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Specific Staining of Wall Mannan in Yeast Cells with Fluorescein-Conjugated Concanavalin A

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A procedure is given for the coupling of fluorescein isothiocyanate to concanavalin A, a protein which specifically combines with a variety of polysaccharides, and for the subsequent isolation of the reactive conjugate. This fluorescent conjugate stains *Saccharomyces cerevisiae* but not *Schizosaccharomyces pombe* or *Rhodotorula glutinis*. The cell walls of the latter two organisms do not contain branched homopolymers of α-linked mannose. Furthermore, the staining of *S. cerevisiae* is competitively inhibited by either unlabeled concanavalin A or methyl-α-D-mannopyranoside. On the basis of this evidence, it is concluded that the staining of *S. cerevisiae* results from the specific interaction of the fluorescein-concanavalin A conjugate with the α-mannan present in the cell wall of this yeast.

Concanavalin A, a protein of the jack bean (*Canavalia ensiformis*), combines specifically with a variety of polysaccharides which have branched structures and α-D-glucopyranosyl, α-D-mannopyranosyl, β-D-fructofuranosyl, or α-D-arabinofuranosyl residues occupying nonreducing terminal positions (7-10). Of the polysaccharides which react with concanavalin A, e.g., glycogen, amylopectin, arabinogalactan, various dextrans, and levans (4, 7, 9, 10, 15, 24, 25), α-linked mannose homopolymers such as those produced by *Saccharomyces* species seem to be the most tenaciously bound (22).

Three types of polysaccharides occur in cell walls of bakers' yeast: major amounts of glucan and mannan and lesser quantities of chitin (16, 17, 21). Since concanavalin A does not react with yeast glucan (10) and would not be expected to react with chitin, a linear β-linked polymer, fluorescently labeled concanavalin A should be a specific staining reagent for the α-mannan present in such cell walls.

In the present communication we report the preparation of fluorescein-labeled concanavalin A and the specificity of the interaction of this conjugate with living yeast cells.

MATERIALS AND METHODS

Purification of concanavalin A. Concanavalin A was isolated from jack bean meal (Worthington Biochemical Corp., Freehold, N.J.) by the method of Agrawal and Goldstein (1), a purification technique based on the specific adsorption of concanavalin A to a cross-linked dextran gel and its subsequent elution with 0.1 M glucose. The concentration of concanavalin A was estimated spectrophotometrically at 280 nm employing an $E^{1\%}_{1\,cm}$ value of 11.4 (3).

Isolation of mannan. Mannan from baker's yeast (Anheuser Busch Inc., Old Bridge, N.J.) was prepared as described by Peat et al. (20) with the modification that, after precipitation with Fehling's solution, the copper was removed by the addition of Bio-Rad cation exchange resin AG 50W-X12, 20 to 50 mesh, hydrogen form (Bio-Rad Laboratories, Richmond, Calif.).

Conjugation of fluorescein to concanavalin A. Fluorescein isothiocyanate (Calbiochem, Los Angeles, Calif.) was dissolved (0.94 mg/ml) in 0.1 M Na_2HPO_4. To 0.48 ml of this solution was added 11.9 mg of concanavalin A in 0.6 ml of 1.0 M NaCl. The reaction mixture, the *p*H of which was 8.35, was incubated at room temperature (ca. 22 C) for 2 hr and then dialyzed overnight at 4 C against 2 liters of 1.0 M NaCl. After the small amount of precipitate which formed on dialysis was removed by centrifugation (44,000 $\times$ *g*, 15 min, 4 C), the mixture was placed on a column (0.9 by 15.5 cm) of Sephadex G-75 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) previously equilibrated with 1.0 M NaCl. The absorbancies of the 1-ml fraction were determined at 280 and 493 nm. When the column was eluted with 1.0 M NaCl, a small peak absorbing at both wavelengths appeared in the void volume. The bulk of the yellow material which remained on the upper third of the column was eluted by a solution of 0.1 M glucose in 1.0 M NaCl. This second peak which consisted of active, fluorescein-conjugated concanavalin A and any remaining unmodified concanavalin A was pooled and dialyzed overnight at 4 C against 2 liters of 1.0 M NaCl to remove the glucose. The optical absorbancy of the dialyzed pool, diluted 1:10 with 1.0 M NaCl, was 0.190 at 280 nm and 0.102 at 493 nm. The dialyzed pool was stored at -20 C and diluted 1:50 with 1.0 M NaCl for use as a staining reagent.

Ouchterlony analysis. Ouchterlony analysis (19),

which was used to determine the concanavalin A activity in various column eluates, was carried out on microscope slides in a supporting medium consisting of 0.85% (w/v) NaCl, 0.01% Merthiolate, and 1.5% Noble agar (Difco) in 0.01 M potassium phosphate buffer, *p*H 7.0. The antigen well was filled with a solution of yeast mannan (0.5 mg/ml).

