Cell Wall Composition of Yeast- and Rod-forms of *Candida krusei*

Shigeyoshi Katohda, Mitsuru Tsukinaga, Yasuo Tobinai and Tsutomu Sato

Laboratory of Applied Microbiology, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997, Japan

Received June 15, 1981

Cell walls were obtained from yeast (Y)- and rod (R)-forms of *Candida krusei*. These Y- and R-form cells were grown in synthetic media containing lysine and alanine as nitrogen sources, respectively. Comparative analysis of their walls showed that the Y-form wall had greater quantities of alkali-soluble glucan, alkali-insoluble glucan and mannan than the R-form wall had.

The glucan from Y-form walls had a larger amount of β-1,3-glycosidic linkage with a few branches at the C-6 position and a smaller amount of linear β-1,6-glycosidic linkage than the glucan from R-form walls had. There was, however, no significant difference in the chemical structure of mannan between the walls of these two forms.

The results are discussed in relation to the chemical composition and structure of polysaccharides of walls in the yeast–rod dimorphism.

The yeast-mycelial dimorphism of certain species of *Candida* has been studied by Skinner and Fletcher,1) and Nickerson.2) They reported that the dimorphism depends on a variety of environmental factors. Moreover, comparison of cell walls of yeast- and mycerial-forms of *C. albicans* by chemical analysis was reported by Chattaway et al.3) and Yamaguchi.4)

The nitrogen source in the culture medium has been shown to affect morphogenesis of *C. albicans* by Mardon et al.5) They showed that pseudohyphal morphology was observed when each of glycine, D- or L-ornithine, L-serine, L-methionine, L-phenylalanine and L-tyrosine was used as a sole nitrogen source. In their subsequent study,6) germ-tube production by *C. albicans* and elongated septa cell production by *Candida krusei* were shown to occur in a glucose–salt–biotin medium containing L-α-amino–n-butyric acid as the nitrogen source. In spite of many investigations on the composition of cell walls in yeast–mycelial dimorphism, there has been no report on the comparison of cell walls in yeast–rod dimorphism. To understand further the role of the structure of polysaccharides in morphological change, it is important to determine whether amino acids control the polysaccharide synthesis and the morphological change of *Candida krusei* or not.

The purpose of the present study was to compare the chemical composition and the structure of cell wall polysaccharides obtained from Y- and R-form cells of *C. krusei* that was grown in synthetic media containing lysine or alanine as a nitrogen source.

**MATERIALS AND METHODS**

Organism and culture methods. *Candida krusei* strain Y-13 was maintained in a refrigerator on a slant of Koji-agar medium. After precultivation at 30°C for 48 hr in a test tube containing 5 ml of a synthetic medium that contained casamino acid (4 mg/ml) as a nitrogen source, the cells obtained were washed and transferred, with an inoculum size of 10^5 cells per ml, to 100 ml of the medium in 500-ml conical flasks. Cultivation was done at 30°C on a rotary shaker (220 rpm, 6-cm amplitude) in a synthetic medium of the following composition in one liter of distilled water: glucose 10 g, NaH₂PO₄·2H₂O 0.35 g, MgSO₄·7H₂O 0.19 g, KCl 0.11 g, FeSO₄·7H₂O 0.15 mg, ZnSO₄·7H₂O 0.4 mg, CuSO₄·4H₂O 0.025 mg, thiamine 1.0 mg.
Enzymic hydrolysis and acetolysis of mannan preparations. 1.0 mg, Ca-pantothenate 1.0 mg, and biotin 1.0 mg. Lysine (2.61 mg/ml) or alanine (2.55 mg/ml) was added to the medium as a nitrogen source.

Photomicroscopic observation. C. krusei cells, prepared as wet mounts, were observed with a Nikon SUR-type optical microscope and photographs were taken with a Nikon EFM-type photographic attachment.

Preparation of glucan and mannan. The preparation and fractionation of cell walls of C. krusei were carried out as described previously.8 Briefly, cell walls were prepared by extraction with 1 N KOH for 30 min at 100°C and then with 2 N H2SO4 for 20 min at 100°C. The insoluble residue obtained with the above treatment was called alkali-insoluble glucan. The large amount of precipitate from the supernatant, that appeared after dialysis, was collected by centrifugation, washed with water and dried: this material was called alkali-soluble glucan. After removal of the alkali-soluble glucan from the alkali extract, mannan was precipitated as a Cu-complex with Fehling’s solution from the residual solution.

