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Preliminary communication

Preparation of a subcellular conjugate with the lipopolysaccharide from *Vibrio cholerae* 01 using β-D-glucan as matrix

Eva Machová [a,*], Slavomír Bystrický [a], Anna Gáliková [b], Grigorij Kogan [a]

[a] *Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 842 38 Bratislava, Slovakia*
[b] *Faculty of Pharmacy, Comenius University, Odbojárov 10, 832 32 Bratislava, Slovakia*

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Abstract

A conjugate consisting of detoxified lipopolysaccharide of *Vibrio cholerae*, a carrier polysaccharide matrix and an immunogenic protein has been synthesised and the reaction conditions have been optimised for obtaining a high degree of conjugation. The obtained construct showed reactivity with the antibodies against *V. cholerae* and can serve as a prospective candidate for preparation of subcellular anti-cholera vaccine.

Keywords: cholera; lipopolysaccharide; β-D-glucan; conjugation; vaccine candidate

1. Introduction

Cholera is an infectious disease that can kill humans within a matter of hours. It has emerged frequently in pandemic waves and is easily transmitted through contaminated water and food. It is a manifestation of a highly efficient and specific toxin delivery mechanism. Prevention of cholera requires good sanitation and application of an effective vaccine. Although infection with *Vibrio cholerae* provides lifelong immunity, attenuated whole-cell vaccines have provided partial, short-lived protection [1]. Parenterally administered cellular vaccines (inactivated *V. cholerae* 01) can cause side reactions, and provide limited protective immunity in adults with a duration of 6 months and confer even lesser efficacy in children [2]. The protein-conjugates of detoxified lipopolysaccharide (LPS) from *V. cholerae* serotype Inaba elicited vibriocidal antibodies. However, the level of LPS antibodies was not as high as that induced with whole cell vaccine [3]. Low immunogenicity of these conjugates could be due to a low content of antigen in the preparations. Two surface bacterial polysaccharides are potential components for construction of subcellular vaccines: capsular polysaccharides and LPS. Recently, *V. cholerae* 0139 capsular polysaccharide with recombinant diphtheria toxin were used for preparation of subcellular vaccine [4].

Immunogenicity of polysaccharide antigen in a conjugate is related to its size. It has been postulated that repetitiveness of the polysaccharide epitope in conjugate allows for the cross-linking of immunoglobulin receptors on the surface of B-cell [5]. Higher number of receptor molecules cross-linked on B-cell surface renders a stronger signal for activation and proliferation of B-cells. We expect that an analogous effect could be achieved by means of the construct loaded with high density with short antigenic structures (LPS of *V. cholerae* 01 contains <15 monosaccharide units). Herein we suggest synthesis of a highly immunogenic preparation via a construction of a sandwich type of conjugate: antigen is initially attached in high density to the polysaccharide matrix (yeast cell-wall $(1\rightarrow3)$-β-D-glucan, followed by the conjugation of such a matrix–antigen complex to the protein carrier, human serum albumin (HSA). As a suitable matrix for the sandwich

Abbreviations: LPS, lipopolysaccharide; HSA, human serum albumin; ADH, adipic acid dihydrazide; TNBS, 2,4,6-trinitrobenzenesulfonic acid; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; CM, carboxymethyl; M_w, weight average molecular weight; HPLC, high performance liquid chromatography; SEC, size exclusion chromatography; MES, 2-(*N*-morfolino)-ethanesulfonic acid; IgG, immunoglobulin G; m.u., monomer unit; KDO, 3-deoxy-D-manno-2-octulosonic acid.

* Correspondence and reprints
E-mail address: chememch@savba.sk (E. Machová).

