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Note

Sandwich enzyme-linked immunosorbent assay of D-mannans of *Candida albicans* NIH A-207 and NIH B-792 strains using concanavalin A and polyclonal rabbit anti-*C. albicans* antisera*

Minehiro Tojo[†], Nobuyuki Shibata[†], Takako Osanai[†], Takeshi Mikami[‡], Masuko Suzuki[‡], and Shigeo Suzuki[†,§]

[†]*Second Department of Hygienic Chemistry and* [‡]*Department of Microbiology, Tohoku College of Pharmacy, 4-4-1 Komatsushima, Aobaku, Sendai, Miyagi 981 (Japan)*

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In the preceding study, Tojo *et al.*[1] investigated the relationship of reactivities between the enzyme-linked immunosorbent assay (EIA) of polystyrene-microtiter plates coated with D-mannans prepared from *Candida albicans* NIH A-207 and NIH B-792 strains (abbreviated as A- and B-strains, respectively), and the quantitative precipitin reaction (QPR) of the same D-mannans in glass test-tubes. The results clearly indicated that the reactivities of the D-mannans in EIA on the solid support were quite similar to those of the same antigens on QPR in phosphate-buffered saline solution (PBS), and that EIA is a useful technique for immunochemical assay of yeast D-mannans or the antibodies of various yeasts in microscale. In a study of the adsorbability of the D-mannans of A- and B-strains onto the surface of plastic microtiter plates, Tojo *et al.*[2] also showed that the hydrophobic peptide moiety existing in each D-mannan participated in the adhesion.

As the succeeding study, we attempted to develop a sandwich–EIA method of the D-mannans of *C. albicans* spp. by use of precoated, plastic-microtiter plates with concanavalin A (Con A), a lectin from jack bean (*Canavalia ensiformis*) possessing a high-binding specificity for the D-mannopyranose unit[3], although it has also been shown to react with D-glucopyranose, D-fructofuranose[4], and 2-acetamido-2-deoxy-D-glucopyranose units to a weaker extent. Suzuki *et al.*[5] reported that Con A precipitates several yeast D-mannans having different chemical structures in their branching moieties, including those of A- and B-strains, in exactly the same amounts in terms of precipitated D-mannan per fixed amount of Con A in QPR. Therefore, this lectin seems to be the best

* Dedicated to Professors Toshiaki Osawa and Nathan Sharon.

§ To whom correspondence should be addressed.

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reagent for the quantitative determination of various yeast D-mannans containing different proportions of phosphate and protein.

In another study by Okubo and Suzuki[6] on the fractionation of the neutral component of D-mannan of bakers' yeast (*Saccharomyces cerevisiae*), fractional precipitation with Con A yielded four D-mannan subfractions having different average-lengths of the branches, although these subfractions gave exactly identical precipitin curves in QPR. This observation provided substantiating evidence for the similarity of the reactivity of the lectin with various yeast D-mannans.

On the basis of these observations, we developed a sandwich–EIA procedure for the quantitative determination of the D-mannans of A- and B-strains in phosphate-buffered saline solution by means of Con A and polyclonal antibodies to A- and B-strains as the trapping and the overlaying agents of these D-mannans, respectively. Firstly, investigation of the reactivity of Con A by sandwich–EIA against Fractions A and B and their subfractions (Fractions A-II–A-V, and B-II–B-V) containing different proportions of phosphate and protein by QPR in glass test-tubes showed an identical reactivity regardless of their structural differences (Fig. 1).

In the preceding paper[1], the four D-mannan subfractions had been shown to react with the homologous-polyclonal rabbit antiserum proportionally to their phosphate content both in the QPR and direct EIA test. Therefore, the same D-mannan subfraction series were investigated by sandwich EIA with Con A (see Fig. 2). The two series of

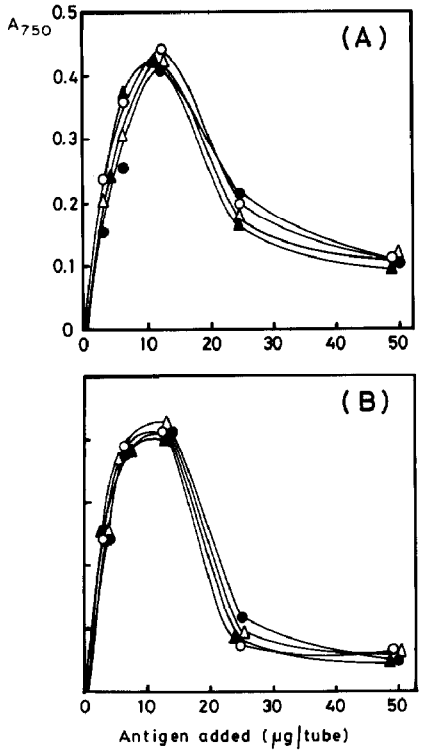

Fig. 1. Quantitative precipitin curves of antigens of Fractions A-II–A-V (A), and B-II–B-V (B) against Con A: Fr. II (●), Fr. III (○), Fr. IV (▲), and Fr. V (△).