Yeast strains and culture conditions. *S. cerevisiae* ATCC 9763, *Schizosaccharomyces pombe* ATCC 2478, and *Rhodotorula glutinis* strain 77 (culture collection of W. J. Nickerson) were grown at 28 C for 24 hr with vigorous shaking in a medium consisting of 0.5% peptone, 0.3% yeast extract, and 1.0% glucose. Stock cultures were maintained on the same medium with agar added to a final concentration of 1.5%. Peptone, yeast extract, and agar were purchased from Difco Laboratories, Detroit, Mich.

Staining of yeast cells with fluorescein-concanavalin A conjugate. Yeast strains were harvested by centrifugation, washed twice with distilled water, and resuspended in distilled water to a final optical absorbancy of 0.1 at 660 nm (50 Klett units, Klett-Summerson Photoelectric Colorimeter, no. 66 red filter). The cells in 0.5-ml portions of the various suspensions were collected by centrifugation and resuspended in 0.5-ml portions of the fluorescein-concanavalin A conjugate diluted 1:50 with 1.0 M NaCl. After 30 min at room temperature (ca. 22 C), the cells were again collected by centrifugation, washed twice with 2-ml portions of 1.0 M NaCl and once with 2 ml of distilled water, resuspended in 0.5 ml of distilled water, and applied to fluorescent-antibody slides (Clay Adams, Parsippany, N.J.). The slides were air-dried and fixed in acetone for 10 min at room temperature. A mounting solution consisting of 9 volumes of glycerol and 1 volume of 0.25 M sodium carbonate-bicarbonate buffer (*p*H 9.0) was employed.

Inhibitor studies. To determine the effects of unlabeled concanavalin A and of methyl-α-D-mannopyranoside on the staining of *S. cerevisiae* by the fluorescein-concanavalin A conjugate, the procedure described in the previous paragraph was modified in only one respect: cells were resuspended in 0.5-ml portions of the 1:50 dilution of fluorescein-concanavalin A conjugate containing either 30 or 150 μg of unlabeled concanavalin A or 3 or 15 μmoles of methyl-α-D-mannopyranoside (Nutritional Biochemicals Corp., Cleveland, Ohio).

Microscopy and photography. Microscopic observations were made with a Zeiss dark-field fluorescence microscope, model GFL, equipped with an Osram mercury arc lamp, BG 12 exciter filter, and OG 4 barrier filter. Photographic records were made with Tri-X Pan film (Eastman Kodak Co., Rochester, N.Y.) developed by the Acufine process (Acufine Inc., Chicago, Ill.). Exposure times of 45 sec and 5 or 10 sec were used for fluorescent and dark fields, respectively.

RESULTS AND DISCUSSION

The reactivity of fluorescein isothiocyanate with proteins generally increases with *p*H in the range 6.0 to 10.0 (6). However, at *p*H values above neutrality, concanavalin A exhibits a tendency to form inactive high-molecular-weight aggregates (2, 18). To minimize this inactivation and aggregation, the reaction of fluorescein isothiocyanate with concanavalin A was carried out at *p*H 8.35. After coupling, the active conjugate was separated from the unreacted fluorescein isothiocyanate and inactivated protein by dialysis and affinity chromatography on Sephadex. The first peak, which was eluted from the dextran column with 1.0 M NaCl, absorbed at 280 and 493 nm but did not precipitate yeast mannan (Ouchterlony analysis). As expected, the material which bound to the Sephadex and eluted as a second peak with 0.1 M glucose in 1.0 M NaCl was highly active in mannan precipitation.

The interaction of the fluorescein-concanavalin A conjugate with *S. cerevisiae*, *S. pombe*, and *Rhodotorula glutinis*, as well as the effect of inhibitors on the staining of *S. cerevisiae*, is shown in Fig. 1 and 2 (left-hand columns). Dark-field photomicrographs are presented for comparison (right-hand columns).

The fluorescence of stained *S. cerevisiae* cells (Fig. 1a) is very intense as compared to that of unstained control cells (Fig. 1g). Furthermore, this staining is partially inhibited by the presence of 30 μg of unlabeled concanavalin A (Fig. 1c) or 3 μmoles of methyl-α-D-mannopyranoside (Fig. 1e). So and Goldstein (22) showed that methyl-α-D-mannopyranoside is a potent inhibitor of the precipitation of yeast mannan by concanavalin A. Increasing the amount of inhibitor to 150 μg of unlabeled concanavalin A or 15 μmoles of methyl-α-D-mannopyranoside decreases the fluorescence to a level only slightly greater than that of unstained controls.

