Neoglycoconjugates of Mannan with Bovine Serum Albumin and Their Interaction with Lectin Concanavalin A

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Neoglycoconjugates were prepared from mannan isolated from yeast *Saccharomyces cerevisiae* and activated by periodate oxidation to create aldehyde groups. Various degrees of oxidation introduced 11−28 aldehyde groups per mannan molecule and simultaneously resulted in a molar mass decrease from 46 to 44.5−31 kDa. The activated mannans were subsequently conjugated with bovine serum albumin forming neoglycoconjugates. Some parameters of these mannan−bovine serum albumin conjugates were characterized: saccharide content 25−30% w/w, molar mass within the range 169−246 kDa, and polydispersion (M_w/M_n) from 2.8 to 3.6. The interaction of these conjugates with lectin concanavalin A was studied using three different methods: (i) quantitative precipitation in solution; (ii) sorption to concanavalin A immobilized on bead cellulose; and (iii) kinetic measurement of the interaction by surface plasmon resonance. Quantitative precipitation assay showed only negligible differences in the precipitation course of original mannan and the corresponding mannan−bovine serum albumin conjugates. Both the sorption method (equilibrium method) and the surface plasmon resonance measurement (kinetic method) demonstrates that the values of dissociation constant K_D of all synthetic neoglycoconjugates were within the range 10^{-7}−10^{-8} mol·L^{-1} (close to $K_D = 10^{-8}$ mol·L^{-1} determined by the sorption method for the original mannan). In conclusion, characterization of synthetic neoglycoconjugates confirmed that the method used for their preparation retained the ability of mannan moiety to interact with concanavalin A.

INTRODUCTION

Carbohydrate moiety in glycoproteins modifies the physicochemical and biological properties of the proteins to which they are attached. By their conjugation with carbohydrate derivatives, the synthetic glycoproteins (neoglycoproteins) can be obtained. Neoglycoconjugates are compounds that in useful ways emulate the behavior of the natural glycoconjugates. Neoglycoproteins can be also prepared from bioactive proteins such as enzymes, immunoglobulins, or growth factors. A variety of strategies have been employed in preparing neoglycoproteins (neoglycoenzymes) using mono-, oligo-, or polysaccharides (Magnusson et al., 1994; Lee and Lee, 1997).

Neoglycoproteins containing mannosylated serum albumin were prepared and used as carriers for selective delivery of drugs to cell membranes. These neoglycoproteins are specifically recognized by membrane lectins of liver (Beljaars et al., 1999; Franssen et al., 1993). Mannosylated bovine serum albumin conjugates were utilized as carriers for receptor-mediated drug delivery to treat macrophage-associated diseases (Chakraborty et al., 1990) and for increase of oligonucleotide internalization in cells (expressing mannose-6-phosphate specific membrane lectins) at inhibition of gene expression (Bonfils et al., 1992). Polysaccharides from certain microorganisms were effectively converted into vaccines by partial hydrolysis and by reductive amination with appropriate protein carriers (Jennings and Sood, 1994).

The biospecific affinity of glycoconjugate to lectins originates from the specific saccharide contained in the protein/enzyme molecule. The immobilization procedures based on binding of glycoenzyme to lectin−matrix (in the case of having a strong affinity to this lectin) are gaining increasing acceptance (Saleemuddin, 1999) in the last years. The synthesis of neoglycoenzymes may therefore serve for preparation of modified enzymes with biospecific affinity to lectins, which could be utilized in such immobilization techniques.

Mannan is a polysaccharide with strong affinity (Jennings and Sood, 1994) to lectin concanavalin A (Con A)[1] and therefore is important for glycoprotein synthesis for their prospective use in lectinology. It was found out that

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[1] Abbreviations: BSA, bovine serum albumin; *SC* mannan, mannan from *Saccharomyces cerevisiae*; Con A, concanavalin A; BC, bead cellulose; CHTBC, chloro-1,3,5-triazine bead cellulose; Con A-TBC, Con A-triazine bead cellulose; α-MMP, methyl α-D-mannopyranoside; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); M1−M5, samples of mannan dialdehydes; RM1−RM5, samples of reduced mannan dialdehydes; CM1−CM3, samples of mannan-BSA conjugates; SEC, size exclusion chromatography; HPLC, high performance liquid chromatography; HPSEC, high performance size exclusion chromatography; MALC, multiangle light scattering; SCV, single capillary viscometer; SPR, surface plasmon resonance; M_w, weight-average molecular mass; M_p, molecular mass of the apex of the peak.

