Kluyveromyces bulgaricus Yeast Lectins

ISOLATION OF N-ACETYLGALACTOSAMINE AND GALACTOSE-SPECIFIC LECTINS: THEIR RELATION WITH FLOCCULATION

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Salman Al-Mahmood, Philippe Giummelly, and Roger Bonaly
From the Laboratoire de Biochimie Microbiologique, Faculté des Sciences Pharmaceutiques et Biologiques, 5, Rue Albert Lebrun, F-54001 Nancy Cedex, France

Francis Delmotte and Michel Monsigny
From the Laboratoire de Biochimie Cellulaire et Moléculaire des Glycoconjugués, Centre de Biophysique Moléculaire, Centre National de la Recherche Scientifique, 1, Rue Haute, F-45071 Orleans Cedex 2, France

Kluyveromyces bulgaricus is a yeast which, upon culture in a calcium-enriched glucose-peptone medium, flocculates. Its flocculation can be reversed by the addition of galactose. In this paper, it is shown that two lectins can be isolated either from the concentrated culture broth or from the supernatant of deflocculated cells suspended in galactose solution.

The N-acetylgalactosamine-specific lectin, at pH 7.4, agglutinates untreated sheep red blood cells, but agglutinates neither untreated rabbit red blood cells nor glutaraldehyde-fixed sheep or rabbit red blood cells. Conversely, at pH 4.5, this lectin agglutinates glutaraldehyde-fixed sheep red blood cells.

The galactose-specific lectin, at pH 7.4, agglutinates both untreated and glutaraldehyde-fixed rabbit red blood cells but does not agglutinate untreated or glutaraldehyde-fixed sheep red blood cells. At pH 4.5, this lectin agglutinates both glutaraldehyde-fixed sheep and rabbit red blood cells and induces flocculation of deflocculated K. bulgaricus cells.

In all cases, the agglutination and the flocculation induced by one of these two lectins were inhibited by free or conjugated N-acetyl-D-glucosamine or by free or conjugated D-galactose, respectively.

No glycosylhydrolase activity could be detected in the purified lectins.

Flocculation of yeast cells can be reverted by addition of specific sugars (9) showing that a sugar binding recognition mechanism takes place in addition to the classical calcium bridge mechanism.

Kluyveromyces bulgaricus yeast cells are known to spontaneously flocculate upon growth. Their aggregates are not dissociated by suspension in Helms’s buffer at pH 4.5 (10), but are easily deflocculated by suspension in the same buffer containing D-galactose (11). On the basis of this observation, we have been motivated to look for the presence of a galactose-binding protein produced by this yeast. The results presented here show (i) that two lectins are not only associated with the walls of K. bulgaricus cells but are also excreted by them, (ii) that these two lectins, which have similar molecular weights and are able to specifically agglutinate red blood cells, act as agglutinins at pH 4.5, and (iii) that the galactose-specific lectin induces the flocculation of K. bulgaricus cells at pH 4.5.

MATERIALS AND METHODS

Mycroorganism and Culture Conditions—The flocculent yeast K. bulgaricus was originally isolated from dairy products. The culture medium contains in 1 liter: 4 g of Bacto-peptone (Difco), 40 g of glucose, 1 g of KH₂PO₄, 0.2 g of MgSO₄-7H₂O, and 0.2 g of CaCl₂-2H₂O (Prolabo, Paris, France). The cells were grown aerobically (air, 40 liters/h), during 24 h, at 25 °C in a 2 liter fermenter (Biolafitte, Saint-Germain-en-Laye, France). Cultures were maintained on solid medium containing brewery wort and 2% agar.