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type of conjugate we have chosen carboxymethylated glucan, a soluble derivative of yeast (1→3)-β-D-glucan, that has been shown to exhibit dose-dependent radioprotective [6] and immunostimulatory [7] effects. Carboxymethylated glucan enhanced haematopoietic recovery in sublethally γ-irradiated mice [8] exerted protective antibacterial effect in experimental animals [9], and revealed mitogenic and comitogenic activity on rat thymocytes [10]. Although lacking antigenic activity, native β-glucans from yeasts are reported to exert a wide range of immunomodulatory effects involving humoral, cell-mediated and non-specific immunity as well as antitumour activity [11,12]. However, a mechanism of their function in host immune system still remains unclear [13].

The aim of the present work was to prepare a subcellular conjugate of the sandwich type using purified and detoxified surface LPS from *V. cholerae* 01 bound to yeast immunomodulatory β-glucan matrix and protein carrier, HSA. At the same time, the conjugate was constructed using an immuno-inert polysaccharide matrix—amylose, that has been taken as a reference to evaluate an immunomodulatory effect of the yeast β-glucan.

2. Chemistry

Carboxymethylation of β-glucan was performed using monochloroacetic acid and sodium hydroxide in isopropyl alcohol. This is a standard procedure for obtaining water-soluble derivatives from insoluble polysaccharides. However, in order to prepare the derivatives with even better solubility, fractions of carboxymethylated polysaccharides with decreased molecular weight have been prepared by ultrasonic treatment of the high molecular weight CM-polysaccharides.

To prepare the derivatives of CM-polysaccharides that would bear appropriate functional groups required for binding LPS and protein in the further steps, adipic acid dihydrazide (ADH) linker was introduced into CM-polysaccharide molecule by the reaction of free hydrazide group of ADH with carboxylic group of the polysaccharide.

In the further step, the remaining free hydrazide groups of the introduced ADH linker was brought in a reaction with *V. cholerae* LPS that was detoxified previously with anhydrous hydrazine that removed O-linked fatty acids from the lipid A moiety.

In this way prepared LPS-ADH-CM-polysaccharide was further conjugated with HSA using amide formation between the free carboxylic groups on the polysaccharide and protein amino-groups.

3. Results and discussion

3.1. Preparation and characterisation of ADH-CM-glucan and ADH-CM-amylose

The ultrasonically depolymerised CM-glucan ($M_w \sim 146\,000$) and CM-amylose ($M_w \sim 126\,000$) were derivatised with the optimum amount of ADH. The optimisation experiments were described in our previous article [14]. The proposed structure of ADH-derivatised CM-glucan includes both free and fixed hydrazide groups (Fig. 1). The content of free hydrazide groups in ADH-CM-glucan ($y = 0.13$ mol ADH/m.u.) is considerably reduced in comparison with their total amount ($x = 0.51$ mol ADH/m.u) determined from elemental analysis (Table 1). Calculation of the amount of ADH involved in cross-linking is described in the previous paper [14]. The bound hydrazide groups are involved in the cross-links formed with carboxyl groups of CM-glucan. A similar situation was observed with ADH-CM-amylose. However, there are still enough free hydrazides in both ADH-derivatives to bind the detoxified LPS from *V. cholerae* 01. We suppose that carboxyl groups of 3-deoxy-D-manno-2-octulosonic acid (KDO) present in LPS form covalent bonds with free hydrazides of ADH-polysaccharides.