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the polymer carrying multiple specific mannosyl residues exhibited a several times higher affinity toward Con A than methyl α-D-mannopyranoside (α-MMP) considered to be the most interacting monosaccharide (Mortell et al., 1996). This was proved by synthetic multivalent mannosylated clusters (glycodendrimers) which were shown to be high-affinity ligands for mannose specific lectins (Roy et al., 1998). Yeast mannans are extensively branched polymers (Goldstein and Poretz, 1986) where the presence of extended sequences of α-D-(1 → 2)-linked mannose residues in their macromolecules provides sites for interaction with Con A. The interaction of yeast mannan (as a natural multivalent ligand) with Con A is comparable with affinity of 16-mer mannosyl-dendrimer (Pagé et al., 1996). The mannan from *Saccharomyces cerevisiae* (*SC*) was previously used for determination of binding properties of natural and synthetic α-D-mannosyl ligands, e.g., inhibition effect of these ligands on yeast mannan precipitation with Con A was investigated (Goldstein and Hayes, 1978; Pagé et al. 1996). The idea of mannan conjugation with protein, to incorporate the polysaccharide properties into protein molecule, was applied in the last years to preparation of synthetic antigens. The high-molar-mass mannans exhibit immunogenic activity (Young et al., 1998); therefore, the synthetic mannan−protein conjugates (antigens) induce the protective antibody response (Han et al., 1999; Apostolopoulos et al., 2000), but the mannan−protein conjugates have not yet been investigated from the point of view of their interaction with lectins.

The aim of this study was to develop a method to prepare conjugates of *SC* mannan with bovine serum albumin (BSA) with retained lectin-binding affinity of mannan moiety. Three principally different methods to exhaustively investigate mannan−BSA conjugates interaction with Con A were used.

EXPERIMENTAL PROCEDURES

Materials. The mannan isolated from *Saccharomyces cerevisiae* (average molar mass ≈ 46 kDa) was kindly provided by Dr. Šandula (Institute of Chemistry, SAS, Slovak Republic) (Šandula and Vojtková-Lepšíková, 1974). BSA was purchased from IMUNA, Šarišské Michaľany, Slovak Republic, sodium cyanoborohydride ($NaCNBH_3$) was obtained from Fluka Chemie AG, Buchs, Switzerland, and sodium borohydride ($NaBH_4$) and sodium periodate ($NaIO_4$) were the products of Lachema, Brno, Czech Republic. Lectin Con A for precipitation and sorption experiments was obtained from Lectinola, Prague, Czech Republic. α-MMP was purchased from Fluka Chemie AG, Buchs, Germany. Bead cellulose Perlose MT-50 (BC) was supplied by Lovochemie, Lovosice, Czech Republic. Chloro-1,3,5-triazine bead cellulose (CHTBC) was prepared according to the procedure described in our previous work (Mislovičová et al. 1995). The content of 3% w/w of nitrogen and 1.5% w/w of chlorine were determined (10% w/w of chlorotriazine group/dry sorbent).

Con A and *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) used in the SPR method were purchased from Sigma, St. Louis, USA. The SPR chips CM-5, covered with carboxymethylated dextran and an amine coupling kit, were purchased from BIACORE (Uppsala, Sweden). Other reagents were of the analytical grade.

Preparation of Mannan Conjugates with BSA. 100 mg of *SC* mannan was dissolved in a subsequent volumes of 0.05 mol·L^{-1} aqueous solution of sodium periodate:

1 mL − molar ratio $NaIO_4$/mannosyl unit = 0.054 (sample M1)

2 mL − molar ratio $NaIO_4$/mannosyl unit = 0.135 (sample M2)

3.5 mL − molar ratio $NaIO_4$/mannosyl unit = 0.270 (sample M3)

7 mL − molar ratio $NaIO_4$/mannosyl unit = 0.540 (sample M4)

10 mL − molar ratio $NaIO_4$/mannosyl unit = 0.710 (sample M5).

Reaction mixtures were stirred in the dark at 4 °C during 1 h. Then the reactions were stopped by addition of ethylene glycol (1 mL) and stirred 1 h. Low molecular weight compounds were removed from the mixture by dialysis (Spectra Por dialysis tubing MWCO 6000−8000) against water at 4 °C in the dark. Obtained aqueous solutions of oxidized polysaccharides were lyophilized. The amount of aldehyde groups was determined in lyophilizates by the Park-Johnson method (Park and Johnson, 1949). Mannan dialdehydes M1, M2, and M3 were used for conjugation with bovine serum albumin. The mixtures of each three mannan dialdehydes (M1−M3) in 0.05 mol·L^{-1} phosphate buffer of pH 7 (4 mL, c = 10 mg·mL^{-1}) with a solution of BSA (4 mL, c = 10 mg·mL^{-1} of the same buffer) and a solution of $NaCNBH_3$ (2.5 mL, c = 10 mg·mL^{-1} of the same buffer) were stirred at room temperature. The reaction was stopped after 24 h by addition of $NaBH_4$ in 0.05 mol·L^{-1} borate buffer of pH 9.5 (ca. 50 mol of $NaBH_4$ were added to the determined aldehyde groups, c = 5 mg·mL^{-1}) to reduce the remaining aldehyde groups. The reduction was proceeded by stirring at room temperature for 6 h. The products were adjusted with 4 mol·L^{-1} HCl to pH 6−7 and dialyzed against H_2O. Unreacted mannans and BSA were removed by ultrafiltration (YM 100, Amicon Inc., Beverly, USA), and the resulting water solutions of conjugates were lyophilized. The contents of saccharides (Saha and Brewer, 1994) and proteins (Lowry et al., 1951) in lyophilizates were determined. The content of nitrogen was determined by elemental analysis of conjugates (EA 1108 CHN-O analyzer, Milan, Italy).

