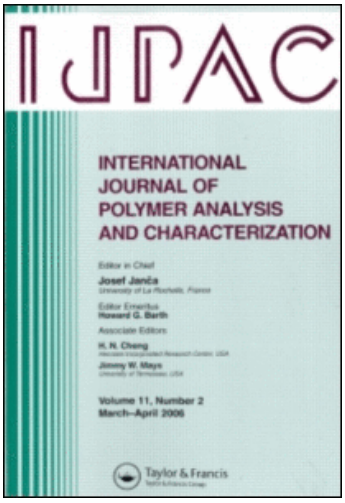

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Investigation of the Conjugation Reaction of Mannan with Albumin

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Investigation of the Conjugation Reaction of Mannan with Albumin

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The conjugation reaction of mannan, originated from Saccharomyces cerevisiae*, with bovine serum albumin (BSA) was investigated. Mannan was functionalized by periodate oxidation and then grafted to albumin by reductive amination. Three types of mildly oxidized mannan and four various weight ratios mannan:albumin were used. The time course of conjugation was measured as a decrease of elution volume using HPLC. The molecular weight (M_{app}) as well as the content of saccharides and proteins in the conjugates were determined. The dependence of M_{app} and molar ratio mannan : BSA in products on the type of mannan used (difference in content of aldehyde groups as well as M_{app}) was observed. Parameters that provide for $\geq$80% of nonoxidized monosaccharide rings are deciding factors for maintaining the original mannan features in conjugates with albumin, which is responsible for their interaction with lectins.*

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Keywords: Mannan from yeast; Periodate oxidation; Bovine serum albumin; Neoglycoproteins; HPSEC; Mannan-albumin conjugate characterization

Methods of the chemical modification of proteins with polysaccharides have been studied in the past two decades. Proteins/enzymes with bound saccharides are more resistant against heating and organic solvents and against proteolytic attack in general[1–3]. Glycosylation of proteins (or enzymes) lead to the change of their physicochemical properties[4–8] to improve antimicrobial[9] and emulsifying behaviors[10] as well as their stability. The changes of biological features of synthetic glycoproteins were utilized in immunological studies[11] in cytochemistry and in biochemical detection of lectins[12]. The new properties of synthetic glycoproteins in comparison with the nonmodified proteins are a result of the saccharide part, where composition, structure, size and other physicochemical features of this moiety are important.

Before the synthetic introduction of polysaccharides into the protein molecule, they are activated by various reagents, for example cyanogen bromide[13], carbonyl diimidazole[14] or epichlorohydrine[15]. The periodate oxidation is also an effective method for introduction of new reactive aldehyde groups into the polysaccharide molecule[16,17]. By such means, prepared dialdehyde polysaccharide is then bound to the protein through the primary amino groups of lysine or asparagine (*N*-linked saccharide) by a reductive amination method[18,19]. In most work involved with conjugation of polysaccharide with proteins, dextran was used[3,8,9,17–19]. In our present study, conjugates of mannan from *Saccharomyces cerevisiae* with bovine serum albumin were prepared. The sequences of mannan are very important at physiological immunointeractions (B cell antigen) as well as at pathological immunoresponse (antigen in Crohn's disease) of the glycoprotein antigen[20]. The significant biospecific behavior of mannan is its interaction with lectin, especially with Concanavalin A[21]. The binding of mannan with proteins or enzymes is needed for the preparation of synthetic glycoconjugates having affinity to Concanavalin A. The mild periodate oxidation of mannan and affinity of modified samples to Concanavalin A was described in our preceding work[22]. The present paper deals with the investigation of the reaction of mannan dialdehyde conjugation with bovine serum albumin by using a reductive amination method. The content, size and structure of mannan in conjugates was studied at various reaction conditions. The aim of this work is to obtain experience that can be used in the preparation of neoglycoproteins with affinity to α-mannosyl-specific lectins.