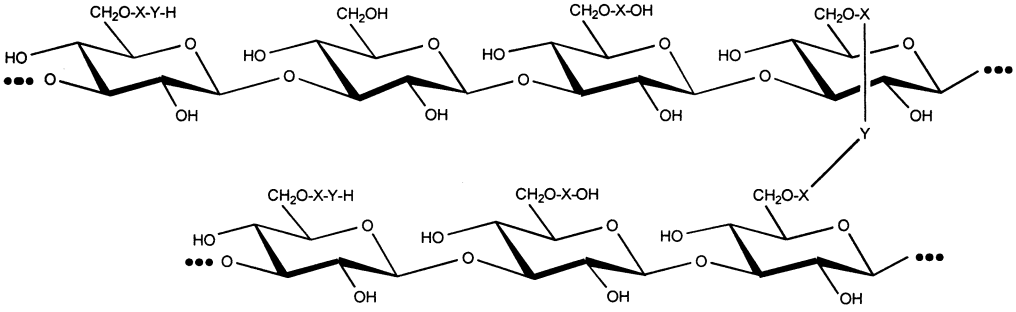

Fig. 1. Proposed structural segment of ADH-CM-glucan. x: –CH_2–CO– (i.e. part of carboxymethyl). y: –NH–NH–CO–$(CH_2)_4$–CO–NH–NH– (i.e. ADH).

Table 1
Characterisation of ADH-CM-glucan, ADH-CM-amylose derivatives and their LPS derivatives

Sample	mol ADH/m.unit [a]	M_w of m.u. [b]	x [c] (ADH_{el}/m.u.)	y [d] (ADH_{TNBS}/m.u.)	$x-y$ (cross/m.u.)
ADH-CM-glucan	7.4	310.9	0.51	0.13	0.38
ADH-CM-amylose	9.81	421.6	0.78	0.23	0.55

[a] Molar ratio in chemical reaction.
[b] Average M_w of repeating monomer unit (m.u.) of ADH-derivatised CM-polysaccharides.
[c] Calculated from elemental analysis.
[d] Free hydrazide groups estimated by TNBS method.

3.2. LPS derivatives of ADH-CM-glucan and ADH-CM-amylose

Derivatisation of ADH-CM-polysaccharides with detoxified LPS of *V. cholerae* 01 was accompanied by formation of slightly opalescent matter in reaction mixtures. The opalescence-causing substances (fractions S* in Table 2) representing a large portion of the total obtained derivative, 62% for LPS-glucan derivative and 40.2% for LPS-amylose derivative, were removed from the soluble LPS conjugates by centrifugation. Upon freeze-drying, samples S* were completely water-insoluble. SEC on Sepharose CL 6B column was used for elimination of non-reacted LPS from reaction mixtures. Three fractions (A–C) were collected, desalted and freeze-dried (Fig. 2).

While fraction C was not of saccharide origin, the amounts of free hydrazide groups in fractions A, B of LPS-ADH-CM-glucan or LPS-ADH-CM-amylose derivatives were markedly lower in comparison with free hydrazide content in initial ADH-CM-polysaccharides. In fraction A, a considerable decrease of free hydrazides was observed: from 36.0 μg ADH/1 mg LPS-ADH-CM-glucan (corresponding to molar ratio 0.13, Table 1) to 0.85 μg ADH/1 mg LPS-ADH-CM-glucan (Table 2). For amylose derivatives, the observed decrease was of a similar magnitude. We assume that these hydrazide groups were used for binding of ADH-CM-glucan, or ADH-CM-amylose with LPS through its free KDO carboxyl groups. Taking into consideration the significant decrease of free hydrazide content in both LPS derivatives, we suppose that at least one half of them got involved in binding with LPS. M_w of LPS determined by HPLC was 7330 Da. Since the molecule of LPS contains several free carboxyl groups, it can bind a number of free hydrazides present on ADH-CM-polysaccharides. Fraction A of LPS-ADH-CM-glucan with $M_w \sim 132$ kDa and the corresponding fraction of LPS-ADH-CM-amylose with $M_w \sim 151$ kDa are well soluble in water and contain sufficient amounts of hydrazide groups for preparation of conjugates with HSA. HPLC profiles of both LPS derivatives are presented in Fig. 3.

3.3. Characterisation of subcellular conjugate HSA-LPS-ADH-CM-glucan and amylose

Fractions A of the LPS derivatives of both polysaccharides were used in the conjugation with HSA. The absolute yields of conjugates were 7.3 mg (glucan matrix) and 8.6 mg (amylose matrix). The protein content was 0.067 mg HSA/mg conjugate for glucan derivative and 0.047 mg HSA/mg conjugate for amylose derivative.

