**Kluyveromyces bulgaricus** Yeast Lectins

ISOLATION OF TWO GALACTOSE-SPECIFIC LECTIN FORMS FROM THE YEAST CELL WALL*

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Incubation of galactose-treated *Kluyveromyces bulgaricus* yeast cells in EDTA/phosphate-buffered saline led to an extract possessing hemagglutinating and yeast flocculating properties. Purification of this extract by affinity chromatography and gel filtration gave two lectin forms, Kb-CWL I and Kb-CWL II, with an apparent molecular mass of 38,000 and 150,000 Da, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that Kb-CWL I and Kb-CWL II were dimeric and octameric of a subunit of 18,900 Da. At high concentration, purified Kb-CWL I associated to give Kb-CWL II. This association seemed to be independent on pH. The two lectin forms were glycoproteins, the peptide counterpart was very rich in Lys, Glu, and Gly, and the carbohydrate part represented 1% of the whole molecule and was composed of Glc, Man, and Ara. The two lectin forms (KB-CWL I and Kb-CWL II) agglutinated human red blood cells and flocculated EDTA-treated *K. bulgaricus* yeast cells. The activity of both lectin forms required Ca²⁺ ions, while Sr²⁺ showed some competitive inhibition. Overall activity was obtained within a pH range of 4–6.5 for both forms. Temperatures of 80–90 °C for 20 min, or proteolytic treatment reduced irreversibly the activity of Kb-CWL I and Kb-CWL II. The role of the cell wall phosphopentrudamman as a ligand and a potential physiological receptor of these lectin forms was demonstrated.

Flocculation and sexual agglutination are the two types of specific cell-cell recognition phenomena reported in yeasts (1). The isolation of sexual agglutinins from many yeast species led to a better understanding of the mechanism of the sexual agglutination phenomenon (2–5). The sexual agglutinins were characterized by their covalent linkages to the cell wall, their monovalency, and their hapten (monosaccharides) attached cells when they were liberated from the cell wall, therefore their activity was always evidenced by an agglutination inhibition test in which cellular receptors of one mating type were saturated with the isolated sexual agglutinin of the other mating type before cellular contact of the two mating types (6, 7).

Although flocculation was shown to be a specific cell-cell recognition phenomenon, since it is hapten-specific, it's mechanism until now is poorly understood because of nonveined yeast cell flocculating factors. Hypothesis of lectin-carbohydrate interaction based on the deflocculating effect of sugars was described by many workers (8–11). This lectin-carbohydrate interaction was presumed to occur between the hypothetic lectin factor and its presumed receptor, i.e. the cell wall peptidomannan. Although this wall heteropolymer has been the subject of many chemical analyses, showing different structural modifications between the flocculent and the non-flocculent molecule, it's role in the flocculation phenomenon has not yet been directly demonstrated.

In previous studies (12, 13) we reported the isolation of a galactose-specific lectin from the cultures broth of the yeast *Kluyveromyces bulgaricus*. This lectin was excreted by the cells when this yeast was cultivated aerobically in a calcium-enriched medium. It's participation in the flocculation of this yeast was established (12, 13), although chemical analysis failed to demonstrate the presence of galactose residues in cell walls of this yeast (14). The aim of the present study was (i) to isolate and to determine the molecular form of a cell wall linked lectin, and (ii) to demonstrate the role of phosphopentamanns as the receptors of this lectin.

MATERIALS AND METHODS

Reagents—all chemicals were obtained from Sigma unless otherwise noted.

Microorganisms and Culture Conditions—The flocculent yeast *K. bulgaricus* was grown in a bacto-peptone-glucose liquid medium as previously described (13). Saccharomyces cerevisiae X2180 was grown aerobically in a glucose, yeast extract, and ammonium sulfate medium.