Lectin Isolation from the Culture Medium—The cells were harvested at 4 °C by centrifugation at 3,000 × g, for 10 min, and the supernatant was neutralized to pH 7.4 with 2 M NaOH. The precipitate obtained upon neutralization was removed by centrifugation at 10,000 × g for 15 min, and the supernatant was kept up to several weeks at −20 °C without loss of activity. The solution so obtained was applied onto an affinity chromatography column (1.5 × 5 cm; flow rate, 0.3 ml/min) of Ultragel AcA 22 (IBF-Reactifs, Villeneuvre-la-Garenne, France) substituted with 1,6-diaminohexane, then succinylated, and finally activated with a water-soluble carbodiimide and reacted with p-amino benzyl-thio-β-D-GlcNAc (12). After washing with 200 ml of Tris buffer (pH 7.4, 50 mM tri(hydroxymethyl)aminomethane, 0.1 M NaCl, 3.75 mM CaCl₂, 3 mM Na₂HPO₄), elution was started with the same buffer supplemented with 1 M NaCl (100 ml) then with 0.1 M acetic acid (100 ml), and finally with 0.05 M hydrochloric acid (100 ml). The protein content of the eluate was monitored at 280 nm. Five-milliliter fractions were collected. Fractions eluted with the washing buffer, which are free of β-D-GlcNAc binding protein, were pooled. This solution was chromatographed on an affinity column (1.5 × 7 cm) of Ultragel AcA 22 activated and substituted with p-aminophenyl-α-D-Gal, as described above, and sequentially eluted as described above.

Lectin Isolation from the Yeasts—The yeasts were extensively washed with Helms’s buffer (pH 4.5, 150 mM CH₃COONa, 3.75 mM CaCl₂, 5 mM Na₂HPO₄) which is known to keep them flocculent (10).
The cells were then deflocculated in a 0.2 M D-Gal solution in the same buffer and centrifuged (11). The supernatant could be kept several weeks at −20 °C without any loss of activity. This deflocculated cell extract was chromatographed on β-D-GlcNAc affinity column under the previously described conditions. In order to eliminate D-Gal from the D-GlcNAc nonbinding fraction, proteins were precipitated with absolute ethanol at 4 °C overnight (ethanol/extract ratio = 4) and suspended again in Tris-HCl buffer, pH 7.4, prior to chromatography on p-aminophenyl-α-D-Gal coupled to Ultrogel AcA 22, as described above.

Oxidase Activity Determination—Glycosylhydrolase activities were checked either by using p-nitrophenyl-D-glycosides as substrates (Sigma) according to Conchie (13) 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 No. 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 (14).

Hemagglutination and Hemagglutination Inhibition Tests—Two-fold serial dilutions of lectin solution (50 μl) in wells of a microtiter plate were incubated for 1 h at room temperature with 5% erythrocyte suspension (50 μl) in phosphate-buffered saline (pH 7.4, 8 mM NaCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 3 mM Na₃Cit) and examined for agglutination. Sheep and rabbit erythrocytes were used for titration of D-GlcNAc specific and D-Gal specific lectins, respectively. Activity was expressed as titer, the reciprocal of the maximal dilution of lectin that gave complete agglutination. Hemagglutination was also tested at pH 4.5, in Helm’s buffer with sheep and rabbit erythrocytes pretreated with 0.25% glutaraldehyde to resist to pH and osmotic shocks (15). Inhibition tests were performed in the same way, but serial dilutions of inhibitors solution (50 μl) were previously incubated for 15 min at room temperature with 50 μl of a lectin solution giving an agglutination titer of 4.

Flocculation and Flocculation Inhibition Tests—Yeast flocculation was assayed in microtiter plates by mixing together 50 μl of 2-fold serial dilutions of lectin solution in the Helm’s buffer, pH 4.5, and 50 μl of yeast suspension (2 × 10⁴ cells/ml) in the same buffer. A positive result was recorded in formation of aggregates (flocs), whereas nonfloculating cultures settled to the bottom of the well in a diffuse layer (16), as determined either by visual reading or under a microscope.

Other Biochemical Methods—Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed on a 8- cm-long slab gel containing 5% acrylamide (17). Molecular weight was calculated from calibration with standard markers kit (Sigma). Gel electrophoresis under nondenaturing conditions was done according to Ornstein (18) or by their absorbance at 280 nm. Gel electrophoresis was performed in 12.5% acrylamide gels. Proteins were estimated by using the method of Bradford (19) or by their absorbance at 280 nm.

