MERCURY-LABELLED CONCANAVALIN A AS A MARKER IN ELECTRON MICROSCOPY-LOCALISATION OF MANNAN IN YEAST CELL WALLS

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Received 27 August 1971

1. Introduction

Concanavalin A (con A, the jack bean phytohemagglutinin) will agglutinate polysaccharides providing they contain multiple α-D-glycopyranosyl (or its 2-acetamido-2-deoxy derivative), α-D-mannopyranosyl, or β-D-fructofuranosyl units at their non-reducing chain ends [1–3]. Such polysaccharides are glycogens, levans, certain bacterial lipopolysaccharides and mannans. Bivalent metals (Mn^{2+} or Ni^{2+} and Ca^{2+}) are necessary for this protein carbohydrate binding [4, 5].

Ultrastructural visualization of cellular polysaccharides by means of con A has already been reported [6]. This method was based on the fact that con A has two reactive groups, one of which was bound to the cell polysaccharides, while the other acted as a receptor for the carbohydrate containing peroxidase. The presence of peroxidase was detected by the diaminobenzidine reaction [6]. This multiple step method is limited by its low resolution.

It was therefore considered promising to label con A with a heavy metal to enable direct ultrastructural visualization of the polysaccharide–protein complex. The success of such a technique would depend on the modified protein keeping its agglutination properties.

The principle is as follows: as free amino groups are not essential for the binding activity of con A [7], these groups were used to thiolate the cystein-free protein [8, 9]. The thiolated con A was reacted with p-chloromercuribenzoic acid. The resulting mercury-labelled con A still had good carbohydrate binding activity and was found to be a good marker for the localization of *Saccharomyces cerevisiae* cell wall mannan, the only polysaccharide of these walls susceptible to binding by con A.

2. Material and methods

2.1. *General*

Jack bean meal was purchased from Schuchardt GMBH-Co. All chemicals used were of reagent grade. Cellulose acetate electrophoresis was carried out in a Beckman R-100 MicroZone apparatus (15 min, 250 V, 0.5 M acetate buffer, pH 4.5). The strips were stained with Ponceau S. Con A was prepared according to Doyle et al. [10] and was shown to be homogeneous by cellulose acetate electrophoresis. Agar gel diffusion studies were made according to Goldstein and So [3], with the addition of 0.001 M $MnCl_2$ to the agar gel. The agglutination of mercury-labelled con A with mannan was assayed turbidimetrically at 420 nm [11] in 0.05 M acetate buffer pH 6.0, containing 1 M NaCl and 0.001 M $MnCl_2$ (buffer A). Mercury was determined according to Lindstedt [12] by cold-vapour atomic absorption measurement. Cell walls of cerevisiae were prepared by the method of Mill [13]. They were judged clean by light and electron microscopical examination (fig. 1).

2.2. *Mercaptosuccinylation [9] and mercury labelling of concanavalin A*

Con A (400 mg) was dissolved in 0.2 M phosphate buffer (pH 7.5, 10 ml). The solution was stirred at 25° and a six fold excess (calculated on the lysine content of con A [8]) of S-acetylmercaptosuccinic anhydride (197 mg, 1.13 mmole) was added stepwise over a period of 1 hr. The pH was kept at 7.5 in a pH-stat by addition of 0.1 N NaOH (16 ml).

The protecting S-acetyl groups were removed by addition of 0.75 M hydroxylamine (pH 7.5, 4 ml) [9]. The pH was maintained at 7.5 by addition of 0.1 N

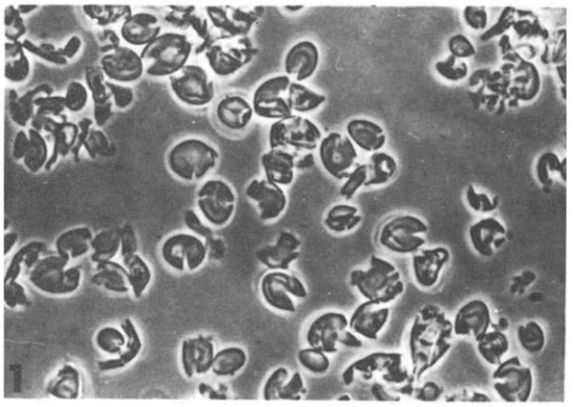

Fig. 1. Phase contrast micrograph of isolated *S. cerevisiae* cell walls. ×850.

NaOH (11.5 ml). After 5 min, Na p-chloromercuribenzoate (455 mg, 1.2 mmole) was added slowly, while maintaining the pH at 7.5 by addition of 0.1 N HCl. After 30 min, the excess of p-chloromercuribenzoate was reacted with mercaptoethanol (0.1 ml). The solution was then dialyzed against distilled water at 5° and centrifuged at 20,000 *g* for 30 min. The supernatant was freeze-dried; yield: 359 mg; Hg content: 3.2%. The Hg-labelled con A was found to be homogeneous by cellulose acetate electrophoresis at pH 4.5. It migrated toward the anode, while unlabelled con A migrated toward the cathode.