Extraction of a Galactose-Specific Lectin from the Cell—The cells were harvested at 4 °C by centrifugation at 5,000 × g, for 10 min, and treated with Gal, as previously reported (13). Cells obtained hereby were washed extensively with 0.01 M phosphate buffer (pH 7, 3 mM NaH₂PO₄) and suspended at a concentration of 4% (w/v) in the same buffer containing 5 mM EDTA (Prolabo). Cell suspension was incubated at 37 °C for 60 min under moderate agitation. Supernatant was collected by centrifugation, and cells were tested in Helm's buffer for residual flocculating activity (13). Supernatant was dialyzed at 4 °C for 48 h against distilled water (4 × 2.5 liters) and then lyophilized.

Preparation of Affinity Support—Unless otherwise noted, the cells of *K. bulgaricus* treated as described above were included in a non-denaturing 7.5% polyacrylamide gel preparation (15). Before the polymerization step, cells were introduced as 10% w/v suspension in distilled water in the place of water, then polymerization was realized in the presence of crosslinking agent N,N,N',N'-tetramethylethylenediamine. The gel containing the immobilized cells was cut into small pieces, extensively washed with Helm's buffer, pH 4.5, and stored at 4 °C in the same buffer containing 3 mM NaCl. A column of Sepharose 4B was also used for affinity chromatography.

Purification of the Gal-specific Lectin—20 mg of lyophilized extract were solubilized in 50 ml of Helm's buffer and applied onto an affinity chromatography column (20 × 1.5 cm; flow rate, 0.2 ml/min) of immobilized yeast cells equilibrated with Helm's buffer. After washing with 200 ml of Helm's buffer, elution was started with the same buffer supplemented with either 10 mM EDTA or 0.2 M galactose. The protein content of the eluate was monitored at 280 nm. EDTA

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or galactose eluted fractions (4 ml/fraction) were dialyzed separately against Hel'm's buffer and titrated. The dialyzed fractions were lyopholized.

Oxidase Activity Determination—Glycosylhydrolyase activities were examined either by using p-nitrophenyl-D-glucosides as substrates according to Conchie (16) or N-acetyl-β-D-glucosamine 1-phosphate. D-GlcNAc and N-acetyl-β-D-glucosamine 1-phosphate were separated by descending paper chromatography on Whatman 3 filter paper, using a mixture of ethyl acetate/pyridine/water (5:3:2, v/v) as solvent. D-GlcNAc was detected with an alkaline silver nitrate reagent (17).

Extraction of Phosphopeptidomannans—The extraction of the phosphopeptidomannans from the flocculent and nonflocculent yeast K. bulgaricus was performed by autoclaving in a neutral citrate buffer (18). Crude extracts were chromatographed onto a column (85 × 2.5 cm; flow rate, 0.16 ml/min) of Tris-acryl GF-2000 (IBF-Reactifs, Villeneuve la Garenne, France) equilibrated with a 3 mM NaN3 solution. Molecular mass estimations and chemical compositions of these molecules were the same as already reported (14). Phosphopeptidomannan of the yeast S. cerevisiae X2180 was also obtained by autoclaving the cells in a neutral citrate buffer (18). The crude extract was fractionated using cetyltrimethylammoniobromide (19), fraction II corresponding to the PPM1 was used.

Preparation of RBC Bearing Yeast PPM—Human (A, B, or O) or rabbit RBC were trypsinized and treated with glutaraldehyde (20). Before neutralization of the nonreacted aldehyde groups present on RBC surfaces, RBC were incubated at room temperature (22°C) for 1 h as a suspension of 1% v/v in phosphate-buffered saline containing 0.1% PPM, RBC were then washed three times with PBS, and nonreacted aldehyde groups were neutralized prior to glycine treatment (20).

pH and Temperature Stability—The pH range over which the lectins (Kb-CWL I and Kb-CWL II) exhibit optimal binding to human RBC and to K. bulgaricus cells were examined as described (21) by titrating Kb-CWL I and Kb-CWL II samples (25 μg/ml) in the following buffers; 0.01 M sodium acetate/ acetic acid (pH 3.7–5.4), 0.01 M sodium cacodylate/HCl (pH 6.0–7.0), 0.01 M Tris/HCl (pH 7.0–8.8), 0.01 M glycine/NaOH (pH 9.0–10.6). All buffers were made in saline/CaCl2 and test cells were RBC and K. bulgaricus yeast cells.