RESULTS
Isolation and Purification of the GlcNAc Specific Lectin from the Culture Medium—K. bulgaricus cells flocculated at the beginning of the exponential growth phase (after 24 h in culture) and the medium neutralized to pH 7.4 was able to agglutinate human erythrocytes to a similar extent irrespective of the A, B, or O blood group. This result prompted us to isolate the responsible factor for hemagglutination. We previously demonstrated that cell wall mannans of this flocculent isolate the responsible factor for hemagglutination. We precipitated with 0.2 M hydrochloric acid (IV) and 0.05 M hydrochloric acid (IV). Protein was monitored by continuous 280 nm absorbance. All the fractions were tested for hemagglutination; activity was expressed as titer (vertical bars).

Isolation and Purification of the GlcNAc Specific Lectin from the Cells—Deflocculation of the yeasts with 0.2 M D-Gal and centrifugation as described under “Materials and Methods” led to an extract which was chromatographed on the GlcNAc affinity column under the conditions described above. Elution profile is quite similar to that of Fig. 1a. The capacity to agglutinate sheep erythrocytes was observed in the fractions eluted with 0.1 M acetic acid; these fractions did not flocculate yeasts at pH 7.4 or 4.5. From 1 g of wet yeast, the yield in β-D-GlcNAc specific lectin was 0.15 mg.

Isolation and Purification of the Gal Specific Lectin from the Cells—The unretarded fraction was found to agglutinate rabbit erythrocytes at pH 7.4 and glutaraldehyde-fixed rabbit erythrocytes at pH 4.5, and to flocculate yeasts at pH 4.5. In order to eliminate D-Gal from the β-D-GlcNAc nonbinding fractions, proteins were precipitated with absolute ethanol and resuspended in Tris-HCl buffer, pH 7.4, prior to chromatography on Ultrogel AcA 22 substituted with D-Gal. Calculation of activity before and after precipitation showed that less than 50% of the lectin activity was lost during this step. The elution pattern is quite similar to that of Fig. 1b. The fraction eluted with 0.05 M HCl had the same agglutination properties as the homologous fraction isolated from the culture medium (Fig. 1b). The D-Gal specific lectin was obtained with a yield of 0.2 mg from 1 g of wet yeasts.

Purity and Molecular Weight Estimation of the Lectins—
Purity of the lectins was substantiated by polyacrylamide (9% and 7% acrylamide) gel electrophoresis at pH 8.3 in the gel slab, in the presence or in the absence of 15 mM mercaptoethanol and 0.1% sodium dodecyl sulfate. One major band and several minor bands were observed for the four lectin preparations, and a second step of affinity chromatography was required to achieve complete purification. Lectins were eluted from their respective affinity gels with 0.2 M D-GlcNAc or D-Gal. Under these conditions, only one band was observed in both cases for the two lectins, prepared either from the culture medium or from the deflocculated cells (Fig. 2a). Molecular weights were estimated under denaturing conditions to be 61,000 and 65,000 for the D-GlcNAc specific lectins, respectively (Fig. 2b). Oxidase Activity Determination—The following sugar derivatives, in their pyranoside structure, were tested in triplicate in citrate buffer at pH 3.8 or 4.5: p-nitrophenyl-α-D-mannoside, p-nitrophenyl-β-D-mannoside, p-nitrophenyl-α-D-glucoside, p-nitrophenyl-β-D-glucoside, N-acetyl-β-D-glucosamine 1-phosphate, p-nitrophenyl-α-D-galactoside, p-nitrophenyl-β-D-galactoside, p-nitrophenyl-N-acetyl-β-D-glucosamine, p-nitrophenyl-N,N′-diacetyl-β-D-chitobioside, p-nitrophenyl-β-lactoside. No oxidase activity could be detected with either D-GlcNAc or D-Gal specific lectins prepared either from the culture medium or from deflocculated cells.

