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Inhibition of Yeast Growth by Broadly Cross-Reactive Antisera Elicited by Heterologous Mannan-Protein Conjugate

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A new approach to obtain broadly cross-reactive antisera against important yeast pathogens by intensive hyperimmunization with polysaccharide-protein conjugates is described here. Surface mannan of *Candida albicans* and capsular galactoglucoxylomannan of *Cryptococcus laurentii* were isolated and chemically linked to human serum albumin. Antisera elicited by a 7-week vigorous immunization of rabbits with the conjugates showed effective cross-reactive growth inhibition of different representatives of *Candida* spp. as well as *Cryptococcus* spp. IgG antibodies are evidenced as the effective component of the antisera.

Keywords: *Candida*, *Cryptococcus*, mannan conjugate, cross-growth inhibition

The mainstream of vaccine development is focused on glycoconjugates prepared by chemical reaction of saccharide antigens with protein carriers. In the case of *Candida* pathogenic yeasts, mannan represents the main surface antigen [13], and in the case of zoo-pathogenic yeast-like *Cryptococcus*, the main surface antigen is represented by capsular mannan derivatives [1]. So far, a lot of information concerning the different types of yeast glycan conjugates and immune response in experimental animals has already been published [5].

Investigation of the immune protection is one of the key parts of vaccine research and development. Besides highly sophisticated methods focused on the evolution of immune-cell memory, there are challenge experiments using the direct application of an infective microorganism applied to immunized animals. However, nowadays, the last mentioned approach is not easy to implement since it demands special approval from veterinary enforcement authorities.

Our previous papers described the antiserum-mediated inhibition of yeast growth on agar plates. We present here a growth inhibition study with broadly reactive antibodies elicited by the *Candida albicans* mannan-HSA conjugate [3, 10] as well as the *Cryptococcus laurentii* galactoglucoxylomannan-HSA conjugate [4]. Additionally, the efficacy of intensive immunization of rabbits with the conjugates is shown. The aim of this paper was also the characterization of an effective component of antisera causing growth inhibition.

The yeast strain *C. albicans* CCY 29-3-32 (Culture Collection of Yeasts, Institute of Chemistry of Slovak Academy of Sciences, Bratislava, Slovakia) was used for the preparation of mannan using Fehling's reagent [6]. Galactoglucoxylomannan from *Cr. laurentii* was isolated by precipitation with cetyltrimethylammonium bromide [8].

C. albicans mannan-HSA and *Cr. laurentii* galactoglucoxylomannan-HSA conjugates were prepared and characterized as described elsewhere [2], using CDAP (1-cyano-4-dimethylaminopyridinium tetrafluoroborate) as a hydroxyl group activator. Rabbits (male, 8 weeks old) from the Research Institute of Animal Production (Nitra, Slovakia) were injected seven times in 1 week intervals [3].

A dense suspension of yeast cells (*C. albicans* CCY 29-3-32; *C. tropicalis* CCY 29-7-6; *C. parapsilosis* CCY 29-20-1; *C. glabrata* CCY 26-20-1; *Cr. laurentii* CCY 17-3-5; *Cr. neoformans* CCY 17-1-5; and *Cr. albidus* CCY 17-4-6) was poured onto the 1% malt agar. Whatmann No. 1 rings (d = 5 mm) soaked in rabbit antisera after the 7th injection of *C. albicans* mannan-HSA and *Cr. laurentii* galactoglucoxylomannan-HSA conjugates (dilutions of serum with saline were 1:10; 1:1,000; and 1:100,000) were placed onto the surface of the agar. The growth of yeasts was monitored during 3 days at 37°C. The inhibition effect of the rabbit sera was evaluated

as a diameter of clear zones around the soaked rings.

Fractionation of *C. albicans* mannan-HSA serum after the 7th injection was performed by fast protein liquid chromatography (FPLC; Pharmacia, Sweden) on a Superdex 75, 10/300 GL column (GE Healthcare) in 0.05 M phosphate buffer, pH 7.0, 0.15 M NaCl. Fractions were rechromatographed and analyzed by SDS-PAGE under reductive conditions (with β-mercaptoethanol) using Kaleidoscope prestained standards (Bio-Rad). The silver-staining method was used for band visualization.

It is well known that mannans are characteristic cell-surface antigens of *Candida* species. *C. albicans* mannan consists of an α-(1,6)-linked backbone moiety branched with α-(1,2)-, α-(1,3)-, and β-(1,2)-linked mannose residues [13]. The *Cr. laurentii* capsule is composed of several complex polysaccharides. One of them, galactoglucoxylomannan, consists of about 70% of mannose [11]. It is composed of an α-(1,3)-linked mannose backbone moiety branched with short mannosides containing also galactose (20%), glucose (6%), and xylose (6%). Both *C. albicans* mannan and *Cr. laurentii* galactoglucoxylomannan were conjugated to HSA (human serum albumin) and used for growth inhibition assay. Rabbit antiserum obtained after multiple injections of the *C. albicans* mannan-HSA conjugate (dilutions 1:10, 1:1,000, and 1:100,000) was used for growth inhibition assay using various *Candida* spp. and *Cryptococcus* spp. (Fig. 1A). Among all the tested *Candida* spp., *C. glabrata* exhibited the weakest inhibition. This finding evidently corresponds with the structure of *C. glabrata* surface mannan [14]. Unlike mannans from *C. albicans* and *C. tropicalis*, mannan from *C. glabrata* contains shorter side chains. These are mainly α-(1,2)-linked triose side chains and fewer tetraose side chains terminated with β-(1,2)-mannose or α-(1,3)-mannose residues. Moreover, the weaker growth inhibition of *C. parapsilosis* was related to its mannan structure, which is similar to *C. glabrata* [12]. On the other hand, the intensive interaction of *C. tropicalis* can be attributed to the beneficial effect of a higher quantity of longer side chains (up to heptaose) containing one or more β-(1,2)-linked mannose residues on the nonreducing ends, as well as frequent occurrence of internal α-(1,3)-mannosides [9]. In terms of mannan structure, the similarity of antigenic factors of *C. albicans* and *C. tropicalis* is evident. However, the inhibition of *C. albicans* growth by the antiserum was weaker than in the case of *C. tropicalis*, possibly due to better accessibility of mannooligosaccharide side chains.