EXPERIMENTAL

Materials

The mannan used (mean molecular weight $\approx 55{,}000$ g/mol), isolated from *Saccharomyces cerevisiae*, was kindly provided by Dr. Šandula[23]. Bovine serum albumin (BSA) was purchased from IMUNA (Šarišské Michal'any, SR), sodium cyanoborohydride ($NaCNBH_3$) was obtained from Fluka Chemie AG (Buchs, Switzerland), sodium periodate was the product of Lachema (Brno, Czech Republic). Other reagents were of analytical grade.

Mannan Functionalization

To 100 mg of mannan, various volumes of 50 mM aqueous solution of sodium periodate was added (Table I). The reaction mixture was stirred in the dark at 4°C for 1 h. Afterwards, the reaction was stopped by the

TABLE I Samples of oxidized mannan obtained by using 50 mM $NaIO_4$.

Sample	Molar ratio ([$NaIO_4$]/[mannan])	Aldehyde content[a] (mol aldehydes/ mol mannan)	Ratio of extinction coefficients[b] (K_{RM}/K_M)	M_{app}[c] (kDa)
M1	10	11	0.89	54.5
M2	25	20	0.81	51.2
M3	50	28	0.65	49.5
M4	100	22*	0.48	46.1
M5	150	13*	0.37	39.6

[a] Determined by Park-Johnson's method[24].

[b] The K_{RM}/K_M ratios of the extinction coefficient of oxidized and subsequently reduced mannan (K_{RM}) to the extinction coefficient of mannose (K_M) were obtained from calibration curves $A_{490} = K \cdot c$ of individual polysaccharides and mannose using the phenol-sulfuric method[25].

[c] M_{app} of oxidized and subsequently reduced mannans were determined by SEC on a HEMA-BIO 100 column. The system was calibrated with a calibration set of dextrans within 1000–70,000 (Fluka AG, Buchs, Switzerland). The solutions of calibrants as well as reduced dialdehyde derivatives ($1\,mg \cdot cm^{-3}$ of water) were injected in $0.02\,cm^3$ volume and eluted with 0.02 M phosphate buffer pH 7.2 at flow rate $0.4\,cm^3 \cdot min^{-1}$. The elution volume was monitored with a differential refractometer and Class-VP-chromatography program.

*Determined in the soluble part of the sample.

addition of ethylene glycol. The resulting solution was dialyzed (Spectra Por® dialysis tubing MWCO 6– kDa) against water at 4°C in the dark for ≈ 24 h and then lyophilized.

Determination of Aldehydes

The aldehyde groups in oxidized samples were determined by the Park-Johnson method[24] based on the reduction of ferricyanide ions in alkaline solution to Prussian blue (ferric ferrocyanide), and by measuring the color intensity ($\lambda = 690$ nm).

Reduction of Aldehydes Groups in Mannans

The aldehyde groups of prepared samples (10 mg) were treated with 0.5 M $NaBH_4$ solution of 0.05 M borate buffer at pH 9.5 for 6 h at room temperature (~50 molar excess of sodium borohydride to determined aldehyde groups in the individual samples was used). The solution was then adjusted to pH 6 with 4 M HCl, dialyzed against H_2O and finally lyophilized. The calibration $A_{490} = f(c)$ of all oxidized and subsequenty reduced mannans M1–M5 was made using the phenol-sulfuric method[25].

