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Note

Affinity chromatography of anti-mannan antibodies on mannan–acrylamide copolymers

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Candida spp. are capable of producing a variety of infections, ranging from superficial skin infections to life-threatening disseminated diseases. The cell wall of *Candida* is composed mainly of the polysaccharides glucan and mannan [1]. Mannan is one of the major antigens and, besides physical protection of the wall, it may serve to hold enzymes to the cell wall and may contribute to cell–cell recognition [2]. The structure of mannan has long been studied by many investigators [2–6]. Immunoaffinity chromatography has been widely employed for purification of proteins [7]. Isolation of specific anti-mannan antibodies is important for (a) investigation of mannan structure, (b) detection of mannan antigens that are sloughed off the cell walls during infection and circulate in blood [8] and (c) elimination of mannan from cytoplasmic

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extracts of *Candida* spp. in order to improve the specificity of serodiagnosis of candidiases. This is important since sera of uninfected persons contain antibodies to cell wall mannan [9] whereas sera of patients with invasive *Candida* infections contain antibodies both to the cell wall (mannan) and cytoplasmic antigens [10].

This paper proposes a new type of mannan-containing immunosorbent and describes the preparation and properties of four types of mannan-based immunosorbent for isolation of mannan-specific antibodies.

EXPERIMENTAL

Preparation of antiserum

Antiserum was prepared by intravenous immunization of rabbits with heat-killed whole *Candida albicans* CCY 29-3-102 (serotype B) cells suspended in saline (5 mg/ml) [11].

Isolation of mannan

Mannan of *C. albicans* CCY 29-3-102 (serotype B) was extracted from cell paste as described previously [5].

Preparation of the immunosorbent

Preparation of allyl-substituted mannan with a higher degree of substitution. Mannan (0.5 g) was suspended in 10 ml of dioxane, and 5 ml of 2.5% (w/v) sodium hydroxide and 0.25 ml of allyl bromide were added. The suspension was mixed at 80°C for 2 h. Methanol (50 ml) was added to the reaction product, and the suspension was filtered. The product was washed, then dried under vacuum. The yield of the allyl-substituted mannan was 40%. The degree of substitution was 0.06, determined using the method of addition of bromine to the double bond of allyl-substituted mannan (with excess of the reagent) and coulometric determination of unchanged bromine.

Preparation of allyl-substituted mannan with a lower degree of substitution. Mannan (0.15 g) was dissolved in 5 ml of boiled distilled water, and the pH was adjusted to 10 at 50°C. Allyl bromide (40 μl) was added to the solution and titrated with 0.01 *M* sodium hydroxide for 60 min using Titrator TTT 60 (Radiometer, Copenhagen, Denmark). The degree of substitution was 0.016 (calculated from the consumption of sodium hydroxide).

Preparation of mannan–acrylamide copolymers. An aqueous solution of acrylamide (30%, w/v) was mixed with methylene bisacrylamide (2%, w/v), and 50 mg of allyl-substituted mannan were dissolved in the mixture. The solution was evacuated, and 20 μl of tetramethylethylenediamine and 80 μl of 10% ammonium persulphate were added. The polymerization was complete in 10 min at 50°C. The polymer was minced in a homogenizer, washed with water,

TABLE I

CHARACTERIZATION OF PREPARED MANNAN-ACRYLAMIDE COPOLYMERS

Allyl-substituted mannan	Degree of allylation	Copolymer	Acrylamide (30%, w/v) (ml)	Methylene bisacrylamide (2%, w/v) (ml)
A	0.060	A_1	2	4
		A_2	1	5
B	0.016	B_1	2	4
		B_2	1	5

ethanol and acetone, and dried. The volumes of the aqueous solutions of acrylamide and methylene bisacrylamide used are listed in Table I.