Cells of *R. glutinis* and *S. pombe* treated with the fluorescein-concanavalin A conjugate (Fig. 2a and 2c, respectively) fluoresce almost as poorly as untreated control cells (*not shown*).

The preferential staining of *S. cerevisiae* by fluorescent concanavalin A can be explained on the basis of the polysaccharides known to occur in the cell walls of the yeast employed in this study. Glucan (β-linked) is present in *S. cerevisiae* and *S. pombe* but not in *R. glutinis*, whereas chitin is found in the walls of *S. cerevisiae* and *R. glutinis* but not *S. pombe* (14, 17). Thus, neither of these wall components correlates with staining. The consideration with reference to mannose-containing polymers is somewhat more complex. The mannose homopolymer of *S. cerevisiae* consists of an α-(1 → 6)-linked backbone and numerous short side chains which contain both α-(1 → 2) and α-(1 → 3) linkages and which are attached to the backbone by α-(1 → 2) linkages (23). Crook and Johnston (5) reported that mannose is a major wall component in *Rhodotorula*, but the nature of the mannose-containing polymer

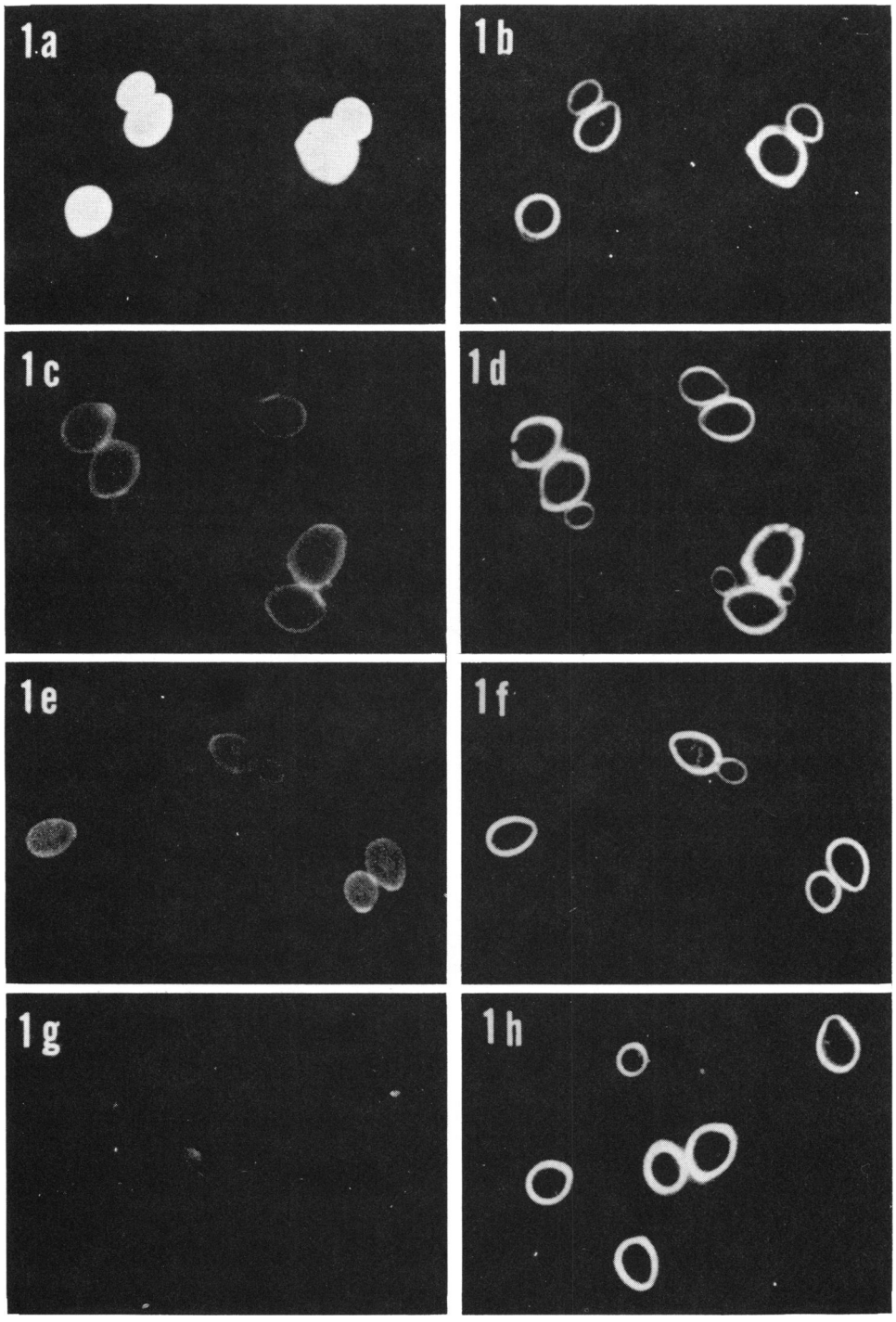

FIG. 1. *Left-hand column: fluorescence of S. cerevisiae treated with (a) a 1:50 dilution of the fluorescein-concanavalin A conjugate in 1 M NaCl; (c) the diluted conjugate containing 30 μg of unlabeled concanavalin A; (e) the diluted conjugate containing 3 μmoles of methyl-α-D-mannopyranoside; (g) distilled water only. b, d, f, h: Corresponding dark-field photomicrographs for comparison.*

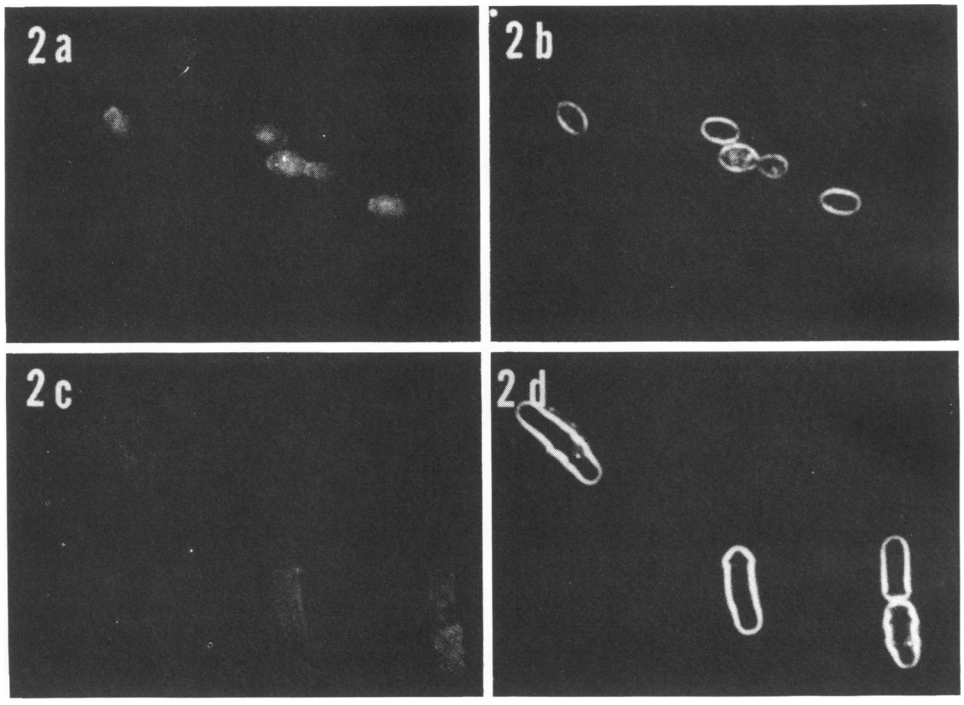