Determination of the Molar Mass of the Mannans. M_w of the original as well as of the oxidized mannan samples (after their reduction with $NaBH_4$) (Masárová et al., 2001) were determined by the HPSEC method. HPLC system (Shimadzu, Wien, Austria) was composed of a high-pressure pump LC-10AD, a membrane degasser GT-104, an injector Rheodyne 77251, and a differential refractometer RID-6A. The system was calibrated with a set of pullulans with M_w = 5−100 kDa (Macherey-Nagel, Düren, Germany). The solutions of calibrating pullulans as well as of tested mannans (c = 1 mg·mL^{-1} of water) were applied onto a HEMA BIO 100 column (8 × 250 mm, 10-μm particle size) obtained from Tessek, Prague, Czech Republic, and eluted with 0.02 mol·L^{-1} phosphate buffer at flow rate 0.4 mL·min^{-1} at ambient temperature. The volume of injected sample was 0.02 mL. The chromatographic data were processed by Class-VP-chromatography software (Shimadzu, Wien, Austria).

Determination of the Molar Mass of the Mannan−BSA Conjugates. The molecular characterization of the starting (nonconjugated) mannan and BSA and of the mannan−BSA conjugates was performed by a multidetector size exclusion chromatography (SEC) system. The system consisted of an Alliance 2690 separation module, a 410 differential refractometer from Waters (Milford, MA), a homemade single capillary viscometer (SCV), and an additional multiangle laser light scattering

Table 1. Characteristics of Modified Mannans and Their Interaction with Con A Determined by SPR

sample	content of aldeh. groups before reduc. n(aldeh.)/n(polysach.)	M_p[a] [kDa]	polydispersion index M_w/M_n	k_a [M^{-1} s^{-1}]	k_d [s^{-1}]	K_D [M]
mannan	1	46.0	1.3	3.64×10^2	3.93×10^{-4}	1.08×10^{-6}
RM1	11	44.5	1.4	5.25×10^2	2.58×10^{-4}	4.91×10^{-7}
RM2	20	42.0	1.4	2.56×10^2	3.77×10^{-4}	1.47×10^{-6}
RM3	28	40.0	1.7	1.69×10^2	3.86×10^{-4}	2.28×10^{-6}
RM4	22[b]	37.0	1.8	1.08×10^2	3.06×10^{-4}	2.83×10^{-6}
RM5	13[b]	31.0	2.0	0.42×10^2	1.15×10^{-3}	2.74×10^{-5}

[a] Determined by HPSEC (HEMA BIO 100 column) and RI detection. [b] Determined in a soluble part.

(MALS) Dawn DSP-F photometer from Wyatt (Santa Barbara, CA). The SEC-MALS-SCV system was described in detail elsewhere (Wyatt, 1993; Mendichi et al., 1995; Mendichi and Giacometti Schieroni, 1999). The experimental SEC conditions were the following: 0.5 mol·L^{-1} NaCl at 35 °C as mobile phase, 0.8 mL·min^{-1} of flow rate, and 200 μL of injection volume. The column set was composed of two OHpak Shodex columns (KB-806 and KB-805) (8 × 300 mm, 13-μm particle size) from Showa Denko (Tokyo, Japan).

The differential refractive index increment, dn/dc, of the mannan−BSA conjugates with respect to the mobile phase was measured by a KMX-16 differential refractometer from LDC Milton Roy (Riviera Beach, FL).

Quantitative Precipitation Assay. Precipitation of *SC* mannan as well as mannan−BSA conjugates (CM1−CM3) with Con A was assayed in phosphate buffer of pH 7 containing 0.05 mol·L^{-1} sodium phosphate, 0.1 mol·L^{-1} NaCl, 0.1 mmol·L^{-1} $CaCl_2$, and 0.1 mmol·L^{-1} $MnCl_2 \times 4$ H_2O. The total volume of precipitation tests was 1 mL containing 230 μg·mL^{-1} of Con A and various concentrations of mannan (within the range from 20 to 190 μg·mL^{-1}) or conjugate (within the range from 10 to 120 μg·mL^{-1} of the conjugate saccharide). The samples were shaken at 25 °C for 2 h. The precipitates were separated in a microcentrifuge MPW-310 (Mechanika Precyzyjna, Warszawa, Poland) at 10 000 min^{-1} (8400*g*) during 10 min, washed twice with 1 mol·L^{-1} NaCl, centrifuged, and subsequently dissolved in 0.05 mol·L^{-1} phosphate buffer adjusted to pH 10.5 with 1 mol·L^{-1} NaOH. These solutions were analyzed for saccharides (Saha and Brewer, 1994) and proteins (Lowry et al., 1951). In each experiment (at the peak of maximum precipitation), the test was done in parallel in the presence of 0.05 mol·L^{-1} of methyl α-D-mannopyranoside (α-MMP) to confirm the specific interaction.

