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Conjugation of yeast mannans with protein employing cyanopyridinium agent (CDAP) – an effective route of antifungal vaccine preparation

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The possibility of using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) for activation of saccharide hydroxyl groups (instead of hazardous cyanogen bromide) is examined with cell-surface mannans of the yeasts *Candida albicans*, *Candida tropicalis*, *Candida lambica* and galactoglucoxylomannan of *Cryptococcus laurentii*. Direct conjugation with human serum albumin yielded soluble products with increased molecular size in comparison with the original polysaccharides. Immunodiffusion experiments revealed that conjugation did not affect the immunospecificity of the antigen epitope.

Keywords: **mannan conjugate, galactoglucoxylomannan conjugate, CDAP, antifungal vaccine**

Introduction

A key problem associated with preparation of the glycoconjugate vaccines is choosing an appropriate, simple and clinically acceptable method of conjugation chemistry. With the acidic bacterial polysaccharides used for preparation of the synthetic protein-polysaccharide antibacterial vaccines, the chemistry of activation of carboxyl or phosphate groups as the sites of attachment, is well explored [1,2]. Much less is known regarding the neutral polysaccharides where hydroxyl groups serve as the sites of binding and the conjugation usually involves a linker such as adipic acid dihydrazide. Despite its toxicity, cyanogen bromide (CNBr) has been used as the only activation agent [3–8]. Recently, less hazardous and easier to use activator 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) was introduced [9,10] to pneumococcal polysaccharide conjugates preparation. CDAP specific activation method requires milder reaction conditions and as the only prerequisite is the presence of hydroxyl groups, the method could become the most prominent in the chemistry of glycoconjugate preparation.

We examined the use of this promising method for activation and conjugation of a series of neutral polysaccharides, structurally different yeast mannans prepared from pathogenic strains *Candida albicans* and *Candida tropicalis*, and a technologically important strain *Candida lambica*, as well as galactoglucoxylomannan (GalGXM) isolated from a pathogenic yeast *Cryptococcus laurentii*.

Mannans are the major surface antigens that define immunological specificity of yeasts and mediate their interaction with the environment [11]. These carbohydrate antigens of candidacea are built of the α-(1 → 6)-linked backbone with the side chains of different length composed of α-(1 → 2)-linked D-mannose units, some of them capped with terminal α-(1 → 3)- or β-(1 → 2)-D-mannose residues [12]. The length of these side chains, their structure, as well as degree of branching of the backbone are the major factors determining immunological properties and virulence of the *Candida* species [13,14]. One of the antigenic polysaccharides of cryptococci is galactoglucoxylomannan (GalGXM). GalGXM composed of D-galactose (22%), D-glucose (6%), D-xylose (7%), and D-mannose (65%) was isolated from *Cryptococcus laurentii* [15].

We examined application of CDAP activation to direct conjugation of different structurally related mannans and GalGXM with human serum albumin (HSA) without using any linker. Structural and immunological properties of the conjugation products were analyzed.

Materials and methods

Chemicals

Sepharose 6B (Pharmacia, Uppsala, Sweden); set of pullulan standards (Shodex Standard P-82, Macherey-Nagel, GmbH,

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Düren, Germany); human serum albumin (HSA, Fluka); 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP, Sigma); acetonitrile, triethanolamine, β-mercaptoethanol and agar were from Merck; acrylamide (BDH Chemicals Ltd, England); N,N′-methylene bisacrylamide and N,N,N′,N′-tetramethylethylenediamine (TEMED), Protein Test Mixture 4 and sodium dodecylsulphate (SDS) were from Serva; NaCl, $NaNO_3$, $AgNO_3$ were from Lachema (Prague, Czech Republic).

Microorganisms

C. albicans CCY 29-3-32, *C. tropicalis* CCY 29-7-6, *C. lambica* CCY 29-97-1 and *Cr. laurentii* CCY 17-3-5 strains were from the Culture Collection of Yeasts (CCY), Institute of Chemistry, Slovak Academy of Sciences. Yeast cells were grown on a semi-synthetic liquid medium containing 2% D-glucose for 4 days at 28°C [16].

