO_4)/n(Man)$ is plotted in Fig. 2. The highest increase of the aldehyde groups was detected by using the mole ratio $n(NaIO_4)/n(Man) = 0.270$. The quantity of the carbonyl groups was reduced using a higher molar excess of periodate, which is connected with a more extensive degradation degree as well as with a slight dissolution ability of the dialdehyde mannans prepared.

It can be said that on the basis of the evaluation of all the experiments from the point of view of the degree of oxidation and degradation, the optimum (the maximum growth of oxidation at the minimum degradation) was achieved by using of 0.880 and 0.270 molar equivalents of periodate to the monosaccharide unit for the dextran and mannan, respectively.

The influence of structure changes (caused by periodate oxidation) of modified polysaccharides on their interaction with Con A was investigated by precipitation experiments. To eliminate the chemical reaction of aldehyde groups of polysaccharides with NH_2 groups of Con A, they were reduced with sodium borohydride before the interaction experiments (the reactivity of the aldehyde groups, in expected application of oxidized polysaccharides – in the conjugation with

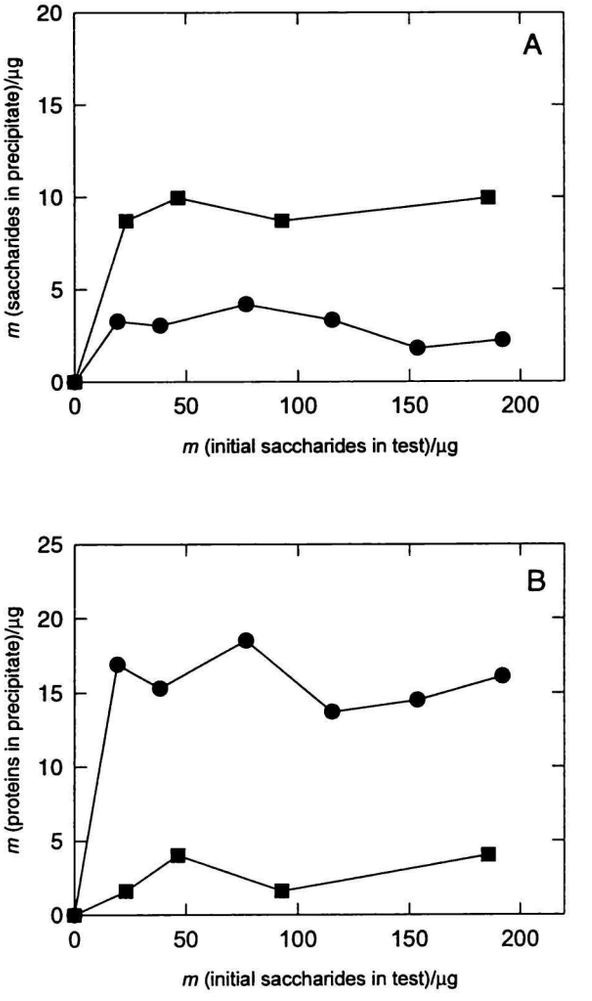

Fig. 3. Precipitation profiles of dextrans with Con A. A – dependence of polysaccharides in precipitates on initial amounts of dextran, B – dependence of Con A in precipitates on initial amounts of dextran, ● dextran, ■ D-4 in reduced form.

proteins, will be eliminated by reductive amination).

Fig. 3 shows the quantitative precipitation profile of dextran and its oxidized and then reduced dextran D-4 (see Table 1) with Con A in dependence of increasing concentration of polysaccharide in the tests. The saccharide (Fig. 3A) as well as protein (Fig. 3B) precipitation profiles appear to be substantially different from that described in literature [14]. A very low interaction of dextran with Con A at conditions used in our experiments was observed. The weak increasing of D-4 precipitation (Fig. 3A) could be caused by nonspecific aggregation, because it was not proved by precipitation course expressed as Con A in the same experiment (Fig. 3B). This assumption was confirmed also by experiment, where the specific inhibitor α-MGP (of concentration 20 mmol dm^{-3}) was added to precipitation mixture Con A with D-4 and the competitive displacing which resulted in dissolution of precipitate