Preparation of Mannan Conjugates with BSA

To the solution of mannan dialdehyde in 0.05 M phosphate buffer pH 7 ($4\,cm^3$, $c = 10\,mg \cdot cm^{-3}$) was added a solution of BSA ($c = 10\,mg \cdot cm^{-3}$ in the same buffer): (i) 1 cm^3; (ii) 4 cm^3; (iii) 6 cm^3; (iv) 10 cm^3, and a solution of $NaCNBH_3$ ($2.5\,cm^3$, $c = 10\,mg \cdot cm^{-3}$). The reaction mixtures were stirred at room temperature. Samples ($0.1\,cm^3$) were withdrawn after 1, 7, 16 and 24 h. To each sample was added 0.2 cm^3 of 0.05 M borate buffer pH 9.5 and $0.2\,cm^3$ of solution of $NaBH_4$ ($c = 5\,mg \cdot cm^{-3}$ of borate buffer) to reduce the remaining aldehyde groups. The reduction was performed at room temperature with stirring for 6 h. The products were adjusted with 4 M HCl to pH 6–7 and then injected into the HPLC system. The reaction mixtures obtained after 24 h of all conjugation experiments were dialyzed against H_2O. Unreacted mannans were removed by ultrafiltration (YM 30, Amicon Inc., Beverly, Massachusetts) and the resulting water solutions of conjugates were lyophilized. The content of saccharide[25] and proteins[26] in lyophilizates was determined. The nitrogen content was determined by elemental analysis of the conjugates (EA 1108 CHN-O analyzer, Milan, Italy). The molecular weights of isolated glycoconjugates were estimated by SEC.

Size Exclusion Chromatography

The molecular-weight parameters of the biopolymeric samples (the reduced mannans as well as the mannan-BSA conjugates) were determined by SEC. The apparatus used (Shimadzu, Wien, Austria) was comprised of a high-pressure pump LC-10AD, a membrane degasser GT-104, an injector Rheodyne 77251, a differential refractometer RID-6A and a UV-VIS detector SPD-10AV. All separations were performed by using size exclusion chromatographic columns HEMA-BIO (Tessek, Prague, Czech Republic). Mannan samples were analyzed by operating one single column (HEMA-BIO 100, of dimensions 8×250 mm and particles size 10 μm). Mannan-BSA conjugates were run through a tandem of two columns (HEMA-BIO 100 followed by HEMA-BIO 1000, both of dimensions 8×250 mm and particles size 10 μm). The volumes 0.02 cm^{-3} of concentration 1 $mg \cdot cm^{-3}$ for injection samples and calibrants were used. The mobile phase was 0.02 M phosphate buffer of pH 7.2. Flow rates 0.4 $cm^3 \cdot min^{-1}$ for HEMA BIO 100 column and 0.5 $cm^3 \cdot min^{-1}$ for the system two columns HEMA BIO 100 and HEMA BIO 1000 were used. Dextrans (of M_w 1, 5, 10, 25, 40, 70 and 150 kDa) and proteins standards (ferritin, katalase, γ-globulin, aldolase, BSA and ovalbumin of M_w450, 240, 169, 158, 68 and 45 kDa) were used to calibrate the SEC system. The differential refractometer as well as the UV detector output signals were processed on-line using Class-VP-chromatography software.

RESULTS AND DISCUSSION

The oxidation of mannan with sodium periodate leads to the cleavage of the mannosyl ring and to the formation of dialdehyde groups. According to the amount of the sodium periodate used, oxidized mannans (M1 to M5) with various aldehyde group content and molecular weights (Table I) were prepared. As evident, the aldehyde content in the oxidized mannan samples increased proportionally up to the molar ratio $(NaIO_4)/(\text{mannan}) = 50$. It should be noted, however, that the solubility of samples M4 and M5 were different from those of M1, M2 and M3. The extent of cleavage of the sample mannosyl rings is seen in the fourth column of Table I. The K_{RM}/K_M values determined by applying the phenol-sulfuric method correspond to the content of the uncleaved mannosyl rings in the macromolecule of the individual mannan samples.

The oxidized mannan samples (M1, M2 and M3) were then conjugated with BSA by reductive amination. Figure 1 illustrates the changes of the elution profiles of the conjugates prepared by using the mannan sample M2 (weight ratio M2:BSA = 4:1). As compared to the unmodified albumin that eluted at 23.3 min (Figure 1), the SEC elution profiles of the