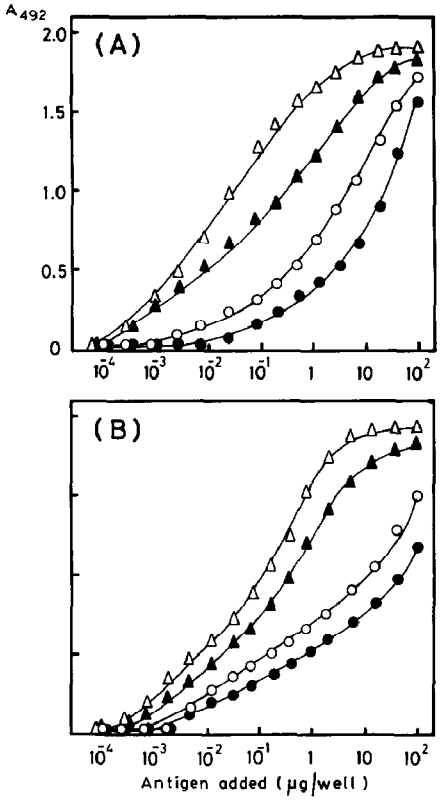

Fig. 2. Sandwich-EIA test with Con A of the reactivity against antigen of Fractions A-II–A-V (A) and B-II–B-V (B), and homologous rabbit polyclonal antibodies. Symbols: see legend to Fig. 1.

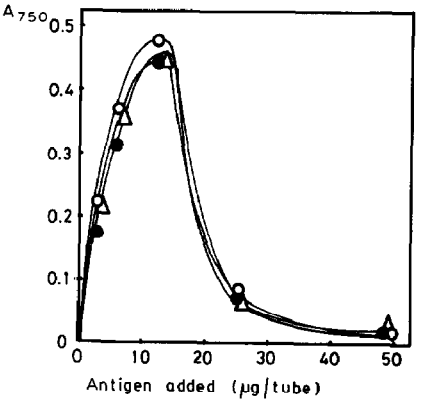

Fig. 3. Quantitative precipitin curve of antigens of Fractions A, B, and S against Con A: Fr. A (●), Fr. B (○), and Fr. S (△).

subfractions from Fractions A and B gave each four reaction curves, the slopes of which are apparently proportional to the phosphate content of the corresponding D-mannan subfraction. This provides evidence that Con A is able to trap these subfraction D-mannans in nearly identical amounts, regardless of the structural heterogeneity due to the different phosphate group proportions. In the QPR test of Fractions A, B, and S with Con A, the amounts of precipitated Con A were obviously the same for the three precipitin curves (see Fig. 3).

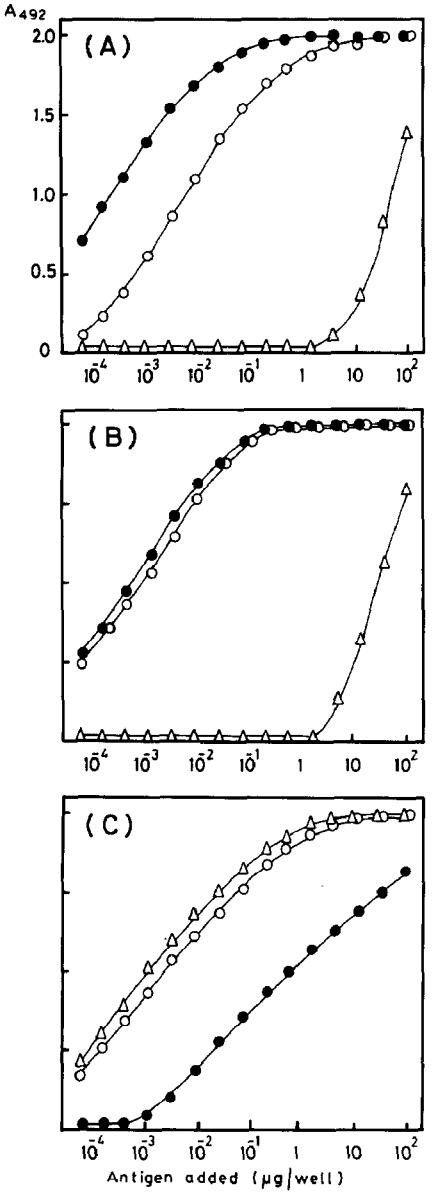

Fig. 4. Sandwich–EIA with Con A of cross-reactivity of Fractions A, B, and S against antisera *A* (A), *B* (B), and *S* (C). Symbols: same as in Fig. 3.

To provide additional evidence for the similarity of the reactivities in both sandwich–EIA and QPR tests, the cross-reaction between Fractions A, B, and S, and antisera *A*, *B*, and *S*, was investigated on Con A-precoated microtiter plates (see Fig. 4). The reactivity between Fraction B and antiserum *S* was higher than that observed between Fraction A and antiserum *S*, as reported previously by Hasenclever and Mitchell[8], and Shibata *et al.*[9] It is worth noting that the results of the sandwich–EIA test with Con A are also consistent with those obtained previously by the direct EIA test with the same antigens and antibodies[1].