Isolation of polysaccharides from yeasts

Cellular mannans were isolated and purified from the yeast biomass (*Candida* strains) using precipitation with Fehling's reagent according to the procedure described previously [17]. Galactoglucoxylomannan from *Cr. laurentii* was isolated by differential precipitation with cetyltrimethylammonium bromide followed by Fehling solution precipitation to remove mannoproteins [15].

Conjugation of polysaccharides with human serum albumin

The same basic protocol [9,10] was utilized with all *Candida* mannans as well as with cryptococcal GalGXM. Briefly, solution of 20 mg CDAP in 0.2 ml acetonitrile was added slowly to a solution of 30 mg polysaccharide in 3 ml 0.15 M NaCl. After 30 s stirring, 0.3 ml of 0.3 M aqueous solution of TEA was added to raise pH ($pH_{max} = 9.5$). The pH was adjusted to 8 by 1 M sodium acetate buffer, and 15 mg of HSA in 0.5 ml of 0.15 M NaCl was added. The reaction was carried out overnight at 4°C and quenched with 1 ml 1 M ethanolamine. Reaction mixture was dialyzed against distilled water and centrifuged 10 min at 14 000 rpm. Significant sediment of insoluble part (pellet) was observed only with GalGXM. This sediment was removed. Soluble products of conjugation reaction were analyzed and isolated by size-exclusion chromatography. The yield of conjugation varied from 20 to 60%.

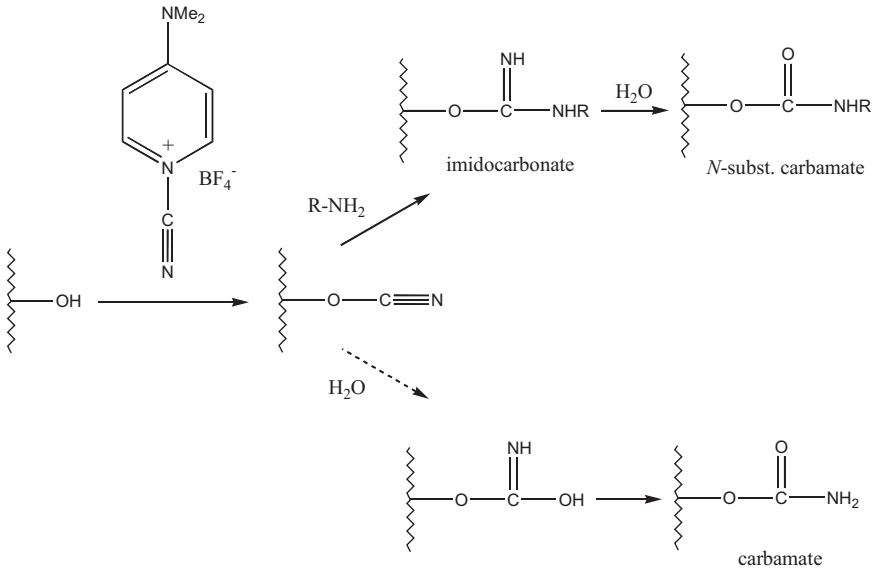
Analyses

The amount of polysaccharides and protein in the conjugation products was measured by colorimetric methods. Saccharides were determined by phenol-sulfuric acid method with respective mannans or GalGXM as reference compounds [18]. Proteins were determined by Lowry method with HSA as reference compound [19].

Products of conjugation reactions were assessed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (with β-mercaptoethanol). Silver-staining method was used for band visualization [20]. As the markers of molecular size the following proteins (Serva) were used: Phosporylase B (92.5 kDa), bovine serum albumin (67 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa).