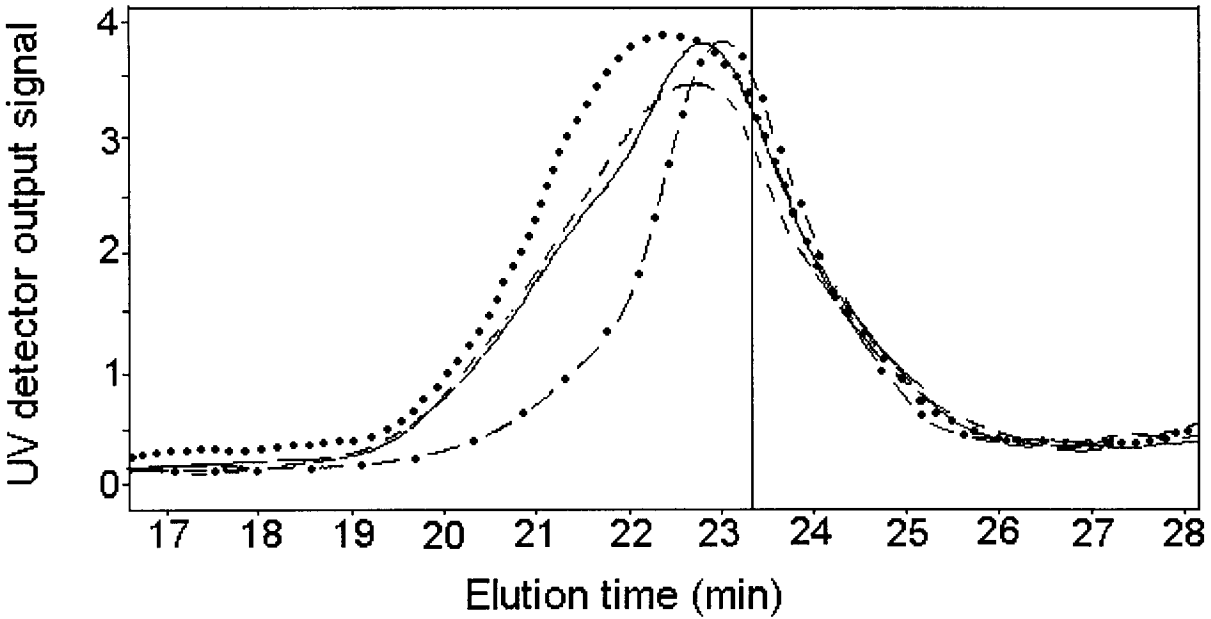

FIGURE 1 Elution profiles of conjugates CM2a prepared by reacting the mannan sample M2 with BSA at a weight ratio of 4:1 for up to 24 h. SEC conditions: columns: HEMA-BIO 100 + HEMA-BIO 1000; sample volume injected, 20μL; eluent: 0.02 M phosphate buffer pH 7.2; flow rate = $0.5\,cm^3 \cdot min^{-1}$. The vertical line indicates the peak position of unmodified BSA. The SEC tracing of the conjugates prepared correspond to the reaction times: 1 h (– • –); 7 h (—);16 h (– – –); 24 h (• • •).

conjugates clearly exhibited changes in elution times of the peaks as well as shapes. These changes, namely the earlier elution of the sample, suggest an increase of the (apparent) molecular weight of the conjugates. To demonstrate this observation more clearly, it can be pointed out that during the conjugation reaction (lasting in total 24 h) with albumin ($M_w = 67\,\text{kDa}$), the mannan sample M2 molecular weight ($M_{app} = 46\,\text{kDa}$) increased to the final value of $M_{app} = 256\,\text{kDa}$ (CM2a; table II). Figure 2 documents the time course of the conjugation reaction, namely the elution volume of samples of oxidized mannan M1 (panel A), M2 (panel B) and M3 (panel C) and BSA with various weight ratio mannan:BSA (4:1, 1:1, 2:3 and 2:5). Figure 2 shows that the size of the

TABLE II Mannan-BSA conjugates obtained at various weight ratio mannan: BSA after 24 h reaction.