Affinity chromatography

The mannan–acrylamide copolymers (0.5 g) were suspended in 0.02 *M* phosphate buffer (pH 8.0) and packed into columns. After equilibrating with 20 ml of buffer, the columns were loaded with 200 μl of *C. albicans* antiserum (containing 21 mg of proteins). After incubation for 2 h at room temperature, the columns were washed with 2 ml of 0.02 *M* phosphate buffer (pH 8.0). The effluent was allowed to pass slowly twice through the same column in order for the whole column capacity to be utilized. Afterwards the column was washed with 15 ml of 0.02 *M* phosphate buffer (pH 8.0).

Desorption of the adsorbed antibody was carried out according to Fornstedt [12]. Desorption was started with elution by 0.1 *M* Gly–sodium hydroxide (pH 9.8) (6 ml) and continued with 100% ethylene glycol. The amount of ethylene glycol necessary for desorption of anti-mannan antibodies ranged from 8 to 13 ml. The absorbance of 1-ml fractions was measured at 280 nm. The fractions with anti-mannan activity were combined, dialysed against 0.02 *M* phosphate buffer (pH 8.0) and concentrated using an Amicon ultrafiltrator, Model 52 (membrane PM 10). The concentration of antibodies for indirect enzyme-linked immunosorbent assay (ELISA) was adjusted to 1 mg/ml.

Indirect ELISA technique

Indirect ELISA technique was performed as described in ref. 11. Mannan was adsorbed on the surface of the wells of microtitre plates from the solution with concentration 6.2 μg/ml. Specific anti-mannan antibodies (1 mg/ml) were used at a dilution of 1:100. The swine antiserum specific to rabbit immunoglobulins labelled with peroxidase was used at a dilution of 1:5000 (v/v). The *o*-phenylenediamine was used as substrate for peroxidase, and the absorbance was read at 492 nm with Uniskan II (Flow Labs., Ayrshire, U.K.).

RESULTS AND DISCUSSION

In order to prepare an immunosorbent suitable for isolation of specific anti-mannan antibodies, the polymerization of lower and higher allyl-substituted mannans *C. albicans* serotype B with acrylamide and methylene bisacrylamide was used. Four mannan copolymers with alternating amounts of acrylamide and methylene bisacrylamide and with different degrees of substitution of allyl-substituted mannan *C. albicans* serotype B were prepared (Table I).

With the copolymers used as immunosorbents, unbound proteins were eluted with 15 ml of phosphate buffer (pH 8.0). Specifically bound anti-mannan antibodies were desorbed according to Fornstedt [12]. This method uses the preserving effect of ethylene glycol on proteins [13,14] and its marked desorptive effect when it is added to the high-pH buffer [12]. As can be seen from Table II, the lowest amount of anti-mannan antibodies (2.97 mg/ml) was desorbed from the copolymer B_1. The highest amounts of antibodies (11.12 and 9.99 mg/ml) were eluted by chromatography on copolymers A_2 and B_2, respectively. ELISA of the combined fractions from elution peaks showed that all the serum anti-mannan antibodies were adsorbed on the column only in the case of copolymer A_2 [ELISA value of the first peak (I) was zero]. The elution profile of *C. albicans* antiserum after affinity chromatography on copolymer A_2 is presented in Fig. 1.

Our results showed that the efficiency of adsorption of anti-mannan antibodies depended on the amounts of acrylamide and methylene bisacrylamide in the copolymer. By decreasing the amount of acrylamide and by increasing that of methylene bisacrylamide (a cross-linking agent), the binding capacity of the copolymer was increased (Table II). A higher degree of mannan allylation also stimulated the binding capacity of the copolymer.