Enzyme preparation. Exo-α-1,2-mannosidase and endo-β-1,6-glucanase were prepared from Acinetobacter sp. grown in the salt-medium with baker’s yeast as a carbon source. These enzymes were purified as described by Katohda et al.8,9 The mannosidase, that split α-1,2-mannosyl-linkages from the non-reducing terminal site, was active on Saccharomyces cerevisiae mannan and on some oligosaccharides produced on acetylation of the yeast mannan. The glucanase hydrolyzed pustulan which is known to contain mainly α,6-glucanase. The mannosidase resistant residues from the non-reducing terminal site, was active on Saccharomyces cerevisiae mannan and on some oligosaccharides produced on acetylation of the yeast mannan. The glucanase hydrolyzed pustulan which is known to contain mainly β-1,6-glycosidic linkages, giving a series of gentio-oligosaccharides and glucose. Gentiotriose and gentiotetraose were hydrolyzed by this enzyme yielding glucose and gentiobiose, and glucose, gentiobiose and gentiotriose, respectively. Gentiobiose was not hydrolyzed.

Enzymic hydrolysis and acetolysis of mannan preparations. A 40-ml reaction mixture containing 50 mg mannan and 0.1 mg enzyme preparation in 0.017 M phosphate buffer (pH 7.0) was incubated at 30°C. The digestion mixture after 60 hr-hydrolysis was applied to a Bio-gel P2 column (2 x 200 cm). The mannosidase resistant residues appeared in the void volume region. Acetolysis was carried out as described previously.8

Enzymic hydrolysis of glucan preparations. Enzyme solution was added in 0.017 M phosphate buffer (pH 5.5) so that, in all cases, the final concentration of glucan was 1.25 mg/ml and that of the enzyme was 42.5 μg/ml. All suspensions were made to 0.01% (w/v) with respect to sodium azide as a precaution against microbial contamination. Suspensions were incubated at 30°C with slow rotation. When required, solubilized carbohydrate was determined with DNPA10 (reducing sugar) and phenolsulfuric acid reagent11 (total sugar) using glucose as a standard. For methylation analysis of alkali-soluble and alkali-insoluble glucan after degradation with β-1,6-glucanase, suspensions were centrifuged (10,000 rpm, 15 min). The insoluble product was repeatedly washed with water followed by centrifugation. The samples were subjected analysis after lyophilization.

Methylation analysis. Polysaccharides were methylated by the Hakomori method.12 The methyl-sulphinyl sodium reagent was prepared according to the procedure of Sandford and Conrad.13 Fully methylated polysaccharides were obtained after four cycles of methylation. The methylated glucan was lyophilized for subsequent hydrolysis, or remethylation, if necessary. The materials were treated with 2 N methanol-hydrochloric acid at 100°C for 16 hr. The methylated monosaccharides were converted into their trimethyl silylates for analysis by GLC using a Hitachi gas chromatograph. Model 063, fitted with a stainless steel column (3000 x 4 mm) packed with 5% OV-17 on Chromosorb W AW (60 ~ 80 mesh). The column temperature, programmed from 150 to 200°C, was raised at a rate of 2°C per min. Nitrogen gas was used as carrier gas and the flow rate was 30 ml per min.

Analytical methods. The determinations of glucose and mannose contents were done by gas chromatography after methanolysis of the neutralized extract. Methanolysis and gas chromatographic determination were described under Methylation analysis. Glucosamine was determined by the modified Elson-Morgan procedure of Boas.14 After cell walls were hydrolyzed with 4 N HCl at 110°C for 16 hr, the resulting hydrolyzates were diluted, passed through a column (0.8 x 5 cm) of Dowex 50 (x 8, H form, 200 ~ 400 mesh) and the retained substance containing glucosamine was eluted with 2 N HCl after washing with deionized water.

RESULTS

Photomicroscopy

Candida krusei cells showed various morphological changes from yeast-like to pseudohyphae in the synthetic medium containing casamino acid as a nitrogen source. When stationary phase-cells (48 hr-culture) in casamino acid were transferred to fresh synthetic medium containing lysine as a nitrogen source and grown aerobically for 36 hr, almost all of the cells were the Y-form (Fig. 1-A). In contrast, when transferred to fresh synthetic medium containing alanine as a nitrogen source, R-form cells were yielded after 32 hr-
incubation (Fig. 1-B). The ratio of the long axis to the short axis of 200 cells was measured for each preparation. The mean value was 1.8 for the lysine cell preparation and 3.0 for the alanine cell preparation.