Conjugate[a]	Weight ratio (mannan/ BSA)	M_{app}[b] of conjugates (kDa)	Saccharides in conjugate (wt%)	Proteins[c] in conjugate (wt%)	Proteins[d] in conjugate (wt%)
CM1a	4:1	237	65	35	33
CM1b	1:1	144	34	66	63
CM1c	2:3	124	41	59	59
CM1d	2:5	112	23	77	74
CM2a	4:1	256	58	42	43
CM2b	1:1	244	32	68	62
CM2c	2:3	134	28	72	67
CM2d	2:5	125	25	75	75
CM3a	4:1	188	47	53	50
CM3b	1:1	156	29	71	66
CM3c	2:3	148	27	73	70
CM3d	2:5	126	19	81	75

[a] Codes 1–3 indicate the type of mannan, codes a–b indicate the weight ratio mannan:BSA.

[b] M_{app} were determined by SEC using two columns: HEMA-BIO 100 followed HEMA-BIO 1000. The system was calibrated with a calibration set of proteins (Boehringer Mannheim GmbH, Germany). The injection volume of the solutions of protein calibrants as well as of prepared conjugates was $0.02\,\text{cm}^3$. 0.02 M phosphate buffer pH 7.2 was used as the mobile phase at a flow rate $0.5\,\text{cm}^3 \cdot \text{min}^{-1}$.

[c] Determined by Lowry method[26].

[d] Calculated from the nitrogen content determined from the conjugate elemental analysis.

