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Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 521–525
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

Evaluation of different mannan polysaccharide usage in enzyme-linked immunosorbent assay for specific antibodies determination

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Received 8 March 2007; received in revised form 4 May 2007; accepted 7 June 2007
Available online 13 June 2007

Abstract

Polysaccharides as antigens impose the problem of the proper ELISA assay. The indirect coating using biotin-avidin or lectins are therefore used. We tried to clarify the efficiency of different approaches to this problem. Our experiments clearly demonstrate that direct coating with mannan polysaccharide on high binding ELISA plates is superior to any other combination using any intermediate protein. On the other hand, the direct coating of the normal ELISA plate with mannan was of significantly lower ($P = 0.008$) efficiency. The use of protein cannot be avoided in normal microplates. All previous statements are supported by experimental data in the paper.
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Keywords: ELISA; Polysaccharide; Immobilization; Coating technique; *Candida*

1. Introduction

Recognition of polysaccharide (PS) structures plays an important role in biological systems. Mannan polysaccharide forms outermost layer of fungi. This location predetermines specific antibody response of host immune system when exposed to yeast and yeast-like microorganisms. There is evidence that antibodies elicited by immunization with yeast mannan structures protect experimental animals against candidiasis [1–3].

In the past years the role of disease-specific antibodies directed against yeast mannan or its oligomannose sequences was extensively studied in the pathogenesis and *in vitro* diagnostics of inflammatory bowel diseases including Crohn's disease and ulcerative colitis [4–6]. To continue research on diagnostics of *Candida* infections it is important to have simple and reliable enzyme-linked immunosorbent assay (ELISA) for quantitative evaluation of anti-mannan antibodies.

Mannan, as antigen, is considered not to be suitable for direct coating of hydrophobic microtitration ELISA plates. Different authors use different approaches to this problem. The problem diminished with rough PS isolates containing certain percentage of protein in their complex structure [3].

Here we present our attempt to summarize the experience with purified and structurally defined *Candida albicans* mannan serotype A [7,8]. *C. albicans* mannan consists of α-1,6-linked mannopyranose backbone and many branches composed of α-1,2; α-1,3 and/or β-1,2-linked mannopyranose units that are connected to the backbone. This mannan was used as a model of neutral polysaccharide antigen in antibody assays using three different indirect ELISA methods.

2. Experimental

2.1. Materials and reagents

PBS (phosphate buffered saline, pH 7.2); Tween 20 (Sigma); carbonate–bicarbonate coating buffer (pH 9.6); Avidin, Biotin (Sigma); Concanavalin A (Con A Type IV, Sigma); Protein A (Alkaline Phosphatase Conjugate, Sigma); Goat Anti-Mouse IgG (γ) antibodies (KPL); BluePhos, Milk Diluent Block (KPL); Rabbit serum normal control (SEVAPHARMA a.s.); Rabbit Reference Serum (6.3 mg/ml IgG), Mouse Reference Serum (9.5 mg/ml IgG) (BETHYL LABORATORIES, INC); disodium ethylenediaminetetraacetate dihydrate (MERCK Titriplex®); Immulon 4HBX microplate (representative high-binding microtiter plate, Dynex) and Microtiter microplate (representative normal-binding microtiter plate, Microtiter®).

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doi:10.1016/j.jpba.2007.06.007

2.2. Isolation and purification of mannan

Mannoprotein was extracted from the fresh *C. albicans* biomass by autoclaving for 1 h at 120 °C three times with 0.2 M NaCl. The supernatant extracts were combined and mannoprotein was precipitated with ethanol, dissolved in distilled water, dialyzed against distilled water for 24 h. For deproteinization of mannoprotein the freeze dried sample was suspended in 2% KOH and heated for 1 h at 100 °C. Insoluble residue was separated by centrifugation, and mannan was precipitated from supernatant with Fehling's reagent [9]. Sedimented mannan-copper complex was dissolved in 3 M HCl, and added dropwise to methanol–acetic acid. The precipitate was centrifuged, dissolved in distilled water, dialyzed and freeze dried. No protein content was determined when the mannan was analyzed for the carbon, hydrogen, and nitrogen content by elemental analysis using the EA 1108 device (FISONS Instruments, UK). The content of nitrogen in carbohydrate is sufficient evidence for the no occurrence of protein in the sample. Double check by Bradford method confirmed no protein content in the mannan sample.