FIG. 2. *Fluorescence of R. glutinis (a) and S. pombe (c) after treatment with a 1:50 dilution of the fluorescein-concanavalin A conjugate. b, d: Corresponding dark-field photomicrographs for comparison.*

has not been elucidated. It is known, however, that the cell wall of *R. glutinis* (unlike that of *S. cerevisiae*) does not contain a polysaccharide capable of forming an insoluble complex with copper (14). Gorin et al. (11) reported that *R. glutinis* elaborates an exocellular mannose homopolymer, but this is β-linked. The cell wall of *Schizosaccharomyces octosporus* contains a galactomannan which consists of an α-(1 $\rightarrow$ 6)-linked mannopyranosyl backbone and α-galactopyranosyl side chains (13). It is probable that the galactomannan of *S. pombe* has a very similar structure since the nuclear magnetic resonance spectra of the galactomannans from these two organisms are identical (12). A heteropolysaccharide of this kind would not be expected to complex extensively with concanavalin A, because α-galactopyranosyl residues occupy most of the nonreducing terminal positions. In summary, of the three types of yeast employed in the present study, only *S. cerevisiae* produces a wall polysaccharide (viz., the mannan) rich in one of the four types of nonreducing terminal residues with which concanavalin A reacts (7–10).

Since the observed staining of *S. cerevisiae* is inhibited by unlabeled concanavalin A or methyl-α-D-mannopyranoside and since *R. glutinis* and *S. pombe* are not strongly stained, it is concluded that the fluorescein-concanavalin A conjugate acts as a specific staining reagent for the highly branched α-mannan in the cell wall of *S. cerevisiae*.

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