Sorption Experiments. Con A was bound to the chloro-1,3,5-triazine bead cellulose (CHTBC) matrix by the method described earlier (Mislovičová et al., 1995). The sorption of mannan−BSA (CM1−CM3) conjugates on Con A-TBC matrix (containing 3.61 mg of Con A/g of wet sorbent) was performed in 0.05 mol·L^{-1} phosphate buffer (pH 7) with 0.1 mol·L^{-1} NaCl containing 0.1 mmol·L^{-1} $CaCl_2$ and 0.1 mmol·L^{-1} $MnCl_2 \times 2$ H_2O. Suspensions of Con A-TBC prepared from 0.1 g of sorbent and 1 mL of individual mannan−BSA conjugate solutions (40−200 μg of conjugate or 10−70 μg expressed as the saccharide in the above-mentioned buffer) were shaken slowly at 25 °C for 2 h. At the same time, the blank experiment was performed, where the nonderivatized bead cellulose and maximum amount of pertinent conjugate in suspension was used. Then the supernatants were separated by twice centrifugation at 10 000 min^{-1} (8400*g*). Saccharides and proteins in the obtained supernatants were determined. The amounts of the adsorbed conjugate were calculated as the difference between the contents of saccharides and proteins in supernatants obtained in sorption on BC and on Con-A TBC. The same adsorption experiment was performed with *SC* mannan to compare the behavior of original mannan with mannan−BSA conjugates. Dissociation constants and binding capacities were calculated by rearrangement of the adsorption isotherm equation to the Scatchard plot $Q^* = Q_m - K_D Q^*/C^*$ (Chern et al., 1999), where C^* is the concentration of sorbate in solution in equilibrium, Q^* is the amount of sorbate adsorbed on the sorbent at equilibrium, Q_m is the maximum adsorption capacity of the adsorbent, and K_D is the dissociation constant of sorbate.

Surface Plasmon Resonance Measurement. The SPR measurements were performed with a BIACORE 2000 (Biacore AB, Sweden) operated with a BIAcore Control Software 1.2. All measurements were performed at 25 °C, at a flow rate of 5 μL·min^{-1}. The buffer of pH 7.4 consisted of 0.01 mol·L^{-1} *N′*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid) (HEPES), 0.15 mol·L^{-1} NaCl, 1 mmol·L^{-1} Ca^{2+}, and 1 mmol·L^{-1} Mn^{2+}. The chip surface was modified by covalently bound Con A using the amine coupling kit. The interaction with Con A was investigated by the injection of 50 μL of sample containing 1 mg·mL^{-1} mannan (RM1−RM5) or mannan−BSA conjugate (CM1−CM3). The chip surface was regenerated by the injection of 20 μL of 5 mg·mL^{-1} *p*-aminophenyl-α-D-mannopyranoside after each measurement. The association (k_a) and dissociation (k_d) rate constants as well as dissociation equilibrium contants (K_D) were calculated (Shinohara et al., 1994).

RESULTS AND DISSCUSION

Oxidized Mannans and their Interaction with Con A. The first step at the conjugation of mannan with BSA was the oxidation of *SC* mannan with periodate to obtain its reactive form. Mannan structure is changed after oxidation, and mannose rings and side chains can be cleaved, which results in a molecular mass decrease. A set of five mannans (M1−M5) oxidized to a different degree (from 11 to 28 of the aldehyde groups per mannan molecule; Table 1, second column) having partially degraded chains (M_p decreases from 46 to 31 kDa; Table 1, third column) and values of polydispersion index within the range from 1.4 to 2.0 (Table 1, fourth column) were prepared. HPSEC chromatograms of these samples are shown in Figure 1.

The oxidation of *SC* mannan was described in more detail in our previous work (Masárová et al., 2001). Quantity of produced aldehyde groups (degree of oxidation) and decrease of M_p of prepared mannan dialdehydes (degree of degradation) were observed. Practically no degradation of polysaccharide chain was observed at the lowest periodate concentration. Degree of degradation was increased with increasing amount of periodate. Samples M4 and M5 were partly insoluble, probably in consequence of the formation of intramolecular hemiac-

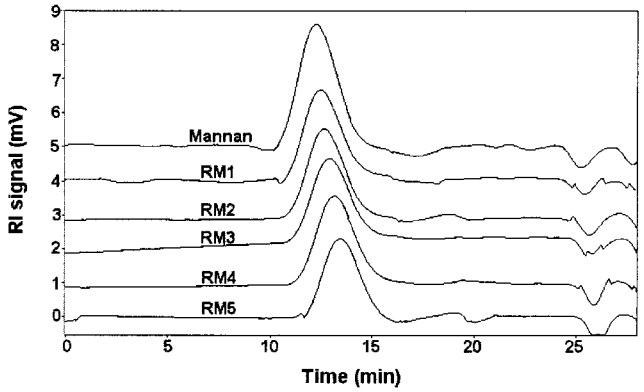

Figure 1. Chromatograms of mannan and oxidized mannan (after reduction) samples obtained by HPSEC chromatography on column HEMA BIO 100. The solutions of calibrating pullulans as well as of tested mannans (c = 1 mg·mL^{-1} of water) were applied onto a HEMA BIO 100 column (8 × 250 mm, 10-μm sorbent of particle size) and eluted with 0.02 mol·L^{-1} phosphate buffer at a flow rate of 0.4 mL·min^{-1} and at ambient temperature. The volume of injected sample was 0.02 mL. The chromatographic data were processed by Class-VP-chromatography software.