Size-exclusion chromatography

Molecular size was examined by gel chromatography as well as using HPLC. At preparative chromatography, the samples were passed through Sepharose 6B column (110 × 1.5 cm, flow rate 0.2 ml/min) in 0.1 M phosphate buffer, pH 7.5. The process was monitored with a differential refractometric detector (RIDK-101, Laboratorní přístroje, Prague, Czech



Scheme 1.

Table 1. Characterization of yeast mannans and mannans-HSA conjugates

Source of mannan: Yeasts, CCY	*Mannan M_w*	*Mannan-HSA conjugate M_w*	*The ratio of saccharide and protein in conjugate*
Candida albicans	1st peak >800 000*	>800 000*	3.12
CCY 29-3-32	2nd peak 67 000	145 000	3.39
Candida tropicalis	57 000	200 000	1.93
CCY 29-7-6			
Candida lambica	32 000	100 000	1.17
CCY 29-97-1			
Cryptococcus laurentii	28 000	80 000	1.14
CCY 17-3-5			

*due to the broad distribution only approximate value is given.

Republic). HPLC system with two in series connected columns (250 × 8 mm) packed with Biospher GM 300 and GM 1000 sorbent [2] was used for double-check of molecular size separation. A set of pullulan standards was used for the calibration of both systems.

Immunochemical properties of the conjugates

The yeast antisera were prepared by intensive immunization of rabbits with thermally killed whole yeast organisms. The content of mannan-specific antibodies in the antisera was determined by quantitative precipitation [21]. The isolated mannan-antibody complex was dissolved in alkali (1 N NaOH) and protein (antibodies) was assayed using the Lowry method.

Double immunodiffusion according to Ouchterlony [22] was performed in 1% agarose gel in phosphate-buffered saline.

Results and discussion

Cyanylation with CNBr is widely used as an activation method of hydroxyl groups. However, activation could be much more effective if performed with other cyanylation agents such as N-cyanotriethylammonium salt (CTEA) or cyanopyridinium salt (CDAP). The yields of activation obtained with these compounds are 7–20 times higher, than at a conventional CNBr-activation [23]. CTEA is an unstable non-isolable activator, while CDAP is a crystalline commercially available compound. An advantage of CDAP activation in comparison to a CNBr-mediated one lies in a milder treatment sparing the labile groups at the polysaccharides, as the pH is determined only by nucleophilicity of the attaching nitrogen-containing groups.

Activation of hydroxyl groups with CDAP and subsequent nucleophile attachment proceed according to the simple scheme (Scheme 1). The first step is formation of a reactive cyanyl ester. The ester can be hydrolyzed to an inert carbamate, but in the presence of adequately reactive nucleophile it preferentially forms isourea-type derivatives. In aqueous solution these imidocarbonate arrangements are transformed to highly stable N-substituted carbamate groups.

In order to perform conjugation of the selected polysaccharides directly to lysine amino groups of HSA, mildly alkaline pH conditions were chosen. There was no gelling observed indicating that no over-crosslinking of proteins with polysaccharides has occurred. Formation of a small amount of precipitate was observed with GalGXM only. The resultant composition of the conjugates (polysaccharide/protein ratio) as well as their molecular size are presented in Table 1.