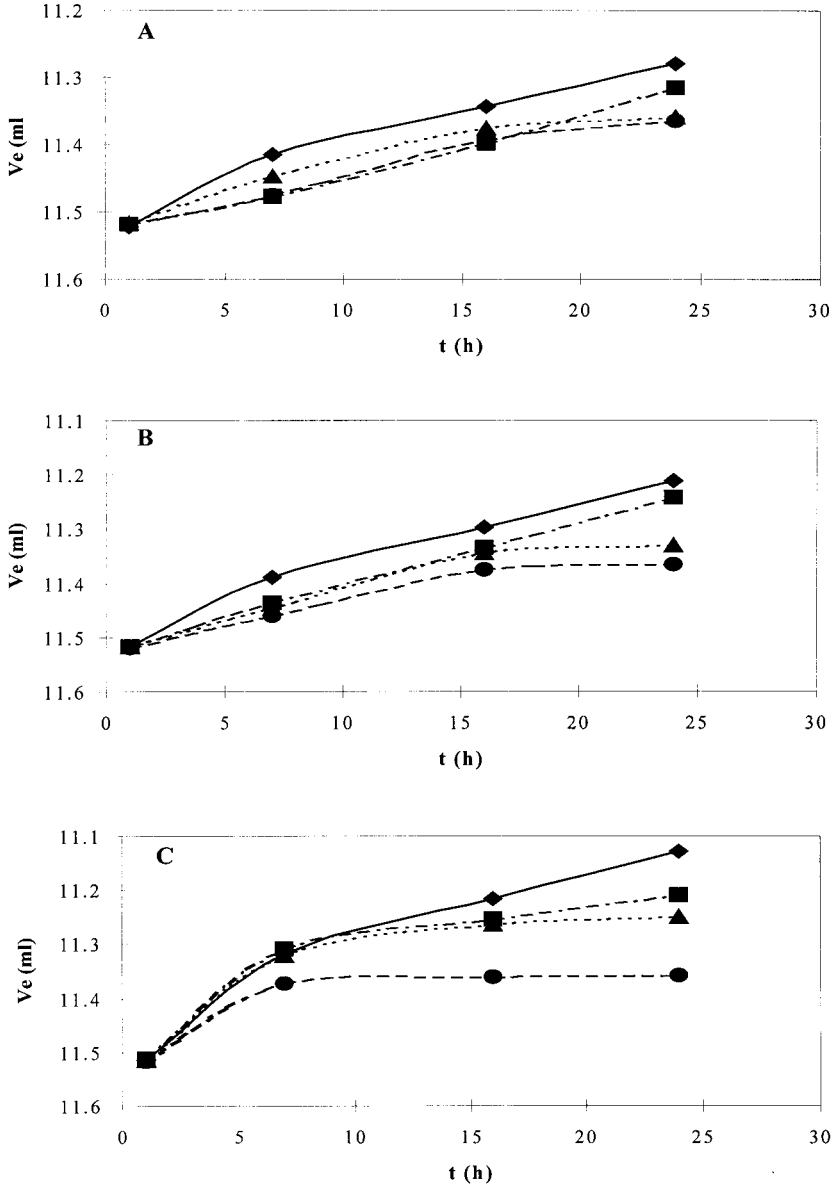

FIGURE 2 Time courses of conjugation of oxidized mannans with BSA at various weight ratios mannan:BSA. Oxidized mannans: M1 (panel A), M2 (panel B), M3 (panel C); weight ratios mannan:BSA 4:1 (—); 1:1 (– · –); 2:3 (· · · · ·); 2:5 (- - - -).

conjugates increases proportionally, depending on the aldehyde content of the mannan and on the weight ratio mannan:BSA used.

The results presented in Table II show the content of the proteins and saccharides (both determined spectrophotometrically) for CM1, CM2 and CM3 conjugates and their M_{app}. It can be seen that M_{app} of the conjugates are within the range from 112 to 256 kDa depending on the weight ratio mannan:BSA and on the type of oxidized mannan used. The saccharides content in the glycoconjugates ranged from 19 to 65%. The values of the protein content in conjugates were determined by nitrogen by elemental analysis (the calculated protein contents are in the last column in Table II). Because a small deviation from values determined by the Lowry method was observed, the spectrophotometric determination of proteins in conjugates can be consider to be sufficient. In Table III are the molar ratios of mannan:BSA in conjugates calculated from the saccharide and protein content (determined in products). A decrease of these molar ratios from CM1 to CM3 conjugates is evident. For example, the molar ratios of conjugates prepared at a weight ratio of 4:1 decreased from 3:1 of the sample CM1a to 1.6:1 of sample CM3a. This can be explained by binding of oxidized mannan, having lower molecular weight and containing more aldehyde groups, to more than one amino group of one macromolecule of BSA. This supposition is supported by the determination of the lower amount of remaining amino

TABLE III The molar ratios mannan:BSA in glycoconjugates.

Glycoconjugate[a]	Weight ratio of conjugation reaction (mannan/BSA)	Molar ratio in conjugate (mannan/BSA)
CM1a	4:1	3:1
CM1b	1:1	1:1
CM1c	2:3	1:1
CM1d	2:5	1:2
CM2a	4:1	2:1
CM2b	1:1	1:1
CM2c	2:3	1:1.3
CM2d	2:5	1:2
CM3a	4:1	1.6:1
CM3b	1:1	1:1
CM3c	2:3	1:1.4
CM3d	2:5	1:1.8

[a] Codes 1–3 indicate type of mannan, codes a–b indicate weight ratio mannan:BSA.

groups in CM3 than in CM1, when both were prepared under the same conditions (results not shown). The dependence of the molar ratio mannan:BSA on the type of mannan used (difference in content of aldehyde groups as well as M_{app}) was observed. Also as expected, the influence of the weight ratios of the initial compounds on the molar ratio in products was observed.

CONCLUSION

This work, devoted to the preparation of neoglycoproteins having an affinity to α-mannosyl-specific lectins, showed that:

Maintaining $\geq 80\%$ of original mannosyl rings and only small changes in the size of the mannan macromolecules in the mannan-BSA conjugates are the main factors responsible for their interactions with the α-mannosyl-specific lectins. Neoglycoproteins of such behavior can be prepared from mildly oxidized mannans (optimally oxidized at the weight ratio of $NaIO_4$/mannan within the range of 10–50).

The conjugation reaction lasting for 24 h along with the weight ratio of the initial mannan/BSA = 1:1 are optimal conditions to obtain the products containing about 30% (w/w) of mannan in the neoglycoproteins synthesized whose M_{app} values are within the range of 144 to 244 kDa.

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