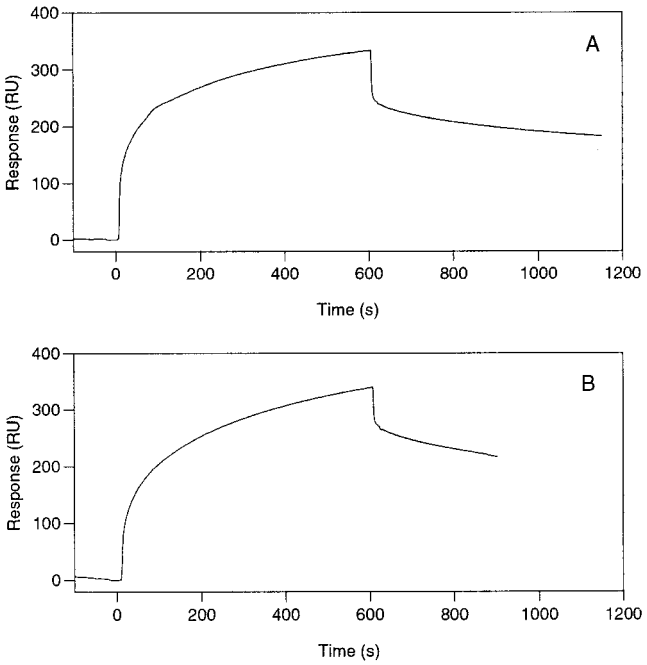

Figure 2. Surface plasmon resonance measurement of the interaction of Con A with mannan. SPR measurements were performed with BIACORE 2000 using the chip with modified surface with covalently bound Con A at a flow rate of 5 μL·min^{-1} with buffer of pH 7.4 consisting of 0.01 mol·L^{-1} HEPES, 0.15 mol·L^{-1} NaCl, 1 mmol·L^{-1} Ca^{2+}, and 1 mmol·L^{-1} Mn^{2+}. The interaction of samples with Con A was investigated by the injection of 50 μL of samples containing 1 mg/mL mannan or mannan-conjugate. Panel A − original mannan; panel B − oxidized mannan M2 after reduction (RM2).

etal bonds. Degree of oxidation and molar mass of these samples therefore could be determined only in the soluble part. By using of higher amount of periodate, higher degradation of polysaccharide chains was going on to the detriment of oxidation.

As it was mentioned above, the activation of mannan has an effect on its structure. The changes of mannan structure has an influence on affinity of mannan samples to lectin Con A. This effect was investigated with mannans that were activated, and subsequently the active groups were reduced. The oxidized mannans were reduced (RM samples) to eliminate chemical reaction of polysaccharide active groups (aldehyde) with amino groups of Con A. The results from precipitation experiments of modified mannans with Con A were presented in a previous paper (Masárová, et al., 2001). Intensive precipitation of mannan and RM1−RM3 samples with Con A was observed. On the other hand, the samples RM4 and RM5 precipitated with Con A weakly. In the present work, the interaction of these modified mannans with Con A was investigated by the SPR method. Figure 2 shows SPR sensorgrams of mannan and the sample RM2 using a chip with surface modified by immobilized Con A. Table 1 shows the calculated kinetic parameters of interactions of original mannan and all the five RM samples with Con A. Gradually reduced mannan affinity to Con A after its modification was observed. There is obvious a correlation of the degree of degradation, and supposed change of mannan structure, with the values of obtained association constants. Surprisingly, the value of k_a of original mannan was not determined to be the highest. We concluded that the oxidized *SC* mannans prepared by mild periodate oxidation (M1−M3) are suitable for the preparation of glycoproteins conjugates. The character (size and valency) of modified mannan in these samples did not change significantly, and therefore they maintained the affinity to Con A.

Preparation and Characterization of Mannan−BSA Conjugates. The three oxidized mannans with the best affinity to Con A (M1, M2, and M3) were used to prepare three BSA conjugates (CM1, CM2, and CM3). The method of reductive amination (described in Experimental Procedures) was used, where the weight ratio mannan/BSA = 1:1 was applied. The content of mannan, M_w, and the polydispersion M_w/M_n in conjugates were determined. The determined characteristics of obtained neoglycoconjugates as well as original mannan and BSA are shown in Table 2.