The temperature stability of Kb-CWL I and Kb-CWL II was estimated by incubation of 300 μl of the lectins in TBS (25 μg/ml) at different temperatures for 20 min, cooling them on ice, and titrating them with rabbit RBC and K. bulgaricus yeast cells.

Divalent Cation Requirements—To examine the divalent cation requirements of Kb-CWL I and Kb-CWL II, the agglutination tests were performed as described above, but using TBS without CaCl2 and in the presence of 2 mM EDTA. Flocculation tests were performed in 0.15 M sodium acetate/acetic acid without CaCl2 and in the presence of 20 mM EDTA. Another series of tests was performed by adding EDTA (20 mM in TBS or in Hel'm's buffer) to the agglutinated cells and monitoring the reversal of agglutination.

Hemagglutination and Flocculation Inhibition Tests—Kb-CWL I and Kb-CWL II were dissolved in TBS or in Hel'm's buffer at concentrations of 40 and 160 μg/ml, respectively. All carbohydrates to be tested for their inhibitory effect were dissolved in TBS or in Hel'm's buffer (at concentration up to 200 mM for mono- and oligosaccharides and 10 mg/ml for polysaccharides and glycoproteins as inhibitors). Serial 2-fold dilutions (50 μl) of the purified lectins were placed in the wells and equal volumes of inhibitor (50 μl) at a certain concentration were added. Mixtures were incubated for 60 min and 50 μl of the RBC suspension (107 RBC/ml) were added to each well. Controls were the substitutions of the inhibitor solutions and of the purified lectins by TBS.

General Analytical Methods—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed under reducing condition (15 mM mercaptoethanol) on a 8-cm long slab gel containing 9% acrylamide as described by Laemmli (22). Gel filtration was performed on a column (97 × 1.5 cm; flow rate, 3.4 ml/min) of Sephadex G150 equilibrated with the Hel'm's buffer. Proteins were estimated by the method of Lowry (23), using crystallin bovine serum albumin as standard, or by their absorbance at 280 nm. Amino acids were determined after 5.6 M HCl hydrolysis under vacuum at 105°C during
TABLE I

| Crude extract | Galactose-eluted fraction | EDTA-eluted fraction |
|---------------|---------------------------|----------------------|
| Q*            | Kb. cells*                | Sepharose 4B         |
| Q             | s.a.                      | Q                    |
| s.a.          | Q                         | s.a.                 |
| 20            | 2                         | 1.1                  |
| 4200          | 1.8                       | 4100                 |
| 1.2           | 4400                      | 2.1                  |
| 4000          | s.a.                      | s.a.                 |

* Purification onto a column of immobilized K. bulgaricus yeast cells.
* Q, quantity of product in mg; s.a., specific activity expressed as K. bulgaricus yeast agglutinating unit/mg of product.

FIG. 3. Gel filtration profile of the affinity chromatography purified fraction on a column of Sephadex G-150 equilibrated with Helm's buffer. Column calibration was realized using a mixture composed of dextran blue (2,000,000 Da), bovine albumin (dimer 132,000 Da), bovine albumin (86,000 Da), egg albumin (45,000 Da), bovine carbonic anhydrase (29,000 Da), a-lactalbumin (14,200 Da).

12 h, using a Technicon NC-2P autoanalyzer. Carbohydrates were determined by the phenol-sulfuric acid method (24) after 2 M HCl hydrolysis under vacuum at 105 °C during 2 h using a mixture of arabinose, glucose, and mannose (20, 40, and 40%, respectively, w/v) as standard. Identification of monosaccharides was performed by descending paper chromatography (17) and as aldito acetate derivatives by gas-liquid chromatography at 210 °C with a 180-cm-long column of 35% Sp2340 on chromosorb WAW-DMCS (100–120 mesh) as described elsewhere (25). A more rigorous identification of the carbohydrate derivatives was performed by gas-liquid chromatography coupled to mass spectrometry using alditol acetate derivatives, in a CPG-SM Carbo Erba 4160-Riber Mag R-10-10C, assisted by a Sider 111A informatic unit. Hexosamines were determined by the reagent of Elson Morgan following the procedure described by Ghuy-

FIG. 4. Polyacrylamide gel electrophoresis of Kb-CWL I and Kb-CWII. in the presence of sodium dodecyl sulfate, with 9% acrylamide. Lane 1 was used for cytochrome c from horse heart. Lanes 2 and 3 contained Kb-CWL I. Lanes 4 and 5 contained Kb-CWII. Lane 6 contained standard proteins (E. coli b-galactosidase, rabbit muscle phosphorylase B, bovine albumin, egg albumin, carbonic anhydrase).