3.4. Immunodiffusion

Immunodiffusion of the prepared LPS conjugates with glucan and amylose matrices, as well as of their HSA-derivatives, with the IgG antibody against *V. cholerae* 01 (type Ogawa) was accomplished according to the Ouchterlony's method in 1% agar (Fig. 4). The precipitation zones of LPS conjugates are clearly visible: LPS-ADH-CM-glucan (sample **1**, Fig. 4), LPS-

Table 2
Characterisation of the fractions of LPS-ADH-CM-derivatives obtained by SEC as well as of HSA-conjugates of glucan and amylose

Sample	Free hydrazide groups (μg/mg derivative)	Yield (mg)
LPS-ADH-CM-glucan fractions		
A	0.85	5.6
B	0.74	4.6
C	n.d.	2.1
Insoluble S*	n.d.	19.3
LPS-ADH-CM-amylose fractions		
A	1.02	7.0
B	0.95	11.3
C	n.d.	3.1
Insoluble S*	n.d.	14.4
	Protein (mg HSA/mg conjugate)	
HSA-conjugate of glucan	0.067	7.3
HSA-conjugate of amylose	0.047	8.6

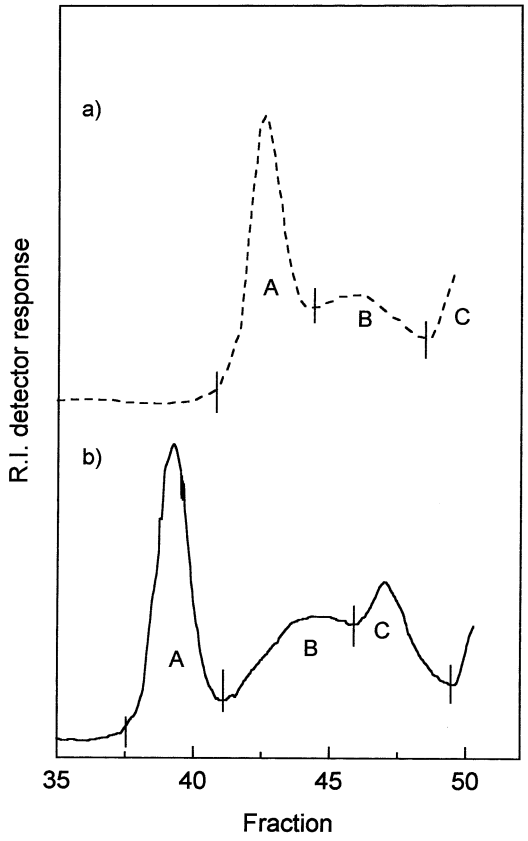

Fig. 2. SEC of the derivatives LPS-ADH-CM-amylose (a) and LPS-ADH-CM-glucan (b) on Sepharose CL 6B column. A–C: collected fractions.

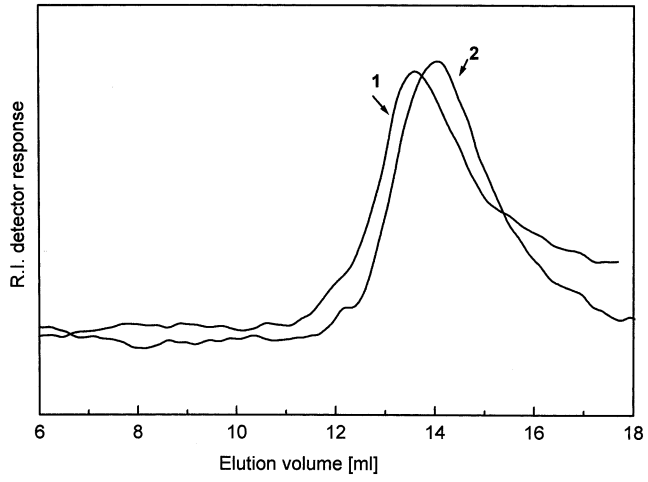

Fig. 3. HPLC profiles of fraction A of LPS-ADH-CM-amylose (1) and LPS-ADH-CM-glucan (2).