Table 2. Molecular Characteristics of Mannan, BSA, and Three Mannan−BSA Conjugates

conjugate	content of sacch. [%]	content of prot. [%]	content of N [%]	M_w[a] [kDa]	polydispers. index M_w/M_n
BSA	0	100	14.00	66.7	1.0
mannan	100	0		53.0	1.3
CM1	25	75	10.96	176.2	2.8
CM2	28	72	10.49	246.3	3.2
CM3	30	70	10.33	169.1	3.6

[a] Determined by HPSEC (two Shodex columns) and LS detection.

The conjugation method used produces a mixture of neoglycoconjugates of variable M_w, due to the polydispersion of oxidized mannan, and various stoichiometric mannan to BSA ratios. The polydispersion and modality of the prepared conjugates were estimated by HPSEC experiments on the tandem columns HEMA BIO 1000 + HEMA BIO 100 using UV detection (chromatograms are shown in Figure 3) as well as on the Shodex OH Pak column using LS detector. The high polydispersion of all three samples (≈3) was found. The problems with association of these conjugates caused the difficulties with the determination of their M_w. (It is seen in Figure 3—the first high-molar mass peak shows conjugates in associated form). The conformation of the conjugates was very compact because the gyration radius was low, considering the molecular mass, and the signal of the online viscometer was practically absent. The samples were quite polydisperse, and we can only estimate approximate mannan/BSA ratios (from the determined weight ratio

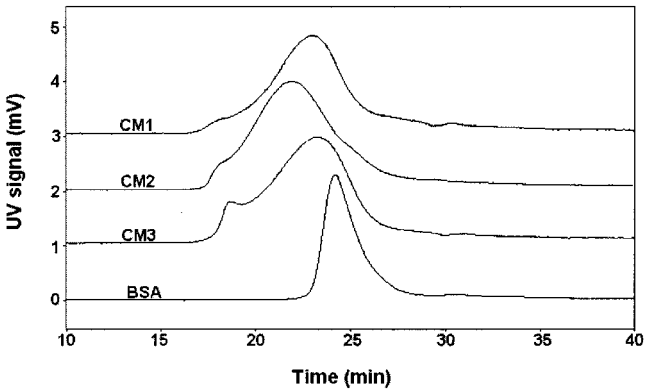

Figure 3. Chromatograms of mannan−BSA conjugates obtained by HPSEC chromatography on two columns HEMA BIO 100 and HEMA BIO 1000. The solutions of tested mannan−BSA conjugates and BSA ($c = 1$ mg·mL^{-1} of water) were applied onto a set of HEMA BIO 100 and HEMA BIO 1000 columns (size of both was 8 × 250 mm with 10-μm particle size of sorbent) and eluted with 0.02 mol·L^{-1} phosphate buffer at a flow rate of 0.5 mL·min^{-1} and at ambient temperature. The volume of injected sample was 0.02 mL. The chromatographic data were processed by Class-VP-chromatography software.

of mannan to BSA in conjugates and M_w of conjugates). We suppose that CM1 contains mainly conjugates with approximately one mannan molecule per two molecules of BSA. The samples CM2 and CM3 are mixtures of 1:1 and 1:2 stoichiometric ratios.

Interaction of Mannan−BSA Conjugates with Con A. The interaction of mannan−BSA conjugates with concanavalin A was studied using three different methods: (i) quantitative precipitation; (ii) sorption method; and (iii) kinetic measurement of the interaction by surface plasmon resonance (SPR).

In the quantitative precipitation method both interacting molecules, neoglycoprotein and Con A, are present in the solution. The measured parameter here is the amount of precipitate obtained at equilibrium. The precipitation profiles of mannan−BSA conjugates with Con A are presented in Figure 4, panel A. The values in precipitation dependence are obtained as the average of two experiments. The similar aggregation course of original mannan and of its conjugates with BSA was determined. Figure 4, panel B, shows dependences of the calculated molar ratios of *SC* mannan and conjugated mannans to Con A in precipitates on the initial concentration of mannan (original or conjugated) in the test. It can be seen that the molar ratio of mannan/Con A in precipitate was achieved to be only 0.5 in the case of original mannan, but the ratios near to 1 were determined for all three conjugates. During precipitation of mannan−BSA conjugates with Con A aggregates with higher content of mannan are formed than in the case of original mannan, what is caused probably by partial association of mannan−BSA conjugates (observed at HPSEC analysis).