12 h, using a Technicon NC-2P autoanalyzer. Carbohydrates were determined by the phenol-sulfuric acid method (24) after 2 M HCl hydrolysis under vacuum at 105 °C during 2 h using a mixture of arabinose, glucose, and mannose (20, 40, and 40%, respectively, w/v) as standard. Identification of monosaccharides was performed by descending paper chromatography (17) and as aldito acetate derivatives by gas-liquid chromatography at 210 °C with a 180-cm-long column of 35% Sp2340 on chromosorb WAW-DMCS (100–120 mesh) as described elsewhere (25). A more rigorous identification of the carbohydrate derivatives was performed by gas-liquid chromatography coupled to mass spectrometry using alditol acetate derivatives, in a CPG-SM Carbo Erba 4160-Riber Mag R-10-10C, assisted by a Sider 111A informatic unit. Hexosamines were determined by the reagent of Elson Morgan following the procedure described by Ghuy-

sen et al. (26), and by ion exchange chromatography using a Technicon NC-2P autoanalyzer.

RESULTS

Extraction and Purification of a Galactose-specific Lectin from the Cells—The flocculent yeast K. bulgaricus excreted Gal and GlcNAc-specific lectins in the culture medium (12, 13). These two lectins were also obtainable from whole cells by incubation with a 0.2 M Gal solution, in Helm's buffer, pH 4.5, in the presence of CaCl2. This treatment induced a dispersion of the flocs, but the cells, after washing in Helm's buffer showed a residual flocculation activity. Treatment of these yeast cells with 5 mM EDTA in 0.01 M phosphate buffer, pH 7.4, and centrifugation described under "Materials and Methods" led to a crude extract which after extensive dialysis showed hemagglutinating activity. This crude extract was chromatographed on the immobilized K. bulgaricus yeast cells in polyacrylamide gel column under the conditions described above. The elution profiles are presented in Fig. 1, A and B. Chromatography of the crude extract on a Sepharose 4B column gave the same results but with a better output (Fig. 2, A and B). (Table I).

Purity and Molecular Mass Estimations—These were examined by both gel filtration and polyacrylamide (9% acrylamide) gel electrophoresis at pH 8.3. Gel filtration on Sephadex G-150 column of the purified fractions led to two products Kb-CWL I and Kb-CWII with an apparent molecular mass of 38,000 Da and 150,000 Da, respectively (Fig. 3). Both products exhibited hemagglutinating and yeast flocculating activities. By polyacrylamide (9% acrylamide) gel electrophoresis, molecular masses of the subunits were estimated from semilog plots of molecular masses versus mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions (Fig. 4). Results were the same for Kb-CWL I and Kb-CWII, the same band observed for the twofractions had a molecular mass estimated to 18,900 Da.

Chemical analysis of Kb-CWL I and Kb-CWII showed that they were glycoproteins (Table II). The peptide part of Kb-CWL I was very similar to that of Kb-CWII. The two lectins were very rich in Lys, Glu, and Gly (Table III). The carbohydrate counterpart of both products represented about 1% of the whole molecule. It was composed of Glc, Man, and a third compound showing, after transformation in alditol acetate, the same retention time as arabinose. Mass spectrum fragmentation of this compound corresponded also to arabin-itol penta acetate. These three monosaccharides Ara, Glc, and Man were present in a molar ratio of 1:2:2 (Table II).