2.3. Monosaccharide composition

Mannan was hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C. Quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates by gas chromatography on a Hewlett-Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m), the temperature program of 110–125 (2 °C min^{-1})–165 °C (20 °C min^{-1}) and flow rate of hydrogen 20 cm^3 min^{-1}. *C. albicans* mannan contained the D-mannose residues and minor traces of D-glucose residues probably originated from cell wall glucan.

2.4. Mannan biotinylation

Periodate oxidation of *C. albicans* mannan was accomplished according to Ďurana et al. [10]. The content of carbonyl groups was determined by Park–Johnson colorimetric assay [11]. The biotinylation of oxidized mannan was performed with biotin-hydrazide using sodium cyanoborohydride ($NaBH_3CN$) as a reducing agent. Degree of biotinylation (∼4%) was selected as optimal for ELISA procedure, not to alter the antigenic structure and was determined by HABA test [12].

2.5. Immunization

Rabbits (male, 8 weeks old, 2000 g of weight, variety HYLA) were from Research Institute of Animal Production (Nitra, Slovakia). Anti-*C. albicans* whole cell rabbit sera were prepared by seven time repeated intravenous injection of heat killed *C. albicans* cells (10^7 cells per ml) in week's intervals.

BALB/c mice (male, 6–8 weeks old) were from Research Institute of Animal Production (Nitra, Slovakia). Anti-*C. albicans* whole cell mouse sera were prepared by eight time repeated intravenous injection of heat killed *C. albicans* cells (10^6 cells per ml) (two times in a week).

2.6. ELISA

Rabbit anti-*C. albicans* mannan IgG antibodies were detected using heterogeneous indirect enzyme-linked immunosorbent assay (ELISA). Two different kinds of polystyrene-microtiter plates, Immulon 4HBX microplate (representative high-binding microtiter plate, Dynex) and Microtiter microplate (representative normal-binding microtiter plate, Microtiter®), and three different coating techniques (i, ii, iii) were tested as follows:

(i) Wells of microplates were coated with Concanavalin A (1 mg/ml PBS), 50 μl per well and kept overnight at 4 °C. Mannan (100 μg/ml) was dissolved in PBS and 50 μl aliquots was added to the wells and let stay overnight at 4 °C.
(ii) Wells of microplates were coated with avidin (10 μg/ml PBS, 50 μl per well). Then standard amount of biotinylated mannan (100 μg/ml PBS, 50 μl per well) were added. The binding was completed after 20 h at 4 °C.
(iii) Microplates were directly coated with the same volume and amount of mannan as in (i) and (ii) Mannan (100 μg/ml) was dissolved in a carbonate–bicarbonate coating buffer (pH 9.6) and let stay overnight at 4 °C.

Washing procedure was repeated three times next. Non-fat milk (2%, w/v; KPL; 2 h at room temperature) was used as blocking reagent to minimize non-specific binding. Titrations of whole cell anti-sera were performed stepwise with serial dilution starting with 1:100 up to 1:204,800 and the plates were incubated 2 h at room temperature. Plates were washed three times with PBST (PBS (pH 7.2), 0.5% (v/v) Tween 20). Immune complexes were detected by (IgG specific) alkaline phosphatase labelled protein A (dilution 1:3000 (v/v) in PBST) for 2 h at room temperature. Enzyme reaction was done by BluePhos Microwell phosphatase substrate system (KPL). Plates were scanned after 30 min for detection of formazan color production at 630 nm (Microplate reader, Dynex). For IgG quantification appropriate calibration curve based on reference rabbit serum (Rabbit Reference Serum, BETHYL LABORATORIES, INC) in direct ELISA was used. For background levels detection (non-specific binding that is without mannan as antigen) rabbit and mouse whole cell sera (sera dilution 1:100) and reference sera (reference rabbit serum, 63 μg/ml IgG; reference mouse serum, 95 μg/ml IgG) in the same ELISA procedures, with the same substances concentration and the same incubation times, were used. Comparisons of detected specific IgG level of whole cell serum dilution in linear range (dilutions 3200–12,800) obtained by different coating techniques were processed using one-way analysis of variance (ANOVA). Statistical analyses were performed using Origin 7.5 Pro software (OriginLab Corporation, Northampton, MA, USA). Results were expressed as mean ± S.E.M. for each dilution.

