Immunosorbent for the Isolation of Specific Antibodies Against Mannan: Localization of Antigens in Yeast Cell Walls

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Received for publication 29 August 1972

A procedure is given for the insolubilization of yeast mannan by entrapment in a polyacrylamide gel. Specific anti-mannan antibodies were separated by immunosorption from all other antibodies of a rabbit antiserum against whole, Formalin-inactivated Candida utilis cells. Colloidal gold coated with the anti-mannan and anti-non-mannan antibody fractions was used for the ultrastructural visualization of cell wall antigens. A control was performed using normal rabbit serum. Inhibition of anti-mannan antibodies was obtained with mannan oligosaccharides, and none of the antibody fractions showed marking of Saccharomyces cerevisiae cell walls.

Techniques using insolubilized antigens have the advantage over conventional absorption methods that antibodies free of serum components and antigens can be obtained; frequently an insoluble antigen can be reused many times. Insolubilization of proteins by immobilization in the lattice of polyacrylamide gels has been described (e.g., 3, 4, 12). In the present paper we describe the extension of this method to the insolubilization of mannan, a neutral soluble polysaccharide. Colloidal gold coated with specific anti-mannan antibody prepared by this technique was successfully used for marking isolated Candida utilis cell walls.

MATERIALS AND METHODS

Chemicals. Acrylamide, \(N,N'\)-methylene bisacrylamide (bis) and \(N,N,N',N'\)-tetramethylethylenediamine (TEMED) were from Serva, Heidelberg, Germany. Tris(hydroxymethyl)aminomethane (Tris) was from Merck, Darmstadt, Germany. All other chemicals were purchased from Fluka AG, Buchs, Switzerland. The reagents were of analytical purity and were used without further purification.

The phosphate-buffered saline, pH 7.2 (PBS), was made by dissolving NaCl (36.0 g), anhydrous Na\(_2\)HPO\(_4\) (7.4 g), and anhydrous KH\(_2\)PO\(_4\) (2.15 g) in water up to 5 liters.

Preparation of the immunosorbent. The method published by Carrel et al. (4) was modified as follows. In a 12- by 100-mm test tube, 1 ml of mannan solution (50 mg of mannan in 1 ml of water) was mixed with 1 ml of buffer (0.05 M Tris-hydrochloride containing 0.20 M TEMED/100 ml, final pH 7.5) and 1 ml of monomer solution (2.40 g of acrylamide and 0.80 g of bis dissolved in water to 10 ml). Finally, 1 ml of a freshly prepared catalyst solution (0.14 g of ammonium persulfate in 50 ml of water) was added, and the mixture was left undisturbed at room temperature (20-25 C). The white, nontransparent polyacrylamide gel formed within 30 min after addition of the persulfate and was left 18 to 24 h to allow polymerization to go to completion.

The gel block was granulated by forcing it successively through a 20- and 40-mesh stainless steel sieve with a spatula. The particles retained on an 80-mesh sieve were filled to a 9- by 150-mm chromatography column (Pharmacia, Sweden) on top of a 5-mm layer of glass beads (1 mm diameter).

The immunosorbent was then precycled at 5 C by washing at a flow rate of 10 to 20 ml/h with Tris-buffered saline (0.05 M Tris-hydrochloride — 0.15 M NaCl, pH 7.5) and acid buffer (0.2 M glycine-hydrochloride, pH 2.3) until the effluents showed no optical absorbancy at 280 nm and were free of mannan as compared to the eluting buffers. Finally, the gel was neutralized to pH 7.5 by washing with Tris-buffered saline.

Isolation of anti-mannan antibodies. A 4-ml portion of rabbit antiserum against C. utilis was pumped through the precycled immunosorbent at a flow rate of 10 ml/h, and the retained material was recovered by washing with Tris-buffered saline until the optical density of the effluent at 280 nm returned to the value of the buffer.

The bound anti-mannan antibodies were eluted with the acid buffer at a flow rate of 10 to 20 ml/h. The fractions absorbing at 280 nm were pooled and immediately adjusted to pH 7.5 with 2 M Tris.

The effluents collected at neutral and acid pH were reconcentrated to the original volume of 4 ml by pressure dialysis against Tris-buffered
saline. All manipulations were carried out at 5 C. The immunosorbent could be reused after washing with Tris-buffered saline.

Preparation of anti-C. utilis antiserum. Two rabbits (2.5-3 kg) were immunized with a suspension (1.6 x 10^6 cells per ml) of heat-inactivated (15 min at 70 C) C. utilis cells in physiological saline without adjuvant. Each rabbit was given six injections spaced over 1 month of 1.5 to 5 ml of suspension per injection by the intravenous or intravenous and subcutaneous routes. The animals were bled by heart puncture and the sera were lyophilized.