Osidase Activity Determinations—The following sugar derivatives, in the pyranoside structure, were tested in triplicate either in citrate buffer at pH 3.8 or in Helm's buffer at pH 4.5: p-nitrophenyl-a-D-mannoside, p-nitrophenyl-b-D-mannoside, p-nitrophenyl-a-D-galactoside, p-nitrophenyl-b-D-galactoside, p-nitrophenyl-a-D-glucoside, p-nitrophenyl-b-D-glucoside, p-nitrophenyl-N-acetyl-b-D-glucosamine, p-nitrope-
The activity could be detected with either Kb-CWL I or Kb-CWL II on the cell surface was high phosphorylated PPM (14). Low phosphorylated PPM was deficient medium as described (14). Main structure on the cell surface was high phosphorylated PPM (14). Amino acid composition of Kb-CWL I and Kb-CWL II

| Amino acid | Kb-CWL I | Kb-CWL II |
|------------|----------|----------|
| Asp        | 5.16     | 4.98     |
| Thr        | 1.61     | 1.76     |
| Ser        | 4.98     | 5.11     |
| Glu        | 13.25    | 12.88    |
| Pro        | 0.70     | 0.65     |
| Gln        | 21.32    | 21.66    |
| Ala        | 2.99     | 2.73     |
| Cys        | 0.19     | 0.2      |
| Val        | 1.97     | 1.85     |
| Met        | 0.21     | 0.18     |
| Ile        | 1.32     | 1.82     |
| Leu        | 1.24     | 1.35     |
| Tyr        | 1.25     | 1.18     |
| Phe        | 2.50     | 2.77     |
| Lys        | 29.75    | 30.12    |
| His        | 5.08     | 5.6      |
| Arg        | 6.42     | 6.01     |

Hemagglutinating and yeast flocculating properties of Kb-CWL I and Kb-CWL II

| Cells              | Kb-CWL I titers | Kb-CWL II titers |
|--------------------|-----------------|-----------------|
| K. bulgaricus (flocculent cells) a | 2048 | 2048 |
| K. bulgaricus (nonflocculent cells) b | 256 | 512 |
| S. cerevisiae c | 4 | 2 |
| Rabbit RBC        | 64 | 128 |
| Rabbit RBC (trypsinated) | 256 | 256 |
| Sheep RBC        | 4 | 8 |
| Sheep RBC (trypsinated) | 8 | 8 |
| Human RBC A      | 32 | 64 |
| Human RBC B      | 64 | 64 |
| Human RBC O      | 128 | 256 |
| Human RBC A (trypsinated) | 64 | 64 |
| Human RBC B (trypsinated) | 128 | 128 |
| Human RBC O (trypsinated) | 256 | 256 |

Molecular masses and chemical analysis of the purified Kb-CWL I and Kb-CWL II

| Molecular masses | Proteins | Carbohydrate | Hexosamine | ARA/Glc/Man molar ratio |
|------------------|----------|--------------|-------------|-------------------------|
| Da               |          |              |             |                         |
| Kb-CWL I         | 38,000 a 18,900 b | 867 | 10.4 | Trace | 1/2/2 |
| Kb-CWL II        | 150,000 a 18,900 b | 873 | 10.5 | Trace | 1/2/2 |

* Molecular masses estimated by gel filtration on a column of Sephadex G-150.
* Subunit molecular masses determined in polyacrylamide-SDS gel electrophoresis.

Isolation of Galactose Lectin Forms from K. bulgaricus Walls

**N**-Acetylchitobioside, S. aureus 1-phosphate, 2,3,4,6-tetra-O-acetyl-D-glucosamine, and 2,3,4,6-tetra-O-acetyl-D-galactosamine were used as PPM bearers. Among Ca++, Mg++, Mn++, Zn++, and Sr++, only the presence of Ca++ was required for the agglutination of Kb-CWL I and Kb-CWL II (Table V). Activities of Kb-CWL I and Kb-CWL II samples stored at room temperature (22 °C) for 2 weeks remained unchanged, while at 4 °C activities remained constant for several months. Incubation at 80–90 °C for 20 min reduced drastically the agglutinating activity of Kb-CWL I and Kb-CWL II. Treatment of Kb-CWL I and Kb-CWL II with pronase or trypsin for 4 h did not affect these molecules with their agglutinating activities. Among Ca++, Mg++, Mn++, Zn++, and Sr++, only the presence of Ca++ was required for the activity of Kb-CWL I and Kb-CWL II (Table V). Addition of EDTA to a 10 mM final concentration reversed agglutination or flocculation of the lectin-RBC or lectin-yeast mixtures. EDTA-lectin preincubated solution showed neither RBC-agglutinating nor yeast-flocculating activities. EDTA-inhibited agglutinating or flocculating activity could be restored by