**Quantitative analysis of cell walls**

The compositions of cell walls obtained from the Y-form and the R-form are shown in Table I. Both the cell wall samples contained about 35% of glucose. But the R-form cell wall contained a higher amount of mannose than the Y-form cell wall did. In spite of these results, the contents of mannan, alkali-soluble and alkali-insoluble glucan were much higher

### Table I. Cell Wall Composition and Contents of Yeast- and Rod-Forms

<table>
<thead>
<tr>
<th>Composition and yield (mg/100 mg cell wall)</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Y-form</td>
</tr>
<tr>
<td>Glucose</td>
<td>35.0±4.0</td>
</tr>
<tr>
<td>Mannose</td>
<td>36.0±3.0</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>2.00±0.34</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>1.5±0.4</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>0.75±0.15</td>
</tr>
<tr>
<td>Yield</td>
<td></td>
</tr>
<tr>
<td>Mannan</td>
<td>14.0±2.0</td>
</tr>
<tr>
<td>Alkali-soluble glucan</td>
<td>9.4±1.3</td>
</tr>
<tr>
<td>Alkali-insoluble glucan</td>
<td>11.4±1.3</td>
</tr>
</tbody>
</table>

### Table II. Hexosamine Contents and Recovery of Each Fraction from Yeast- and Rod-Form Cell Walls

Cell wall samples were prepared and fractionated as described in the text. Fractionation was conducted with 1000 mg of cell wall.

<table>
<thead>
<tr>
<th></th>
<th>Y-form</th>
<th></th>
<th>R-form</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield</td>
<td>Hexosamine</td>
<td></td>
<td>Yield</td>
</tr>
<tr>
<td></td>
<td>(mg)</td>
<td>(μg/mg dry wt.)</td>
<td>(%)</td>
<td>(mg)</td>
</tr>
<tr>
<td>Whole cell wall</td>
<td>1000</td>
<td>22</td>
<td>100</td>
<td>1000</td>
</tr>
<tr>
<td>Alkali-soluble glucan</td>
<td>91</td>
<td>16</td>
<td>6.6</td>
<td>82</td>
</tr>
<tr>
<td>Alkali-insoluble glucan</td>
<td>102</td>
<td>98</td>
<td>45.2</td>
<td>92</td>
</tr>
</tbody>
</table>
in the Y-form wall than in the R-form wall. The Y-form wall contained a greater quantity of nitrogen and hexosamine than the R-form wall did, whereas there was no difference in the contents of phosphorus. Hexosamine contents and recovery of each fraction are given in Table II. The results indicated that the extraction procedure caused about 45 to 50% liberation of hexosamine contents of whole cell walls. The insoluble glucan of the Y-form recovered contained a greater quantity of hexosamine than that of the R-form did.

Properties of mannan

Mannan contents have usually been determined after recovery from alkaline extracts as a Cu-mannan complex precipitated with Fehling's solution (Table I). For studying the properties, mannan was prepared as described by Peat et al.15) As compared with the gel filtration pattern of mannan present in the cell walls of the Y-form and R-form, each mannan consisted of polysaccharides of heterogeneous molecular size, as judged from the broad distribution of the elution profile on a Sepharose 2B column (Fig. 2-A). Acetolysis products of each mannan were subjected to gel filtration on a Bio-gel P2 column. The elution profiles are shown in Fig. 2-B. Y-form and R-form mannan exhibited similar patterns for pentasaccharide components in addition to the mono-, di-, tri- and tetrasaccharides. Acetolysis products of α-1,2-mannosidase resistant residues were placed on a Bio-gel P2 column (Fig. 2-C). As compared with the acetolysis pattern of the original mannan (Fig. 2-B), the residues after enzyme digestion indicated that each mannan was degraded into resistant residues which contained mainly tetrasaccharide components. The results indicated that the enzyme digestion led to a large decrease in the amount of mannobiose side chains and a small amount of mannotriose side chains.