The sorption experiments were performed on Con A-TBC MT-50 sorbent with small diameter of pores, to eliminate the sorption of conjugates into pores. Therefore, only the sorption of conjugates on the surface of cellulose beads could be expected. The sorbent with 3.61 mg of Con A/1 g of wet BC was used. The Langmuir isotherm curves (panel A) and the Scatchard plots (panel B) for mannan as well as mannan−BSA samples adsorbed on Con A-bead cellulose are shown in Figure 5. The obtained results are as average of two experiments. Equilibrium concentration of mannan conjugates in solution as well as of their amount adsorbed on Con A-TBC was expressed

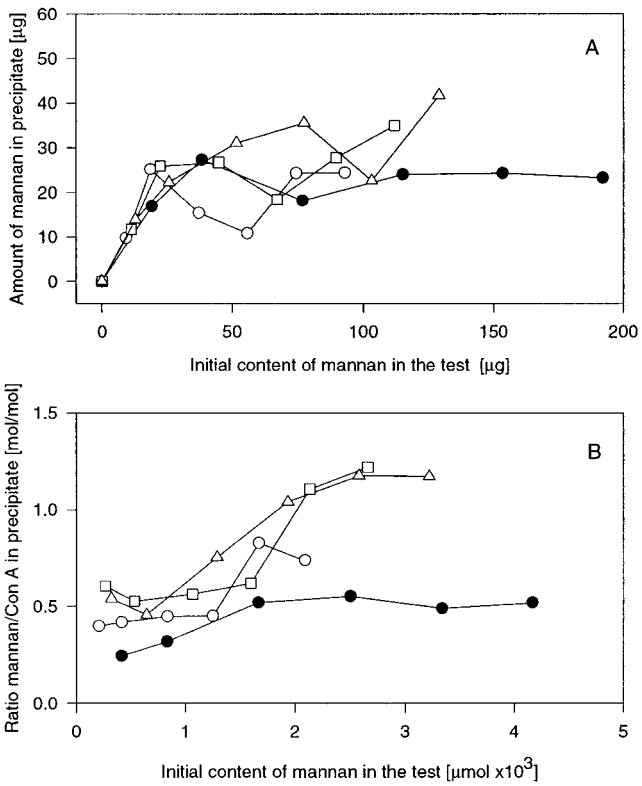

Figure 4. Precipitation of mannan and mannan−BSA conjugates with concanavalin A. Precipitation was performed in 0.05 mol·L^{-1} phosphate buffer of pH 7 containing 0.1 mol·L^{-1} NaCl, 0.1 mmol·L^{-1} Ca^{2+}, and 0.1 mmol·L^{-1} Mn^{2+}. The total volume of precipitation tests was 1 mL containing 230 μg·mL^{-1} Con A and various concentrations of mannan (from 20 to 190 μg·mL^{-1}) or conjugate (from 10 to 120 μg·mL^{-1} of the conjugate saccharide). The samples were shaken at 25 °C for 2 h. The precipitates were centrifugated, washed twice with 1 mol·L^{-1} NaCl, centrifugated, and dissolved in 0.05 mol·L^{-1} phosphate buffer adjusted to pH 10.5. These solutions were analyzed for saccharides and proteins. Panel A, dependences of mannan content (original or in conjugate) in precipitate on the initial content of mannan (original or in conjugate) in the test. Panel B, dependences of the molar ratio mannan (original or in conjugate)/Con A in precipitate on the initial content of mannan (original or in conjugate) in the test. -●- original mannan, -○- CM1, -□- CM2, -△- CM3.

as the mannan concentration. The values of K_D and Q_m estimated from the Scatchard plot and expressed as mannan−BSA conjugates are listed in Table 3. The results obtained by sorption experiments also confirmed that affinity of mannan molecules in conjugates with BSA to Con A was retained. CM2 conjugate showed the highest affinity to immobilized Con A among the three tested conjugates.

Kinetic measurement of the interaction between the neoglycoconjugates and Con A was performed using a biosensor based on surface plasmon resonance. This technique has been previously used for lectin−lipopolysaccharide interaction characterization (Shinohara et al., 1994; Mann et al., 1998; Haselye et al., 1999). SPR uses continuous flow technology and enables one to monitor the interaction in real time in comparison to the sorption method which provides data at equilibrium. In our study, Con A was immobilized on the SPR chip surface, and the interaction of neoglycoconjugates present in solution was monitored. The courses of measurements were similar to that presented in Figure 1; therefore, we present here only the calculated constants summarized in Table 4. The values of dissociation constant K_D are similar to those determined by isothermal adsorption. This demonstrates