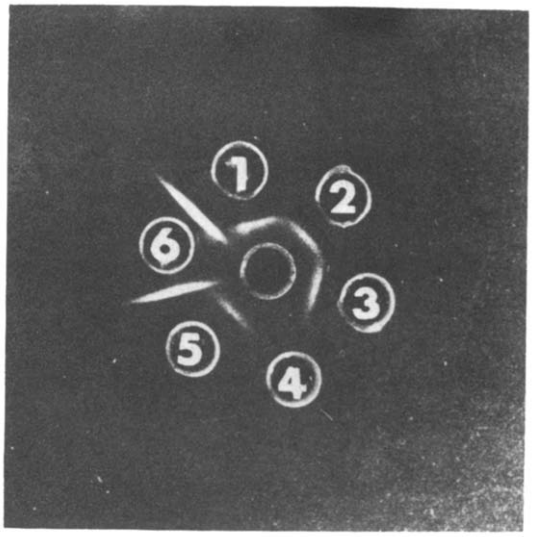

Fig. 3. Interaction in agar gel of con A and Hg-labelled con A with mannan from *S. cerevisiae*. Center well: Hg-labelled con A, 5 mg/ml. Peripheral wells: (1–3, 5) mannan, 5 mg/ml; (4) buffer; (6) con A, 5 mg/ml.

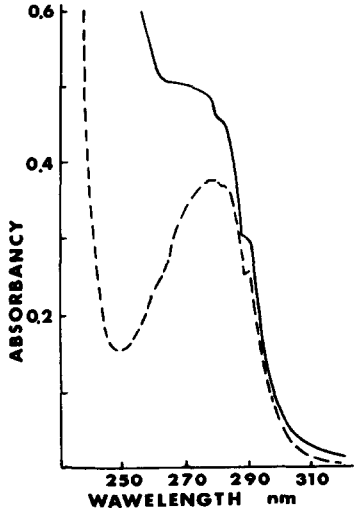

Fig. 2. Absorption spectra of con A (- - - -) and Hg-labelled con A (——) in 0.05 M acetate, pH 6.0; protein content, 0.80 mg of nitrogen per ml.

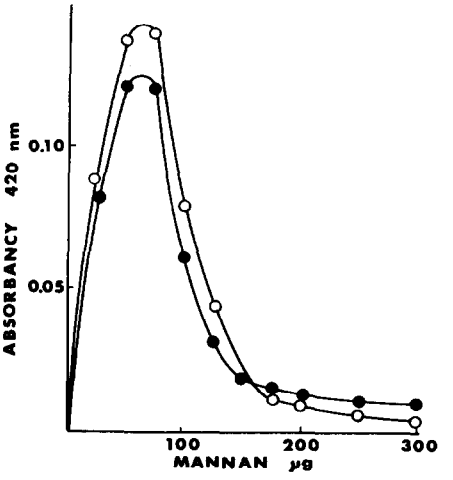

Fig. 4. Interaction of con A (○——○) and Hg-labelled con A (●——●) with *S. cerevisiae* mannan as measured by turbidimetry in buffer A (3 ml); con A and Hg-labelled con A, 0.080 mg of nitrogen per 3 ml.

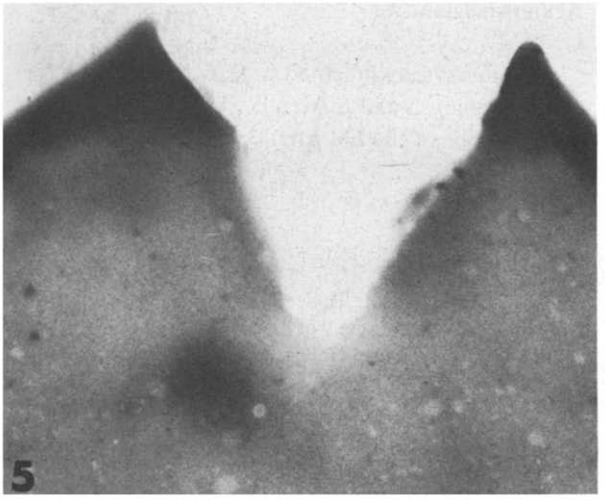

Fig. 5. *S. cerevisiae* cell wall incubated for 4 hr with unlabelled con A (control). ×25,000