Action of β-1,6-glucanase on glucan preparations

In order to confirm the properties of the alkali-soluble and alkali-insoluble glucan, β-1,6-glucanase hydrolysis was carried out. Figure 3 shows the action of the enzyme on each glucan. Very little degradation of each soluble glucan was observed with the enzyme system when hydrolysis was monitored by only the release of reducing power. However, the solubilization of total carbohydrate was much higher, suggesting that large molecules were mostly released during enzymic degradation. The total carbohydrate solubilization was much higher in the R-form, while reducing power was much higher in the Y-form (Fig. 3-
Yeast-Rod Dimorphism of Candida krusei

Fig. 3. The Action of β-1,6-Glucanase on Glucans.

The reaction was carried out as described in the text. Release of total sugar and reducing sugar was expressed as percentage of total carbohydrate. A: alkali-soluble glucan. B: alkali-insoluble glucan. ○, Y-form; ●, R-form; ---, total sugar contents determined with phenol-sulfuric acid reagent; ----, reducing sugar contents determined with DNPA reagent.

![Graph showing the action of β-1,6-Glucanase on glucans](image)

- **Fig. 4.** Bio-gel P2 Column Chromatography of the Hydrolyzates of Alkali-soluble Glucan by the Action of β-1,6-Glucanase.

The hydrolyzates (10 ml) shown in Fig. 3 were placed on a column (2 x 200 cm) and eluted with water at the flow rate of 4 ml per hr. Two ml-fractions were collected. ---, Y-form; ---, R-form; F-1, fractions 60 ~ 70. Vo, void volume; G1, glucose; G2 to G4, gentiobiose to gentiotetraose.

The alkali-insoluble glucan was hydrolyzed only a little by the enzyme; soluble carbohydrate formation was 2~3% of the substrate (Fig. 3-B).

The soluble products hydrolyzed from alkali-soluble glucan were concentrated and fractionated by chromatography on a Bio-gel P2 column (Fig. 4). Analysis by paper chromatography of the carbohydrates released from each soluble glucan after 48 hr-digestion by the enzyme showed glucose, gentiobiose and gentiotetraose. Figure 4 shows that the F-1 fraction (fraction numbers 60 ~ 70), which occupied about 15% of the original glucan, was eluted in the void volume. The F-1 fraction from the Y-form or R-form was considered to be polysaccharide from its properties, and acid hydrolysis gave mostly (80 ~ 90%) mannose with a small amount of glucose. The soluble products hydrolyzed from alkali-insoluble glucan were polysaccharide, and acid hydrolysis gave only glucose.

**Methylation analysis**

Glucan preparations were fully methylated the Hakomori procedure. The alkali-soluble glucan of the Y-form and R-form contained mainly 1,3-glycosidic linkage with branching occasionally at C-6, and a small amount of 1,6-linkage (Table III).

On β-1,6-glucanase hydrolysis, alkali-soluble glucan (50 mg) of the Y-form yielded 35 mg and the R-form yielded 30 mg of an insoluble fraction, suggesting that alkali-soluble glucan was composed predominantly of 1,3-linked glucan. Approximately 50% of 1,6-linkages were resistant to the enzymic hydrolysis (Table III). These occur probably in β-1,6-disaccharide units of main chains of β-1,3-linkages. There was no significant difference in structure of β-1,6-glucanase resistant fractions.

The alkali-insoluble glucan of the Y-form and R-form contains 1,3-linkages having a few branches at the C-6 position, and no 1,6-linked residues. The insoluble glucan obtained after β-1,6-glucanase hydrolysis was subjected to methylation analysis (Table III). A large percentage of the residues were still tri-substituted...
### Table III. Methanolysis Products of Methylated Glucan

<table>
<thead>
<tr>
<th>Methyl glucose</th>
<th>Molar ratios as glucose</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkali-soluble glucan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,4,6-Tetra-</td>
<td>7.0</td>
<td>6.6</td>
<td>6.6</td>
<td>5.1</td>
<td>3.1</td>
<td>2.0</td>
<td>4.3</td>
<td>2.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2,3,4-Tri-</td>
<td>14.0</td>
<td>7.3</td>
<td>17.6</td>
<td>8.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>93.6</td>
<td>92.5</td>
<td>91.9</td>
<td>92.7</td>
<td>91.9</td>
<td>92.7</td>
<td>91.9</td>
<td>92.7</td>
</tr>
<tr>
<td>2,4,6-Tri-</td>
<td>74.0</td>
<td>79.5</td>
<td>70.5</td>
<td>81.0</td>
<td>3.4</td>
<td>5.5</td>
<td>3.8</td>
<td>4.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2,4-Di-</td>
<td>5.0</td>
<td>6.6</td>
<td>5.3</td>
<td>5.1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>93.6</td>
<td>92.5</td>
<td>91.9</td>
<td>92.7</td>
<td>91.9</td>
<td>92.7</td>
<td>91.9</td>
<td>92.7</td>
</tr>
</tbody>
</table>

a, untreated preparation; b, insoluble polysaccharide obtained after β-1,6-glucanase degradation.