The use of affinity chromatography columns in which mannan was used as effector for isolation of anti-mannan antibodies and purification of 1,2-α-mannosidase was reported earlier [15,16]. These adsorbents were prepared by

TABLE II

PROTEIN CONTENT OF THE ELUTION PEAKS OF ANTISERUM *C ALBICANS* ON PREPARED MANNAN-ACRYLAMIDE COPOLYMERS

Copolymer	Peak I		Peak II	
	Amount of protein (mg)	ELISA value	Amount of protein (mg)	ELISA value
A_1	17.09	1.27	3.91	0.69
A_2	9.88	0 00	11.12	1.98
B_1	18.02	1.42	2.97	0.52
B_2	11.01	0.20	9.99	1.57

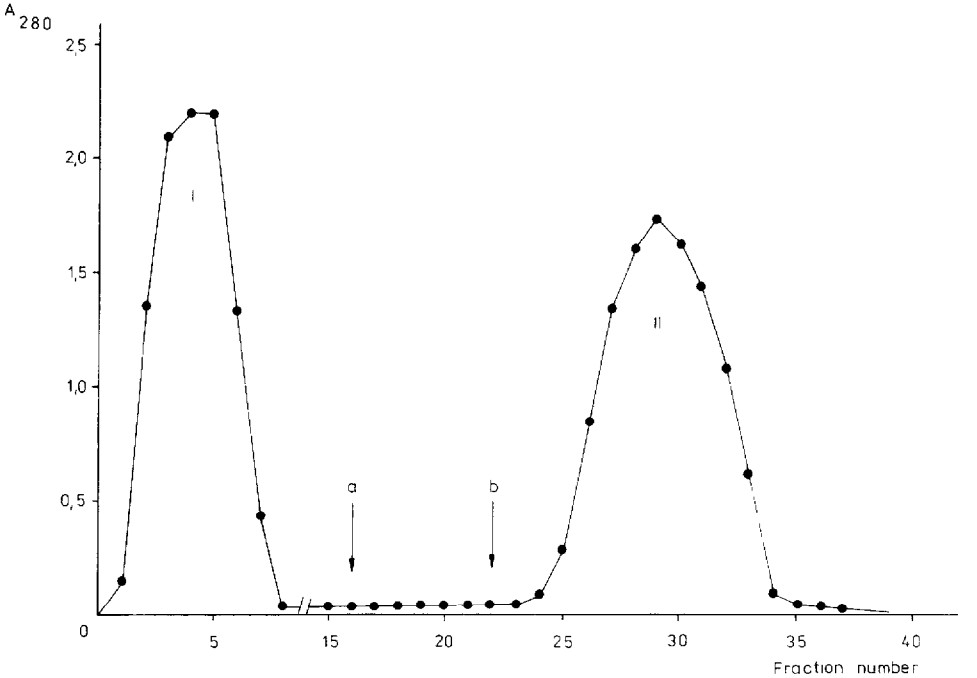

Fig. 1. Elution profile of *C albicans* antiserum after affinity chromatography on prepared mannan–acrylamide copolymer A_2. Affinity chromatography conditions: starting buffer, 0.02 *M* phosphate buffer (pH 8.0); desorption buffers, arrows indicate starting of elution with (a) 0.1 *M* Gly-sodium hydroxide (pH 9.8) and (b) 100% ethylene glycol.

cross-linking with epichlorohydrin. Šandula and Kuniak [15] have described affinity chromatography of yeast antibodies on this cross-linked mannan gel, and the results are comparable with those of affinity chromatography of *C. albicans* antiserum on copolymer A_2. However, the method described here requires a much lower amount of mannan as effector.

The preparation of affinity sorbents for isolation of anti-polysaccharide antibodies presupposes that minimal changes occur in the structure of the reducing ends of the polysaccharide used as a ligand. The proposed procedure for preparing the affinity immunosorbent, by means of allylation of the polysaccharide with allyl bromide and subsequent copolymerization with acrylamide and methylene bisacrylamide, implies only slight modification of the polysaccharide in contrast to the more commonly used immobilization method via the amino-activated adsorbents (e.g. Agarose–adipic acid hydrazide). These require activation of the polysaccharide mostly by periodate oxidation, during which drastic changes or even destruction of the reducing ends or the whole polysaccharide could take place.

The copolymer of mannan with acrylamide and methylene bisacrylamide can be used for isolation of specific anti-mannan antibodies, monoclonal an-

tibodies to mannan from ascitic fluids and for purification of mannolytic enzymes and mannan-specific lectins.

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