ADH-CM-amylose (sample **2**, Fig. 4). On the contrary, extremely large molecules of their HSA-derivatives (samples **3** and **4**, Fig. 4) did not diffuse in 1% agar; they remained at the starting position (as has been proven with protein assay with Coomassie Brilliant Blue G 250).

4. Conclusions

The prepared LPS derivatives in which yeast (1→3)-β-D-glucan or amylose serve as matrices for binding of detoxified LPS from *V. cholerae* 01 formed precipitation zones in agar with IgG antibody against *V. cholerae* 01. It could imply that polysaccharide polymeric chain is a suitable matrix for binding of low-molecular LPS in the quantity sufficient for making it bind and precipitate the antibody. In addition, in the case of using β-glucan derivative as a carrier matrix, we make use of the documented immunostimulatory effect of yeast β-glucan in an organism that can result in enhanced antibody production and stimulation of other components of cellular and humoral immunity. The binding of HSA would probably further boost the immunogenicity of conjugate. However, in our experiments the HSA-conjugates did not diffuse into agar because of their sizes and therefore we were unable to demonstrate their immunoprecipitation with the antibody.

Generally, because of the noxious properties endowed to LPS by its lipid A part, LPS is not considered for utilisation as a vaccine component. Moreover, LPS alone predominantly elicits an IgM antibody response with no memory function. We suggest that detoxifying LPS by removing fatty acids from its lipid A part, and subsequently linking it to the polysaccharide (preferably (1→3)-β-D-glucan matrix) that would multiply the number of short LPS chains in a subcellular vaccine unit, will create an effective vaccine candidate for immunisation against *V. cholerae*. Covalent conjugation of the LPS-polysaccharide construct with a

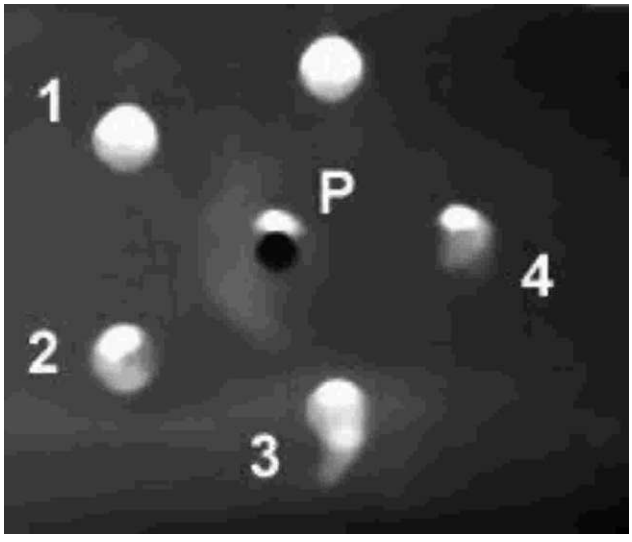

Fig. 4. Immunodiffusion of LPS derivatives of glucan and amylose and their HSA-conjugates contrary to antibody against *V. cholerae* o1 (IgG, type Ogawa). P, antibody IgG, typ Ogawa; 1, LPS-glucan; 2, LPS-amylose; 3, conjugate with glucan; 4, conjugate with amylose.

suitable protein (peptide) would convert it to a T-cell dependent immunogen and presence of a β-glucan immunostimulant as a vaccine component would additionally increase its immunogenicity and cause other beneficial responses in host. Binding O-antigenic polysaccharide (a part of LPS) to an immunogenic carrier protein has been proven to be promising for conferring immunity to Shigella [15] and several other conjugates using detoxified LPS of common Gram-negative infection agents were prepared with tetanus-toxoid as a carrier protein [16–19]. However our proposed method of conjugating whole detoxified LPS to an immunostimulant polysaccharide matrix and a suitable protein represents a new approach that may lead to the formulation of a potentially more efficient vaccine against Gram-negative bacterial pathogens.