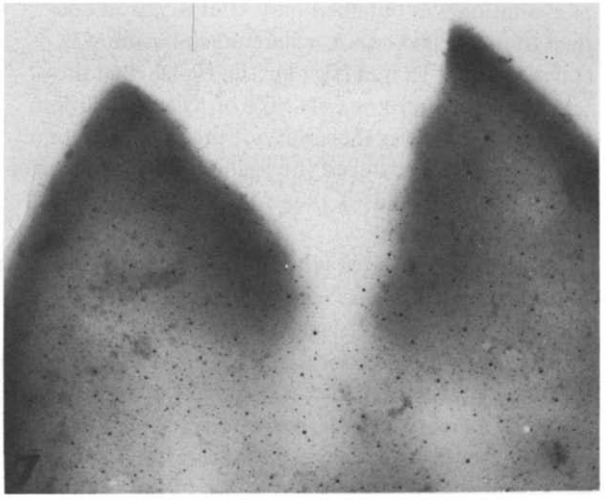

Fig. 7. Glutaraldehyde prefixed cell walls can still be marked with Hg-labelled con A. Again a homogeneous distribution of the mercury is found. ×25,000

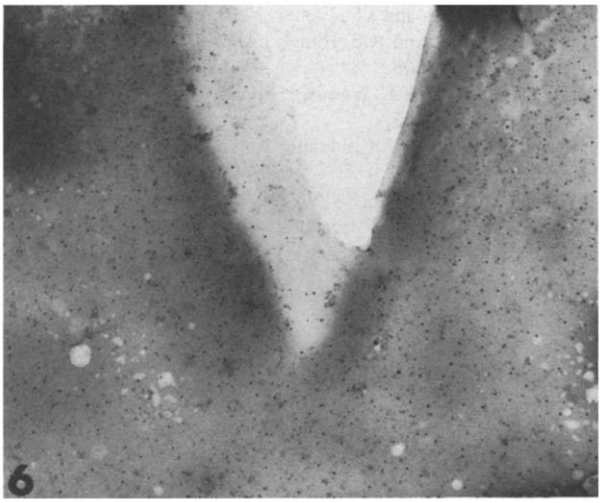

Fig. 6. *S. cerevisiae* cell wall incubated for 4 hr with Hg-labelled con A. An even distribution of the mercury over the whole cell wall is evident. ×25,000

The UV spectrum of Hg-labelled con A is shown in fig. 2.

2.3. *Treatment of S. cerevisiae cell walls with mercury-labelled concanavalin A*

S. cerevisiae walls (5 mg) were dispersed in 3% glutaraldehyde–0.2 M Ca^{2+}-containing cacodylate buffer (pH 7.0, 5 ml). After 5 min fixation, the suspension was centrifuged at 3,000 rpm and washed 3 times with buffer A.

Hg-labelled con A (20 mg) was dissolved in buffer A (10 ml). The solution, containing a drop of toluene, was filtered through a Millipore and added to the cell wall pellet, or in some cases to untreated cell walls. The suspension was slowly shaken at 25° for 4 hr, centrifuged and washed twice with buffer A. The pellet was then examined by electron microscopy.

2.4. *Preparation of samples for electron microscopy*

A drop of cell wall suspension treated with con A (control) or Hg-labelled con A was deposited on formwar-carbon coated grids with a micropipette. The excess material was removed after a few min, and the grids were air dried. The unstained preparations were examined in a Philips EM 300 electron microscope.

3. Results and discussion

Hg-labelled con A gave a single precipitation band with *S. cerevisiae* mannan on agar diffusion plates (fig. 3).

The reactivity of Hg-labelled con A with mannan was also assayed turbidimetrically at pH 6.0. Complete

precipitation was obtained only after 3.5 hr in contrast to unlabelled con A, where precipitation was complete after 15 min (fig. 4). The Hg-labelled protein retained approximately 90% of its agglutination properties. However the ability of the protein to bind Mn^{2+} was greatly reduced (no agglutination of mannan in the absence of Mn^{2+}), therefore Mn^{2+} was included in all incubations.

A cell wall incubated with unlabelled con A is shown in fig. 5. The well preserved shape of the cell wall and the characteristic triangular opening through which the cytoplasm was released are shown. After incubation for 4 hr with Hg-labelled con A, small electron dense, spherical mercury granules were detected (fig. 6). The mercury was evenly dispersed over the whole cell wall indicating homogeneous distribution of the mannan. The average diameter of the mercury grains was 8–10 nm which compares very well with the silver grains observed in the silver methionine technique for the detection of polysaccharides [14]. The resolution, limited by the grain size, is therefore much better than with the peroxidase-con A [6]. A virtually identical picture is obtained with cell walls which have been prefixed with glutaraldehyde, prior to incubation with Hg-labelled con A (fig. 7).

Only whole unstained cell walls have been examined, therefore only the mannan distribution at or near the surface of the cell wall has been visualized. Thin sections of the cell walls are being studied in order to examine the overall location of the mannan.

Acknowledgements

The authors are indebted to Miss A. Estoppey for mercury analysis and to Miss E. Sigarlakie for technical assistance of the EM part.

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