Agglutination assay. Samples (0.5 ml) of double dilutions of antiserum (starting at 1:10 dilution) were mixed with 0.5-ml portions of a suspension of Formalin-inactivated C. utilis cells (1.4 x 10^8 cells per ml). Agglutination readings were taken after 6 and 20 h of incubation at 50 C (Table 1).

Double diffusion assay in agarose. Wells (6.4-mm diameter) in a 1.5-mm-thick layer of agarose (1% in 0.05 M barbitral buffer, pH 8.3) were filled with antisera or mannan solution (0.25 mg of mannan per ml). Photographs were taken after 22 h of diffusion at 18 to 20 C.

Electrophoresis on cellulose acetate. The Beckman Microzone Electrophoresis System (model R-101) was used.

Electrophoresis was carried out in barbital buffer, pH 8.6, µ 0.075, at 250 V for 30 min. The proteins were stained with Ponceau BS.

Sugar analysis. Total sugars were estimated by the phenol-sulfuric acid method (6).

The amount of mannan retained in the polyacrylamide gel was determined either indirectly from the difference between added or unbound mannan or by measuring mannan directly in the gel, or both. For the latter, preexposed immunosorbent was first centrifuged (1,000 X g for 5 min) on a filter to remove liquid between the gel particles. Samples (10-20 mg) of this gel were suspended in 1 ml of Tris-buffered saline and were reacted with phenol-sulfuric acid as with liquid samples. Controls with buffers and polyacrylamide gel without mannan were included.

The sugars present in mannan after acid hydrolysis (2 N H2SO4, 100 C, 4 h) were quantitatively determined as their alditol acetates by gas liquid chromatography at 210 C using a glass column (0.3 by 200 cm) containing 3% (wt/wt) OV-225 (Applied Science Labs, State College, Pa.) on Gas-Chrom Q (100-120 mesh) (10).

Determination of anti-mannan antibodies. Samples (0.1 ml) of antisera were incubated at 37 C for 30 min with optimal proportions of mannan (15 µg) and left 24 to 48 h at 5 C. The washed precipitates were dissolved in 0.1 M NaOH (1 ml), and the protein content was estimated spectrophotometrically at 280 nm, using an E1% at 13.0 for rabbit immunoglobulin.

Isolation of yeast cell walls. C. utilis CBS 567 was grown at 30 C for 24 h in a 2% sucrose medium supplemented with 0.3% yeast extract (Difco, Detroit, Mich.) and mineral salts as inorganic nitrogen source.

Cell walls were prepared from pressed baker's yeast and from cells of C. utilis by the method of Mill (11). They were judged free from cytoplasmic contamination by light and electron microscope examination.

Isolation of mannan. Mannan from C. utilis was prepared as described by Peat et al. (13) and was purified by three precipitations with Fehling solution. The mannan contained mannosc and glucose in the molar ratio 4:1. The mannan composition was found unchanged by further precipitation with Fehling solution. The mannan had [α]D + 100° (water, c 1.2).

Antibody labeling. Antibody fractions were adsorbed onto colloidal gold by the procedure of Faulk and Taylor (7) with one modification. To prevent adsorption of the colloid onto the walls of the polyallomer centrifuge tubes, the colloid was centrifuged twice in PBS containing 0.5% Tween 80. This detergent has been shown not to interfere with antigen-antibody interaction (5). Prior to labeling, the protein content of the anti-mannan antibody fraction was adjusted with normal rabbit serum to the protein level of the original antiserum.

Marking of yeast cell walls with antiserum-coated colloidal gold. Yeast cell walls were suspended in PBS to a final optical absorbancy of 1.0 at 420 nm. The antiserum-coated colloidal gold (0.2 ml) and PBS (0.1 ml) were added to the cell wall suspension (0.2 ml). After incubating at 25 C for 2 h with gentle shaking, the suspension was centrifuged at 2,800 X g for 5 min, washed three times with PBS (6 ml), and finally suspended in the same buffer (0.3 ml).

Inhibition studies. A mixture of oligosaccharides from C. utilis mannan was prepared according to Kocourek and Ballou (9). C. utilis cell walls were marked as described above with colloidal gold coated anti-mannan antibodies in the presence of 1.2, 0.12, and 0.012 mg of oligosaccharides in the incubation mixture (0.5 ml).

Preparation for electron microscopy. A drop of the prelabeled cell wall suspension was placed on a Formvar-carbon-coated grid. After a few minutes, the excess material was removed with a micropipette or a filter paper. The grids were dried in a vacuum desiccator overnight and examined in a Philips EM300 electron microscope.