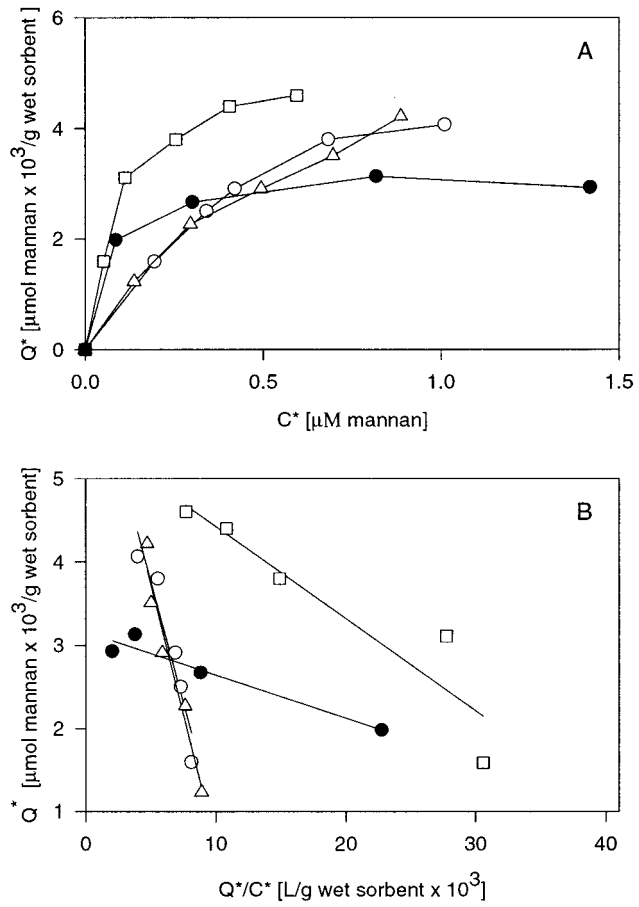

Figure 5. Sorption of mannan−BSA conjugates on Con A-TBC MT-50. The sorption of mannan and mannan−BSA was done in 0.05 mol·L^{-1} phosphate buffer (pH 7) with 0.1 mol·L^{-1} NaCl containing 0.1 mmol·L^{-1} Ca^{2+} and 0.1 mmol·L^{-1} Mn^{2+}. Suspension of Con A-TBC MT-50 (containing 3.61 mg of Con A/g of wet sorbent) prepared from 0.1 g of wet sorbent and 1 mL of individual mannan or mannan−BSA conjugate solutions (from 10 to 70 μg expressed as the saccharide in the above-mentioned buffer) were shaken slowly at 25° C for 2 h. The sorbents were centrifugated, and the saccharide and proteins in supernatants were determined. The amounts of the adsorbed conjugate were calculated as the difference between the contents of saccharides and proteins in supernatants obtained in sorption on blank BC and on Con-A TBC (sorbate is expressed as the content of mannan). Panel A, Langmuir isotherm. Panel B, Scatchard plot; -●- original mannan, -○- CM1, -□- CM2, -△- CM3.

Table 3. Langmuir Parameters for Adsorption of Mannan and Its Conjugates with BSA Determined on Con A Bead Cellulose Sorbent

sorbate	Q_m [μmol of sorbate/ g of wet sorbent]	K_D [mol sorbate·L^{-1}]	R^a
mannan SC	3.16×10^{-3}	5.2×10^{-8}	0.941
CM1	8.68×10^{-3}	5.67×10^{-7}	0.962
CM2	3.44×10^{-3}	8.98×10^{-8}	0.945
CM3	5.71×10^{-3}	2.81×10^{-7}	0.721

[a]Correlation coefficient.

a very good agreement of methods based on measurement at equilibrium (sorption method) and the method based on kinetic measurement (SPR method). We suppose the kinetic behavior of neoglycoconjugates is mainly affected by the two main influences: percentual content of saccharide (Table 2) and modification of mannan which changes kinetic properties of mannan itself (Table 1). The best interaction with Con A was experimentally observed for CM2. CM2 showed the highest kinetic association constant k_a determined by SPR and the lowest equilibrium dissociation constant K_D confirmed by both sorption and SPR method. Another parameter that may influence the interaction of neoglycoproteins are molecular weight and polydispersion. It is interesting that the highest molar mass using HPSEC method was determined for the conjugate CM2.

Table 4. Constants of Mannan−BSA Conjugates Interaction with Con A Determined by SPR

sample	k_a [M^{-1} s^{-1}]	k_d [s^{-1}]	K_D [M][a]
CM1	6.62×10^{2}	3.52×10^{-4}	5.32×10^{-7}
CM2	1.06×10^{3}	3.43×10^{-4}	3.25×10^{-7}
CM3	6.45×10^{2}	3.24×10^{-4}	5.02×10^{-7}

[a]Mol of mannan−BSA conjugate/L.

CONCLUSIONS

Oxidative modification of mannan results in changes in its interaction with Con A and consequently also influences properties of mannan−BSA neoglycoconjugates. Measurement of the interaction of neoglycoconjugates with Con A confirmed that the method used for their preparation retained the ability of the mannan moiety to interact with the lectin concanavalin A. It was observed by both the sorption and the SPR method that binding of mannan moiety of neoglycoprotein is similar as compared to the original mannan; it can be documented by the values of K_D (within the range 10^{-7} and 10^{-8} mol·L^{-1}). The measurements of molar mass and polydispersion suggest that in this conjugation method it is difficult to control structure of prepared conjugates. We suppose that conjugates of various BSA/mannan ratios may be present and also association of conjugates, which influences M_W determination, may occur. Nevertheless, the mannan−BSA conjugates with very similar M_w, polydispersion, composition, and affinity to Con A were obtained at the same condition of their conjugation and separation. This method and obtained experiences may find application in glycosylation of proteins/enzymes, for preparation of biologically active glycoconjugates (e.g., vaccines, markers) or generally as an immobilization technique for glycoproteins.

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