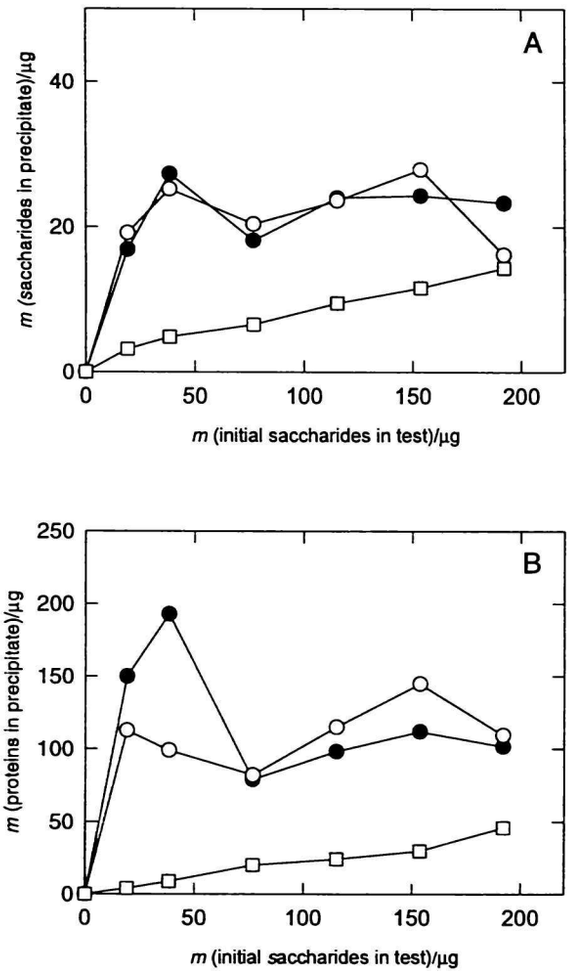

Fig. 4. Precipitation profiles of mannans with Con A. A – dependence of polysaccharides in precipitates on initial amounts of mannan, B – dependence of Con A in precipitates on initial amounts of mannan, ● mannan, ○ M-2, □ M-7 (the latter two in reduced form).

was not observed (not shown).

The results from precipitation experiments of mannan and its oxidized samples M-2 and M-7 (in reduced form) with Con A are shown in Fig. 4A, B. The mannan gave classical precipitation curves with two points of maximum precipitation. One at low content (40 μg) and the second one at higher content (160 μg) of mannan in precipitation tests. The presence of two distinct saccharides as well as protein peaks of maximum indicates that in macromolecules of mannan there are two protein binding sites with different affinity to Con A; the primary site with high affinity and the secondary one with low affinity [23]. The precipitation profiles of modified mannans M-2 (Fig. 4A, B) as well as M-1, M-4 (not shown) – the weak modified samples – were similar to that of the original mannan. Some little changes of extent and shift of the saccharides (Fig. 4A) as well as Con A (proteins) peaks (Fig. 4B) in precipitates were observed. The precipitation profiles of highly modified mannans M-6 (not shown) and M-7 (Fig. 4A, B) appear to be different from those observed before. They are without the peak of maximum of precipitated saccharide (Fig. 4A) as well as Con A proteins (Fig. 4B). The courses of precipitation of both these samples are very similar. The formation of precipitates was increased uniformly with dosage of modified mannans in the experiments, where the total amounts of protein and saccharide in precipitates were achieved substantially lower than at mannan as well as at samples M-1, M-2, and M-4. The biospecificity of interaction of mildly oxidized mannan with Con A was confirmed by the precipitation experiment of sample M-2 (at dosage 20 μg and 40 μg in the test) with Con A in the presence of 20 mM α-MMP as competitive inhibitor (not shown). In this case the precipitation was not observed.

CONCLUSION

The products with sufficient content of reactive aldehydes were obtained by mild periodate oxidation (at mole ratios $n(NaIO_4)/n$(glucose) = 0.440—0.880 and $n(NaIO_4)/n$(mannose) = 0.135—0.270, respectively) of the dextran (n(aldehydes)/n(dextran) = 16—18) and of the mannan (n(aldehydes)/n(mannan) = 20—28). The relatively weak decrease of M_r of both polysaccharides modified in such a way was observed (from 18 400 to 16 500 of the dextran; from 55 000 to 49 500 of the mannan).

Mannan from *Saccharomyces cerevisiae*, the highly branched polysaccharide with α-D-(1→2)-mannopyranosyl units on the terminal ends, showed the good precipitation properties with Con A. The maintenance of mannan structure (the low content of oxidized mannosyl units at the low decrease of M_r and not essential change of branching) in its, at mild conditions, oxidized form (at mole ratio $n(NaIO_4)/n$(Man) = 0.054—0.270) was confirmed by interaction study with Con A. The precipitation of dextran as well as its modified sample with Con A was not observed (caused probably by low-branched structure of dextran molecule).

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