In future work we plan to perform immunisation of experimental animals with the synthesised HSA-LPS-polysaccharide conjugates and to compare immunogenicity of the constructs containing immunostimulator β-glucan and a neutral polysaccharide matrix–amylose.

5. Experimental protocols

5.1. Chemicals and equipment

ADH, 2,4,6-trinitrobenzensulfonic acid, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, 2-(*N*-morfolino)-ethanesulfonic acid and its sodium salt, and CM-amylose were from Sigma; Agar, Coomassie Brilliant Blue G 250 were from Fluka; Sepharose CL 6B (Pharmacia, Uppsala, Sweden); reagents ($Na_2B_4O_7$, NaH_2PO_4, Na_2HPO_4, NaOH, KOH, Na_2SO_4, H_2SO_4, phenol, acetic acid p.a.) were from Lachema s.p., Brno, Czech Republic; pullulans P-5, P-10, P-50, P-100, P-200, P-400, P-800 (Shodex Standard P-82 set) were from Macherey–Nagel GmbH, Düren, Germany). CM-glucan was prepared by alkaline extraction of the yeast cell walls of *Saccharomyces cerevisiae* and subsequent derivatisation of the isolate using monochloroacetic acid [20].

Dialysis Tubing 44146 Servapor, (SERVA) was used. Ultrafiltration was performed using Centricon–100 (AMICON) equipment. HPLC system included high-pressure pump LCP 4000 (Laboratorní přístroje, Prague, Czech Republic), 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), and refractometric detector RIDK 101 (Laboratorní přístroje, Prague). Ultrasound degradation was carried out using ultrasound generator UZD 300 with horn type probe ($\phi = 1.5$ cm), 20 kHz and 100 W (PERSON-Ultragen, Nitra, Slovakia). Elemental analysis was performed with elemental analyser EA 1108 (Fisons Inst., UK).

5.2. Methods

5.2.1. Preparation of the CM-polysaccharides with lowered molecular weight

5.2.1.1. CM-glucan. The high molecular weight carboxymethylated glucan (CM-glucan) with degree of substitution 0.63 and $M_w \sim 324\,000$ prepared from alkali-insoluble glucan isolated from the cell walls of *S. cerevisiae* according to [20] was treated for 10 min by ultrasonication [21]. After dialysis and gel filtration (Sephacryl S-400), the fraction of CM-glucan with $M_w \sim 146\,000$ was chosen for further work.

5.2.1.2. CM-amylose. The commercial carboxymethylated amylose (CM-amylose) with degree of substitution 1.41 and $M_w \sim 288\,000$ was treated by ultrasonication at the above mentioned conditions and the fraction with $M_w \sim 126\,000$ was collected.

The degree of carboxymethylation of both CM-polysaccharides was determined by potentiometric titration [22].

5.2.2. Determination of carboxyl groups by potentiometric titration

The content of free carboxyl groups in the original CM-polysaccharides and their ADH-derivatised products was determined potentiometrically. The solutions of polysaccharides after passing through cation exchanger Dowex 50W × 2 (H^+-form) were titrated with KOH solution to the point of equivalence using a combined electrode. In the case of original CM-polysaccharides, the degree of carboxymethylation was calculated according to an equation suggested by Rinaudo and Hudry-Clergeon [22].

5.2.3. High-performance liquid chromatography (HPLC)

Size-exclusion HPLC experiments were performed at ambient temperature with the system described above. The mobile phase used was 0.1 M aqueous $NaNO_3$, and the flow rate was 0.4 mL min^{-1}. A set of pullulans (P-5 to P-800) was used for the calibration of the HPLC system.