Candida albicans spp., one of the important opportunistic infectious pathogens of man, is a yeast producing a polysaccharide, D-mannan, as the major antigen. In order to detect *C. albicans* D-mannans in patients' sera, Segel *et al.*[11] reported the use of an EIA-inhibition technique, and Suzuki *et al.*[12] a sandwich–EIA method using rabbit polyclonal anti-*Candida* IgG antibody and biotin-linked anti-rabbit IgG antibody. We report herein a sandwich–EIA procedure for the assay of *C. albicans* D-mannans in PBS by means of Con A-precoated microtiter plates and antisera *A*, *B*, and *S*. These mannans showed closely similar reactivities to those displayed by the same D-mannans and antisera in the QPR test. The recovery ratios of Fractions A, B, and S in the concentration range from 10^{-3} to 1 μg/mL were almost quantitative. Thus, this sandwich–EIA procedure may be applied to the determination of various D-mannans and D-mannose-containing polysaccharides in mammalian body fluids. It is important that a Con A of high purity, which is readily available commercially, be used. Tojo *et al.*[1,2,10] have previously demonstrated that anti-*C. albicans* D-mannan antibodies in polyclonal rabbit sera and culture supernatants of monoclonal antibody-producing hybridomas could be determined by means of plastic microtiter plates coated with the D-mannans of *C. albicans*. The mechanism of this adsorption of the D-mannans involves the formation of a hydrophobic bond between the peptide component of the D-mannans and the plastic molecules. Thus, a combination of the two EIA procedures would provide an improved method for the determination of either antigen or antibody of *C. albicans* in various biological specimens.

EXPERIMENTAL

Materials. — The D-mannans of *C. albicans* NIH A-207, *C. albicans* NIH B-792, and *S. cerevisiae* wild type strain, described as Fractions A, B, and S, respectively, were the same specimens as those used in the previous study[1]. The five subfractions of Fractions A and B, which have various contents of protein and phosphate, were prepared by essentially the same manner as that described by Okubo and Suzuki[6] by means of a column of diethylaminoethyl(DEAE)-Sephadex A-50 (AcO^-). They were the same specimens as those used in the previous study[1]. Because of very low yields, further investigations were not conducted on Fractions A-I and B-I, which contained the lowest amount of phosphate in each D-mannan subfraction series. The antisera corresponding to the A, B, and S strains, abbreviated as antisera *A*, *B*, and *S*, respectively, were also the same specimens as those used in the previous study[1].

Ninety six-well, flat, polystyrene–microtiter plates (Linbro/Titerteck, Cat. 76-381-04) were obtained from Flow Laboratories, Rockville, MD, USA).

Sandwich–EIA test with Con A. — Con A (Concanavalin A Type IV, No. C-2010, Sigma, St. Louis, MO, USA; 1 mg/mL) in PBS (pH 7.2) was placed in the wells of a polystyrene-microtiter plate and kept at room temperature for 2 h. The plate was then washed three times with PBS containing 10mM Tween 20 surfactant (PBST). PBST (200 μL) containing bovine serum albumin (10 g/L) was added to each well. The plate was kept for 2 h at room temperature, and then washed three times with PBST. To each well, a solution of D-mannan (100 μL) in PBS containing 1–1000 ng/mL was added. The plate was kept for 2 h at room temperature and then washed three times with PBST. A diluted (1:1000) solution of antiserum *A* or *B* in the same buffer (100 μL) was added to the wells, and the plate was kept for 2 h at room temperature, and then washed three times with PBST. A solution of goat anti-rabbit IgG (H+L) peroxidase conjugate (Sigma, St. Louis, MO) in PBST (diluted 1000-fold; 100 μL) was added to the wells, and the mixture was kept at room temperature for 2 h. The excess of peroxidase-labeled, anti-rabbit IgG (H+L) antibody was removed by washing with PBST three times. Finally, a solution (100 μL) containing 0.02% *o*-phenylenediamine and 0.006% H_2O_2 in 0.15 M citrate buffer (pH 5.0) was added to the wells, and the mixture was kept at room temperature for 30 min. After addition of 2M H_2SO_4 (100 μL), the absorbance at 492 nm was measured with an Immuno Reader NJ-2000 (Inter Med, Tokyo, Japan). Assay of each D-mannan antigen was conducted in triplicate, and the reaction curve was drawn by plotting the average of three values.

QPR test with Con A. — The QPR test with Con A was performed according to the procedure described by Okubo and Suzuki[6], as follows: To a solution of Con A in PBS (100 μL, 1 mg/mL) in small test-tubes (1.6 × 10.4 cm) was added a saline solution (0.5 mL) of D-mannan in serial amounts (1–50 μg). After incubation for 1 h at 37°, the mixture was kept overnight at 4°, and then centrifuged at 1500*g* for 10 min. Each precipitate was washed twice carefully with ice-chilled saline solution (0.5 mL). The amount of protein precipitated was determined by the Folin method of Lowry *et al.*[7]

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