3. Results and discussion

ELISA is a major tool for detection of specific antibodies in a wide variety of diseases. Although the method was initially used

for measurement of antibodies to individual protein antigens, a number of assays for different disease agents have been devised using partially purified or even unpurified non-protein antigens. The assay is carried out on a solid-phase medium (a membrane, well, or bead) in which the reactants have been immobilized mostly non-covalently or covalently in special cases [13]. The most common ELISA procedure involves direct adsorption of an antigen to a solid support, such as polystyrene in 96-well plate. Materials used as solid phase can possess both hydrophobic and hydrophilic properties. Proteins and polysaccharides differ significantly in binding ability to polystyrene microtiter plate. Determined levels of anti-PS antibodies correlate with the ability of antigen to bind to the polystyrene support.

The adsorption characteristics of two types of common commercially available microplates with different binding specificity, representative high-binding plate-Immulon 4HBX microplate (Dynex) and representative normal-binding plate-Microtiter microplate (Microtiter®) were compared. The levels of total anti-mannan IgG antibody in the normal rabbit serum and anti-serum obtained after immunization of rabbits with heat-killed whole *C. albicans* cells were compared by ELISA on different microplates using different coating techniques. For coating we used mannan (direct coating), concanavalin A (Con A, lectin from jack bean (*Canavalia ensiformis*) carrying a high binding specificity for the D-mannopyranose units) and avidin providing specific interaction with biotinylated mannan. The amount of coated substances and the amount of used mannan as antigen were optimized (data not shown). We found no differences in detected IgG level at anti-serum dilution 1:100 by using mannan in range from 200 to 25 μg/ml with optimized amount of Avidin and Con A and also by direct mannan coating in this study.

We did not observe any differences in detected anti-mannan specific antibody levels in normal rabbit serum, except for direct mannan coating with the lowest detection and by all anti-serum dilutions steadily antibody level (Fig. 1).

However, the whole cells anti-serum antibody levels was significantly distinct for mannan coating on different microplates (Fig. 2). By using Immulon 4HBX microplate the highest anti-mannan IgG antibodies level was observed by coating directly by mannan. Lower level of antibodies was detected using coating by Con A for mannan binding and the lowest amount of antibodies was detected using coating by avidin for biotinylated mannan binding (Fig. 2). By using Microtiter microplate, the highest level of antibodies was detected with Con A in coating step. It was also higher than by using Immulon 4HBX microplate (Fig. 2). Slightly lower level of anti-mannan antibodies was detected with avidin-biotinylated-mannan coating. It was also higher than on Immulon 4HBX microplate. The lowest amount of antibodies was detected by directly coated mannan as antigen (Fig. 2).

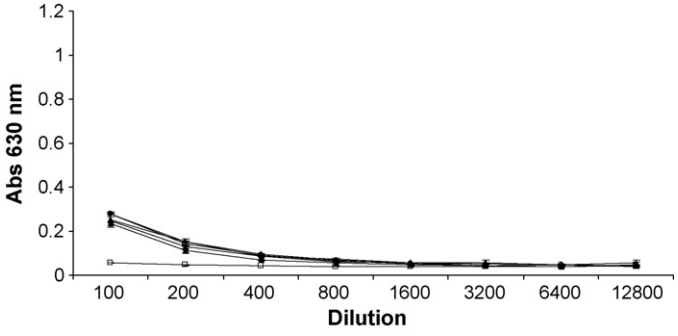

Fig. 1. Serum anti-mannan IgG antibodies in normal rabbit serum at different dilution, plate 4HBX coated with ConA-mannan (●), mannan (■), avidin-biotinylated mannan (▲) and plate Microtiter coated with: ConA-mannan (◯), mannan (□), avidin-biotinylated mannan (△).