| Cells (flocculent cells) a | 2048 | 2048 |
| (nonflocculent cells) b | 256 | 512 |
| (flocculent cells) c | 4 | 2 |
| Rabbit RBC (K. bulgaricus flocculent PPM) | 1024 | 2048 |
| Sheep RBC (K. bulgaricus flocculent PPM) | 512 | 512 |
| Sheep RBC (K. bulgaricus non-flocculent PPM) | 512 | 512 |
| Sheep RBC (S. cerevisiae PPM fraction II) | 4 | 2 |
addition of either Ca\(^{2+}\) (CaCl\(_2\) 30 mM) or, surprisingly, of Sr\(^{2+}\) (SrCl\(_2\) 30 mM).

Inhibition of Hemagglutination and Yeast Flocculating Properties of Kb-CWL I and Kb-CWL II—The sugar specificity of the purified lectins was examined by hemagglutination inhibition tests at pH 4.5 and 7, using rabbit erythrocytes, p-nitrophenylglycopyranosides, and sugars giving the strongest inhibitory effect were reported in Table VII. Among the monosaccharide derivatives tested, p-nitrophenylgalactopyranosides exhibited the strongest inhibitory activity, and the inhibitory potency of their \(\alpha\)-anomeric configuration was better than their \(\beta\) one. The presence of a hydrophobic aglycon also increased the inhibitory efficiency. The hydroxy group in position 6 of galactose could be replaced by a H atom without influencing the inhibitory efficiency, since D-fucose was as potent as D-galactose. Sugars listed below were found to be inactive even at concentrations over 100 mM; L-fucose, L-galactose, D- and L-glucose, D- and L-mannose, D-glucosamine, D-mannosamine, \(\alpha\)- and \(\beta\)-glucopyranosides, and p-nitrophenyl, (\(\alpha\) and \(\beta\))-mannopyranoside.

Inhibition studies on yeast flocculation were realized at pH 4.5 and 7. The most efficient sugar derivatives for inhibition of flocculation were presented in Table VII. These inhibitors were similar to those used to inhibit the agglutination of rabbit erythrocytes at pH 4.5 and 7. However, the minimum amount completely inhibiting the agglutination of rabbit erythrocytes was 4-fold higher than that in flocculation inhibition tests. Similarly, p-nitrophenyl-\(\alpha\)-D-galactopyranoside was a more potent inhibitor than the \(\beta\)-anomeric configuration.

### DISCUSSION

We have shown in previous papers (12, 13) that the yeast K. bulgaricus excreted GlcNAc- and Gal-specific lectins which had molecular masses of 61,000 and 65,000 Da, respectively. These lectins could be eluted from the yeast cell surface with 0.2 M galactose solution in Helm's buffer, which led to temporarily deflocculated yeast cells. Since upon washing with Helm's buffer such cells flocculated again, we postulated the presence of other lectins strongly linked to the cell wall. This study shows that yeast K. bulgaricus possesses beside the 65,000 Da Gal-specific lectin, two other Gal-binding lectins, Kb-CWL I and Kb-CWL II, which are not found in the culture medium. The localization of these two last lectins is essentially on the cell surface. The fact that these two lectins are neither detectable in culture medium nor eluable from cell surface by treatment of yeast cells with a galactose solution, attests that these molecules are associated to the cell surface by any mechanism but carbohydrate-lectin interaction. However, these molecules under their cell surface-associated form recognize the carbohydrate moieties of other cells since, before their extraction, yeast cells flocculate upon elimination of galactose by washing with Helm's buffer.