### Table 1. Agglutination of C. utilis cells by native antiserum against C. utilis, neutral and acid eluates from mannan immunosorbent, and normal rabbit serum

<table>
<thead>
<tr>
<th>Sample</th>
<th>Agglutination titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native anti-C. utilis antiserum</td>
<td>1:320</td>
</tr>
<tr>
<td>Neutral eluate (pH 7.5)</td>
<td>1:80</td>
</tr>
<tr>
<td>Acid eluate (pH 2.3)</td>
<td>1:160</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>&lt;1:10</td>
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</tbody>
</table>
RESULTS AND DISCUSSION

Entrapment of mannan in polyacrylamide gel. Mannan analysis by the direct or indirect method showed that 9.2 to 10.7 mg of mannan were retained in the granulate from a 4-ml gel block. This corresponds to 20% of the total amount of mannan added to the monomer mixture.

Isolation of anti-mannan antibodies on the immunosorbent. The anti-C. utilis antisera was found to contain 2.38 mg of precipitating anti-mannan antibodies per ml. At neutral pH, all anti-mannan antibodies from 4 ml of this antisera were retained on a column filled with the 40- to 80-mesh particles from a 4-ml gel block of immunosorbent (Fig. 1a). No attempt was undertaken to determine the maximal antibody binding capacity of the immunosorbent. The acid eluate from 4 ml of anti-C. utilis antisera contained a total of 9.7 mg of protein of which 6.8 mg (70%) could be precipitated with mannan. Double diffusion analysis against mannan showed complete fusion of the precipitation lines of native anti-C. utilis antisera and acid eluate (Fig. 1a). By electrophoresis on cellulose acetate, we demonstrated that the proteins in the acid eluate consisted exclusively of the immunoglobulin fraction of the antisera (Fig. 1b). This was confirmed by immunoelectrophoresis showing only an immunoglobulin G precipitation line with a polyvalent sheep antisera against rabbit serum proteins.

The results of agglutination tests are shown in Table 1. The agglutination titer of the neutral eluate (antibodies not retained on the immunosorbent) indicates considerable antibody activity against C. utilis antigens other than mannan.

The above results prove that the known
method for insolubilization of protein antigens by entrapment into polyacrylamide gel can successfully be extended to neutral polysaccharides like mannan. On the other hand, the use of an immunosorbent technique in the present study allowed the simple and clear-cut separation of antibodies directed against mannan and non-mannan antigenic determinants.

Electron microscopy. A whole C. utilis cell wall marked with colloidal gold coated with native anti-C. utilis antiserum is seen in Fig. 2. The marking is dense and homogenous. Different densities of marking can be recognized in the sequence of electron micrographs represented in Fig. 3a–f. The highest density is obtained with the native anti-C. utilis antiserum (Fig. 3a). A weaker but still important and random marking can be observed with the neutral eluate from the immunosorbent (anti-nonmannan antibodies; Fig. 3b). An almost identical density and distribution of gold granules is observed with the specific anti-mannan antibodies (acid eluate; Fig. 3c). The two dense lines of gold particles (arrows) correspond to the region of the bud scar, indicating a certain accumulation of mannan at this site. A practically total inhibition of marking can be achieved by the addition of oligosaccharides to the anti-mannan antibody fraction (Fig. 3d). This is in agreement with the fact that side chain oligosaccharides resulting from acetolysis of various yeast mannans strongly inhibit the antiserum-mannan precipitin reaction (1, 14, 15). Total inhibition was achieved with the highest concentration of oligosaccharides of C. utilis mannan (1.2 mg), partial inhibition at lower concentration. The weak marking obtained with normal rabbit serum must be due to nonspecific adsorption of the colloidal gold (Fig. 3e). The same weak, nonspecific adsorption is observed with colloidal gold coated with native anti-C. utilis antiserum when used against Saccharomyces cerevisiae cell walls (Fig. 3f).

The results of the marking experiments only indicate a random distribution of the mannan at the cell wall surface. To determine the location of this polysaccharide within the cell wall, the technique of thin sectioning has to be applied (studies are being undertaken in our laboratory).

Nevertheless, the results obtained with the specific anti-mannan antibodies confirm the observations with mercury-labeled concanavalin A (8), i.e., an even distribution of the mannan at the cell wall surface. The dense marking of the bud scar region (accumulation of mannan) is in excellent agreement with concanavalin A marking experiments of isolated bud scars of S. cerevisiae (2).

ACKNOWLEDGMENTS

We thank H. Hilperti for the antisem against, C. utilis, D. A. Bush for preparing the yeast cell walls, and M. Weber for photographic work.

LITERATURE CITED


Fig. 3. The densest marking of C. utilis cell walls is obtained with the native anti-C. utilis antiserum (a). A randomly distributed marking is observed with the anti-nonmannan fraction (b). About the same density of marking can be detected with the specific anti-mannan antibodies. Note the increased density of gold granules in the bud scar region (arrows) indicating mannan accumulation (c). Oligosaccharides added to anti-mannan antibodies almost totally inhibit marking (d). Some nonspecific adsorption of gold granules can be recognized with normal rabbit serum (e). The same is observed with native anti-E. utilis antiserum when used against Saccharomyces cerevisiae cell walls (f). Magnification, χ40,000.