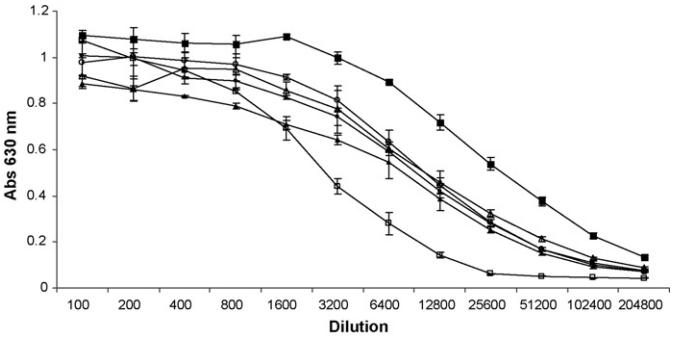

Fig. 2. Serum anti-mannan IgG antibodies after immunization with whole cells of *C. albicans* at different serum dilution, plate 4HBX coated with ConA-mannan (●), mannan (■), avidin-biotinylated mannan (▲) and plate Microtiter coated with: ConA-mannan (◯), mannan (□), avidin-biotinylated mannan (△). Analysis of variance between different coating techniques in linear curve range (dilution 3 200-12 800): mannan coated 4HBX plate (■) vers. mannan coated Microtiter plate (□) $P = 0.008$, mannan coated 4HBX plate (■) vers. avidin-biotinylated mannan coated 4HBX plate (▲) $P = 0.036$, mannan coated 4HBX plate (■) vers. ConA-mannan coated 4HBX plate (●) $P = 0.083$, mannan coated Microtiter plate (□) vers. ConA-mannan coated Microtiter plate (◯) $P = 0.067$, mannan coated Microtiter plate (□) vers. avidin-biotinylated mannan coated Microtiter plate (△) $P = 0.062$.

We observed statistically significant difference in detected IgG level between direct mannan coating on Immulon 4HBX microplate and direct mannan coating on plate Microtiter ($P = 0.008$). Statistically significant difference was also between direct mannan coating and avidin-biotinylated mannan coating on Immulon 4HBX microplate ($P = 0.036$).

The backgrounds were also evaluated by using rabbit and mouse whole cell and reference sera. The highest background was observed by using Con A pre-coating, lower by using avidin pre-coating and the lowest background was observed by mannan-free coating (coating buffer) on both microplates types. The highest background was registered by using mouse whole cell anti-serum, lower by using rabbit whole cell serum and the lowest by using rabbit and mouse reference serum (Fig. 3). Background is known to be higher by high-binding plates [14]. We found that indirect coating (Con A, avidin) even elevate this level.

To compare anti-mannan IgG antibody detection limit of these plates and coating techniques serum dilution of 1:12,800 was selected. Dilution 1:12,800 of whole cell serum is the highest dilution in linear curve range. The absorbance level of mannan specific IgG antibodies in control (normal rabbit serum) was 0.047 ± 0.007 at this serum dilution with all coating techniques. We considered absorbance 0.061 (0.047 + 2S.D.) to be

Table 1
Comparison of different mannan immobilization methods

Plate	Coating technique	C_{IgG}[a] (ng/ml)	Δ[b] (ng/ml)	Ratio[c]
Immulon 4HBX	Con A—Mannan	127.79	114.9	6.90
	Avidin—Biotin. mannan	120.48	107.2	6.33
	Mannan (Direct coating)	187.39	175.0	11.76
Microtiter	Con A—Mannan	132.51	119.8	7.28
	Avidin—Biotin. mannan	134.95	122.4	7.48
	Mannan (Direct coating)	58.11	27.0	2.36

[a] C_{IgG}, detected specific anti-mannan IgG concentration (ng/ml).
[b] Δ, difference between IgG concentration and cut-off value.
[c] Ratio-C_{IgG}/cut-off value.

the cut-off level at dilution 1:12,800, since all of controls had absorbance values of mannan specific antibodies less than this value. We compared the level of IgG antibodies with cut-off value and quantified level of IgG antibodies in whole cell antiserum at the highest dilution in linear range (1:12,800) (Table 1).