5.2.4. Preparation of the ADH-derivatised polysaccharides

5.2.4.1. ADH-CM-glucan. To CM-glucan ($M_w \sim 146\,000$) dissolved in MES buffer (0.05 M, pH 5.1), 6.02 mg of ADH/1 mg CM-glucan was added. After 5 min, 1 mg EDC/1 mg CM-glucan was added and the reaction mixture was stirred for 3 h at pH 5.2–5.5 at room temperature. pH was maintained with 0.1 M MES (pH 3.6). The reaction mixture was exhaustively dialysed in the tubing against distilled water and freeze-dried.

5.2.4.2. ADH-CM-amylose. CM-amylose was derivatised according to the above outlined procedure, with 7.19 mg ADH/1 mg CM-amylose used in the reaction. Obtained products were subjected to the cross-link analyses and HPLC characterisation.

5.3. TNBS analysis

The content of free hydrazide groups in ADH-CM-glucan and ADH-CM-amylose samples was evaluated by the trinitrobenzenesulfonic acid method (with 0.25% TNBS solution) using ADH as a reference [23]. The results are expressed as moles of free hydrazide per repeating unit (m.u.) of CM-polysaccharide (y). The amount of ADH involved in cross-linking ($x-y$) represents the difference of total amount of bound ADH (x) and amount of ADH possessing one free hydrazide group (y) [14]. Combination of at least two analytical methods is required to provide unambiguous data. We have tried a combination of elemental analysis and colorimetric TNBS assay.

5.4. Elemental analysis

The solid samples were analysed for their carbon, hydrogen and nitrogen content using EA 1108 device. The combustion of samples at a very high temperature (1020 °C) and intensive oxygen supply in the instrument ensured the complete gassification without any pyrolytical side products.

5.5. Conjugation of ADH-polysaccharides with detoxified LPS from V. cholerae 01

Detoxified LPS of *V. cholerae* was prepared at the Laboratory of Developmental and Molecular Immunology (NIH, Bethesda, MD, USA) by deacylation of the glycolipid, i.e. cleavage of the ester-linked long chain fatty acids of the LPS with hydrazine. Conjugation reaction: 50 mg of ADH-glucan or ADH-amylose was dissolved in 5 mL 0.05 M MES-buffer, pH 5.3 and 19 mg freeze-dried deacylated LPS was added. The reaction mixture was stirred at room temperature and 58 mg EDC was added slowly. pH was maintained at 5.4–5.5 with 0.1 M MES (pH 3.6). After 2 h the reaction was terminated. The opalescent sediment formed during the conjugation reaction was removed by centrifugation at 2500 rpm. In order to remove non-reacted LPS from the conjugate product, SEC on Sepharose CL 6B column was employed.

5.6. Conjugation of LPS-ADH-polysaccharides with HSA

3.5 mg LPS-ADH-CM-glucan (fraction A), or 3.9 mg LPS-ADH-CM-amylose was dissolved in 0.5 mL 0.05 M MES-buffer, pH 5.5 and EDC was added slowly to 75 mM concentration. Thereafter, 12.0 mg HSA (glucan sample) and 14.0 mg HSA (amylose sample) was added and reaction was carried out at room temperature at pH 4.6–4.7 during 3 h. Non-reacted HSA was separated from reaction mixture by ultrafiltration at 10 000 rpm during 5 min. Supernatant was thrice rinsed with 0.3 mL distilled water and freeze-dried. Protein assay was accomplished according to Lowry et al. [24].

5.7. Immunodiffusion

Immunodiffusion was accomplished according to Ouchterlony's method [25] in 1% agar. The IgG antibody against *V. cholerae* (type Ogawa) was prepared at the Laboratory of Developmental and Molecular Immunology. Precipitation of antigens and antibody was realised during 48 h at 4 °C.

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