Usually, lectins are composed of subunits (27). The molecular basis of the two lectins of K. bulgaricus seems to be identical, since only one type of subunit is detected for both forms. These results are supported by the results of the chemical analysis of the two lectins. Indeed, the similarity in amino acid composition as well as the identity of the osidic counterpart assign the same subunit structure to both lectins. Moreover, the association of Kb-CWL II at higher concentration can evolve Kb-CWL II.

It is of great interest to note that these molecules contain a glycosidic counterpart different from the major polymers building up the cell wall, since arabinose is absent from the cell wall structure (14). This implies at least that these molecules do not undergo the same glycosylation pathway as glycoproteins building up the cell wall.

These two lectins Kb-CWL I and Kb-CWL II show the same agglutination profiles toward A, B, and O blood groups and rabbit erythrocytes. Higher titers are obtainable with RBC-bearing K. bulgaricus yeast PPM. These results corroborate those already reported (12, 14) and demonstrate that PPM represent a potential physiological receptor on the yeast cell wall surfaces for these lectins. The fact that these lectins cannot agglutinate RBC-bearing PPM (fraction II) of S. cerevisiae, shows at least, that these later are not recognized by the two lectins. Nevertheless, RBC-bearing PPM from flocculent yeast K. bulgaricus provide always higher titers than RBC-bearing PPM from nonflocculent yeast. To our knowledge these results are the first direct demonstration of the role of yeast phosphopeptidomannan in flocculation, at the same time they show that this phenomenon is also governed by the structure of these wall polymers. Indeed, structural differences between PPM from flocculent and nonflocculent yeasts have been already described (14, 10, 29), but the

### Table VI

| Cations | Kb-CWL I | Kb-CWL II |
|---------|---------|---------|
| Ca\(^{2+}\) | 2048 | 2048 |
| Mg\(^{2+}\) | 4 | 4 |
| Mn\(^{2+}\) | 4 | 4 |
| Zn\(^{2+}\) | 4 | 4 |
| Sr\(^{2+}\) | 0 | 0 |
| Ca\(^{2+}\)/Sr\(^{2+}\) | 64 | 32 |

*Ca\(^{2+}\)/Sr\(^{2+}\) was 4 mM Ca\(^{2+}\) and 28 mM Sr\(^{2+}\) in their final concentrations.*

### Table VII

| Compound | Minimum amount (mM) completely inhibiting hemagglutination | Minimum amount (mM) completely inhibiting flocculation |
|----------|---------------------------------------------------------|---------------------------------------------------------|
| D-Fucose | 50                                                      | 12.5                                                    |
| D-Galactose | 50                                                    | 12.5                                                    |
| \(\alpha\)-Nitrophenyl-\(\alpha\)-D-galactoside | 0.8                                               | 0.2                                                    |
| \(\beta\)-Nitrophenyl-\(\beta\)-D-galactoside | 1.2                                               | 0.4                                                    |
| Methyl \(\beta\)-D-galactose | 25.0                                             | 6.2                                                    |
| Lactose (Gal-\(\beta\)-1-4-Glc) | 25.0                                             | 3.1                                                    |
| Melibiose (Gal-\(\alpha\)-1-6-Glc) | 12.5                                             | 1.5                                                    |
| Raffinose | 25.0                                                   | 3.0                                                    |
Isolation of Galactose Lectin Forms from K. bulgaricus Walls

Hereby, we show that the flocculation of the yeast K. bulgaricus implicates two galactose-specific lectins, one (65,000 Da) excreted in the culture medium and another present in the cell wall under multimeric forms of a 18,900-Da subunit. The interrelation between these two lectins is not known. However, their biosynthetic chronology shows some regulation between their biosynthesis, and at the moment we cannot discard the possibility that the presently described lectin (18,900 Da), as well as the previously described one (65,000 Da), are proteolytic products of a large cell wall precursor. Such a case was already evidenced for sexual agglutination factors in S. kluveyri (4). Whatever the case, these results reveal that in the same microorganism, at least two galactose-specific lectins are implicated in its aggregation phenomenon.

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