Data in Table 1 show evidently, that the highest anti-mannan specific IgG antibodies level was detected by using direct mannan coated Immulon 4HBX microplate (187.39 ng/ml). It was 11.76 times higher than the cut-off value. Comparable levels of mannan specific IgG antibodies were detected using Con A and avidin coating. They were slightly higher in the case of Microtiter plate (Con A 132.51 ng/ml, which is 7.28 times higher than the cut-off value and avidin 134.95 ng/ml, which is 7.48 times higher than the cut-off value) than in the case of Immulon 4HBX plate (Con A 127.79 ng/ml, which is 6.9 times higher than the cut-off value and avidin 120.48 ng/ml, which is 6.33 times higher than the cut-off value). The lowest specific IgG antibodies level (58.11 ng/ml, which is 2.36 times higher than the cut-off value) was detected using directly mannan coated Microtiter plate.

Humoral responses to various antigens are often quantified by some variation of ELISA. When a biomolecule binds to hydrophobic normal/medium plate, there are hydrophobic interactions between the surface of the plate and the biomolecule. A number of manufacturers produce modified materials in order to increase binding capacity. This is typically accomplished by irradiation or by placing polar or charged groups on the polystyrene plates, facilitating hydrophilic interactions. For high binding plates, the binding can be based on both hydrophobic and polar

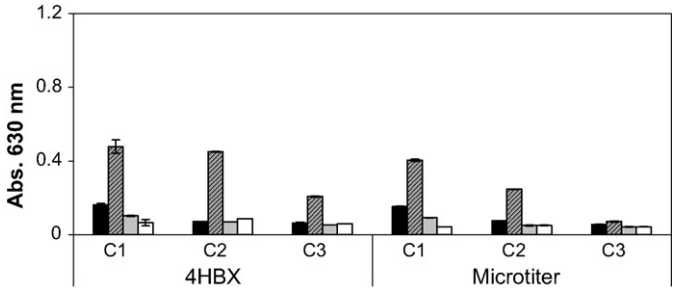

Fig. 3. Background detected on 4HBX and Microtiter plate with rabbit (black bars) and mouse (grey with striped bars) sera after immunization with whole cells of *C. albicans* at dilution 1:100, with reference rabbit serum (63 μg/ml IgG) (grey bars) and mouse reference serum (95 μg/ml IgG) (white bars). C1-coated with ConA, C2-coated with avidin and C3-mannan free.

interactions. Simultaneous interactions are usually needed to stabilize the adsorption when performing the binding. Untreated polystyrene plates (Microtiter plate) have been successfully used in immunoassays. Their binding ability is based primarily on hydrophobic interactions between polystyrene and the molecule to be bound. High binding Immulon 4HBX plates have enhanced binding capacity. Irradiation makes the surface more hydrophilic and both the hydrophilic and the hydrophobic interactions can be realized. It is suitable for positively charged biomolecules of medium size [15].

In the past years the role of yeast mannan and its endogenous receptor mannan-binding lectin (MBL) was extensively studied in the pathogenesis of Crohn's disease (4). More recently the use of disease-specific antibodies directed against oligomannose sequences. Antibodies directed against oligomannose sequences α-1,3 Man (α-1,2 Man α-1,2 Man)n ($n = 1$ or 2) termed anti-*Saccharomyces cerevisiae* antibodies (ASCAs) have begun to be explored for diagnosis of inflammatory bowel diseases (IBD, Crohn's Disease, Ulcerative colitis) (5). ASCAs were found to be present in 50–60% of patients with Crohn's disease, 10–15% of those with ulcerative colitis (6). We found, that high binding microplates (Immulon 4HBX) are able to directly bind polysaccharides very effectively. Immulon 4HBX microplates PS binding ability is even higher, than through the use of binding agent (avidin-biotinylated-mannan and Con A-mannan) on both kinds of microplates. Specific anti-mannan IgG sera antibodies levels by coating mannan directly on a high-binding plate have the lowest probability to be affected by non-specific interaction and have the highest sensitivity, enhanced detection limit and enhanced range of quantitation. Using normal-binding microplate for indirect mannan coating with Con A and coating with avidin-biotinyled mannan is recommended. Our results suggest that the surface of high-binding plate has less hydrophobic character and consequently is able to effectively and directly bind polysaccharide antigens.

Acknowledgements

This work was supported by the Grant Agency of Slovak Academy of Sciences, VEGA No. 2/7029/27 and APVV–0032-06

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