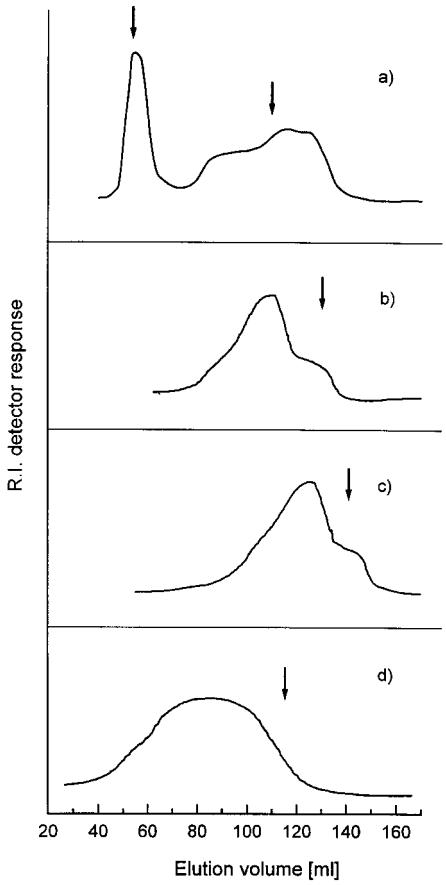


Figure 1. Size-exclusion chromatography on Sepharose 6B of the polysaccharide-HSA conjugates of: *Candida albicans* mannan **(a)**, *Candida lambica* mannan **(b)**, *Cryptococcus laurentii* galactoglucoxylomannan **(c)**, *Candida tropicalis* mannan **(d)**. Arrows denote peak positions corresponding to original polysaccharides.

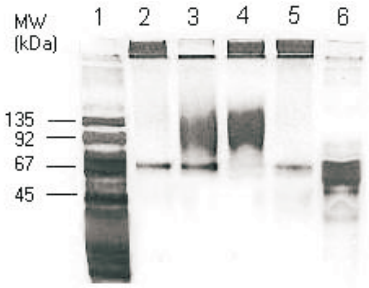


Figure 2. SDS-PAGE analysis of polysaccharide-HSA conjugates and HSA alone. Lanes: **1** – protein standards, **2** – *Candida tropicalis* mannan, **3** – *Candida lambica* mannan, **4** – *Cryptococcus laurentii* galactoglucoxylomannan, **5** – *Candida albicans* mannam, high molecular fraction, and **6** – HSA.

Gel filtration patterns of the conjugate reaction products are presented in Figure 1. The positions of elution maxima of the original mannans and GalGXM are marked with arrows. It can be seen that molecular size of all resulting conjugates is evidently larger than that of the original polysaccharides. The elution volumes of all macromolecules were compared with those of the pullulan standards and relative molecular masses were calculated (Table 1).

Polyacrylamide gel electrophoresis of SDS-denatured conjugation products was used as another analytical method. Silver staining was used for higher sensitivity. The obtained pattern confirmed the presence of chemically conjugated protein-polysaccharide macromolecules (Figure 2). The *C. albicans* mannan-conjugate, high molecular fraction (lane 5) was the one with the biggest mass and did not enter to the gel at all. *C. tropicalis* mannan-conjugate remained at the start position as well (lane 2). On the other hand, *C. lambica* mannan-conjugate (lane 3) entered the gel as well as that of *Cr. laurentii* (lane 4) giving evident smear in the area of molecular masses higher than those of individual HSA and mannans.

Prepared conjugates were tested also by immunochemical method. Immunodiffusion test revealed that conjugates were antigenic, i.e. reacted with homologous antisera except for *Cr. laurentii*. A representative picture (Figure 3) with *C. albicans* antiserum shows precipitation bands formed with mannan as well as with mannan-HSA conjugate. This fact

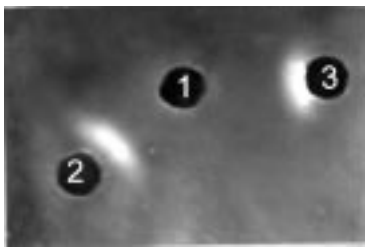


Figure 3. Double immunodiffusion. Wells: **1** – hyperimmune *Candida albicans* antiserum (70 μg antibodies), **2** – *Candida albicans* mannan (40 μg), **3** – *Candida albicans* mannan-HSA conjugate, high molecular fraction (30 μg saccharide).

indicates that conjugation did not alter the polysaccharide epitope. A detailed study of the immunological properties of the prepared conjugates is planned in the future.

The presented preparation of glycoconjugates using CDAP activation of saccharide hydroxyl groups demonstrates the simplicity and efficiency of the method. The synthesized prototype mannans-conjugates outline a new possible way of vaccine preparation against fungal infections that have recently experienced an enhanced dissemination.

Acknowledgements

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