Furthermore, the rabbit antiserum obtained after multiple injections of *Cr. laurentii* galactoglucoxylomannan-HSA conjugate (dilutions 1:10, 1:1,000, and 1:100,000) was used for growth inhibition assay using the same yeasts as mentioned above (Fig. 1B). As expected, intensive growth inhibition was observed with all *Cryptococcus* spp. tested. It is known that mannan embedded in cryptococcal capsular polysaccharides consists of α-(1,3)-linked mannose residues. Interestingly, *C. albicans* growth was also very effectively inhibited by the *Cryptococcus* antiserum. This finding implies that the dominant epitope of yeasts may contain the internal α-(1,3)-linked mannose residues that frequently occur in the side chains of *C. albicans* mannan. The size of the antigen epitope, according to the classical theory tested on

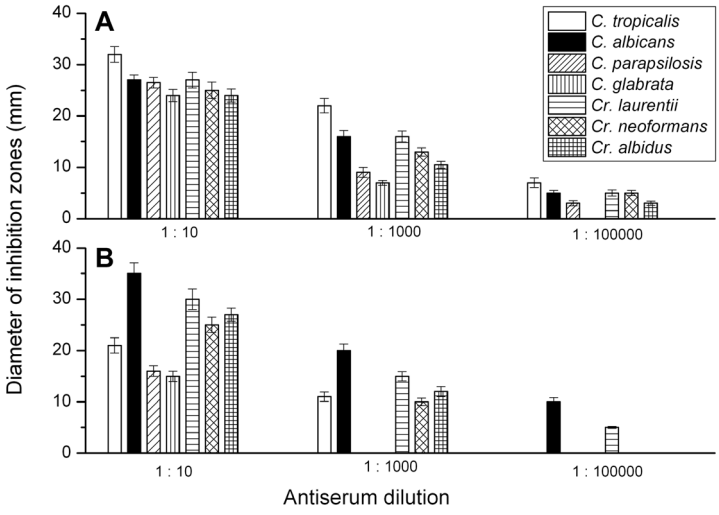

Fig. 1. Growth inhibition of *Candida* and *Cryptococcus* spp. tested on 1% malt agar in the presence of rabbit antiserum elicited after a 7-week immunization with *C. albicans* mannan-HSA conjugate (**A**) and *Cr. laurentii* galactoglucoxylomannan-HSA conjugate (**B**). Equal amounts of rabbit antisera were applied on Whatmann No. 1 rings and the diameter of inhibition zone was measured.

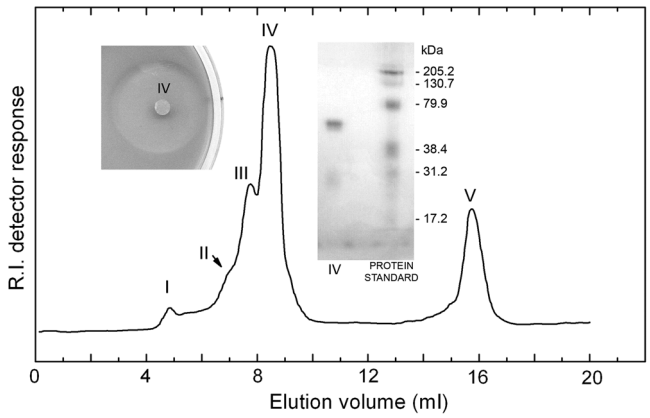

Fig. 2. Size-exclusion chromatographic profile of rabbit antiserum proteins elicited after immunization with *C. albicans* mannan-HSA conjugate; peaks representing individual fractions are labeled consecutively from I to V.

Pictures of the inhibition effect of fraction IV after deactivation of complement on yeast growth (left) and SDS-PAGE analysis of fraction IV visualized by silver staining (right) are inserted.

dextrans, is around six saccharide units [7]. Here, according to the growth inhibition observations, the mannooligosaccharide epitopes can comprise α-(1,2)-, β-(1,2)-, as well as expressive α-(1-3)-linked mannose residues.

The size-exclusion chromatographic profile of rabbit antiserum proteins elicited by *C. albicans* mannan-HSA conjugate comprised five fractions. SDS-PAGE of fraction IV showed two bands at ~25 and ~55 kDa. Apparently, they belong to the heavy and light chains of IgG antibodies, respectively (Fig. 2). The individual fractions purified by double chromatographic separations were used for the determination of inhibition activities on yeast growth. Only one of them, fraction IV, exhibited a pronounced effect. Interestingly, after heating (56°C, 60 min) to deactivate the complement, the inhibition effect of fraction IV still remained (Fig. 2).

From the obtained results above, we can conclude that the heterologous as well as homologous antisera clearly showed similar inhibition zones. There were no significant differences between antisera obtained after intensive immunization with *C. albicans* mannan-HSA or *Cr. laurentii* galactoglucoxylomannan-HSA conjugates. Both antisera elicited by the conjugates contained a portion of antibodies with high affinity to pathogens. These broadly reactive antibodies were present in a titer sufficient to completely inhibit the growth of all tested yeasts and therefore the spreading of the infection. This preparation and the antifungal properties of the hyperimmune antisera may be effectively applied in microbial biotechnology.

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