**DISCUSSION**

Comparison of the composition of the cell wall in the Y-form and R-form of *Candida krusei* showed quantitative variations in contents of glucose, mannose total nitrogen and hexosamine. But, during preparation of cell wall fractions, the extraction procedure caused partial degradation and low recovery of carbohydrates. Especially, the recovery of mannann from the R-form was lower than that of the Y-form as compared with the values calculated from the mannose contents of the cell walls.

Earlier works\(^{16-18}\) showed that acetolysis is a valuable procedure for structural analyses of yeast mannann. In the present experiment on enzyme digestion, mannobiose produced on the acetolysis of mannann followed by deacetylation was hydrolyzed almost completely by α-1,2-mannosidase. The enzyme hydrolyzed mannotriose weakly. Mannotetraose and mannopentaose were not attacked by the enzyme.

The structures of β-1,3- and β-1,6-glucan of *S. cerevisiae* were elucidated in earlier studies\(^{19,20}\) and a subsequent study\(^{21}\) showed that the enzyme degradation method is useful for the elucidation of the structure of yeast glucans. The alkali-soluble glucan of *C. krusei* was extensively degraded by the endo-β-1,6-glucanase with liberation of gentio-oligosaccharides and glucose. The enzyme also released a soluble polysaccharide fraction (F-1, Fig. 4) which contained predominantly mannose with a minor amount of glucose. It is not clear from the data whether this polysaccharide is a covalent-linked glucomannan or not, since further fractionation was not carried out. The action of β-1,6-glucanase and methylation analysis on the alkali-soluble glucan showed that the contents of 1,6-linked residues were much higher in the R-form, while that of 1,3-linked residues was much higher in the Y-form. The polysaccharide of the Y-form resistant to the action of β-1,6-glucanase was shown by methylation analysis to be similar to that of the R-form. In contrast, methylation analysis of the insoluble glucan gave 2,3,4,6-tetra-, 2,4,6-tri- and 2,4-di-O-methylglucose in the molar proportions of 1 : 20 ~ 30 : 1. 2,3,4-Tri-O-methylglucose was not detected in the Y-form or the R-form. The results show that they contain a major proportion of β-1,3-linkage and minor proportion of branched linkage at the C-6 position, agreeing with the results of Manners et al.\(^{19}\) for *S. cerevisiae*-glucan.

Yamaguchi\(^4\) found that the mycelial phase wall of *C. albicans* contains greater quantities of alkali-insoluble glucan but small quantities of alkali-soluble glucan and mannann. On the other hand, Chattaway et al.\(^3\) demonstrated that the mycerial-form cell contained three times as much chitin as the yeast-form cell. The present data show that the Y-form wall of...
C. krusei has greater quantities of these polysaccharides than the R-form wall has. A comparable supply of lysine or alanine as a nitrogen source has been shown to affect morphogenesis and synthesis of the polysaccharides. The wall cultured with lysine contained a higher amount of alkaline and acid-resistant polysaccharides than the wall cultured with alanine.

Sietsma and Wessels reported the proposed model for the structure of the glucan-chitin complex of Shizophyllum commune, in which lysine, glutamic acid, citrulline and N-acetylglucosamine were involved in the linkages between glucan and chitin. Their results provided evidence that the covalent linkages between β-glucan and chitin are indicated by a characteristic change in solubility of the glucan when chitin is specifically removed by first deacetylating the chitin with alkali followed by depolymerization of the deacetylated chitin with nitrous acid. A comparative study of structure of the glucan-chitin complex in the two forms of C. krusei will be the subject of further work.

Acknowledgments. The authors express their sincere thanks to Mr. Hiroaki Hosokawa and Mr. Shigeru Kanaya, and to Miss Kazuko Koto and Miss Mikiko Kondo for their technical assistance.

REFERENCES
13) A. Sandford and H. E. Conrad, Biochemistry, 5, 1508 (1966).