Influence of the pH on Hemagglutination Properties—The results on the study of agglutination of erythrocytes at pH 7.4 in phosphate-buffered saline and at pH 4.5 in Helm’s buffer are summarized in Table I. The D-GlcNAc specific lectin did not agglutinate glutaraldehyde-treated sheep erythrocytes at pH 7.4 but did at pH 4.5. In contrast, the D-Gal specific lectin did agglutinate glutaraldehyde-treated rabbit erythrocytes at both pH values. In addition, the D-Gal specific lectin, which did not agglutinate untreated or glutaraldehyde-treated sheep erythrocytes at pH 7.4, did agglutinate the glutaraldehyde-treated sheep erythrocytes at pH 4.5.

Hemagglutination Inhibition Test of the D-GlcNAc Specific Lectin—The sugar specificity of the lectin purified on agarose coupled with D-GlcNAc was examined by hemagglutination inhibition tests using sheep erythrocytes. p-Nitrophenyl glycosides and sugars giving the strongest inhibitory effect are presented in Table II. The similarity of the results obtained with lectins isolated either from the culture medium or from cells supports the view that there is only one D-GlcNAc binding protein. α- and β-p-Nitrophenyl derivatives of D-GlcNAc had the same efficiency as hemagglutination inhibitors. Conversely, the lectin was inhibited with a lower concentration of p-nitrophenyl-β-D-glucoside in comparison with p-nitrophenyl-α-D-glucoside. The p-nitrophenyl-N-acetyl-D-glucosaminides are more efficient than free D-GlcNAc, showing the importance of the hydrophobic moiety of the ligand.

**TABLE I**

*Influence of the pH on agglutination of glutaraldehyde-treated (T) or untreated (U) sheep and rabbit erythrocytes, by the D-GlcNAc and D-Gal specific lectins*

| Lectins                        | pH 7.4 (phosphate-buffered saline) | pH 4.5 (Helm) |
|--------------------------------|------------------------------------|---------------|
|                                | U Sheep | T Sheep | U Rabbit | T Rabbit | U Sheep | T Sheep | U Rabbit | T Rabbit |
| D-GlcNAc (medium)              | +       | -       | +        | -        | +       | -       | +        | -        |
| D-GlcNAc (cell wall)           | +       | -       | +        | -        | +       | -       | +        | -        |
| D-Gal (medium)                 | +       | -       | +        | -        | +       | -       | +        | -        |
| D-Gal (cell wall)              | -       | +       | +        | +        | +       | +       | +        | +        |

*Because some aggregates are visible in the presence or in the absence of the lectins, there is no evidence of a lectin-mediated hemagglutination.*

**TABLE II**

*Hemagglutination inhibition test of the purified D-GlcNAc specific lectin from the culture medium of *K. bulgaricus* or released upon incubation in the presence of D-Gal from the yeast cell wall, at pH 7.4, using untreated sheep red blood cells*

| Sugars                          | Minimum amount completely inhibiting hemagglutination |
|---------------------------------|------------------------------------------------------|
| Culture medium bound lectin     | Cell wall bound lectin                               |
| N-Acetyl-D-glucosamine          | 72.0                                                 |
| p-Nitrophenyl-α-D-glucoside     | 11.0                                                 |
| p-Nitrophenyl-β-D-glucoside     | 4.5                                                  |
| p-Nitrophenyl-N-acetyl-α-D-glucosamin | 0.2                                              |
| p-Nitrophenyl-N-acetyl-β-D-chitobioside | 0.2                                             |
| p-Nitrophenyl-N,N′-diacetyl-β-D-chitobioside | 0.15                                              |

Fig. 2. Polyacrylamide gel electrophoresis of the four lectin preparations in the presence of sodium dodecyl sulfate, with 9% acrylamide (a) and in the absence of sodium dodecyl sulfate, with 7% acrylamide (b). Lanes 1 and 2 contained the D-Gal specific lectin isolated from the culture medium and from the deflocculated yeast extract, respectively. Lanes 4 and 5 contained the D-GlcNAc specific lectin with the same origins. Lanes 3 and 6 of a were used for standard proteins (*E. coli* β-galactosidase, rabbit muscle phosphorylase B, bovine albumin, egg albumin, bovine carbonic anhydrase). Lane 3 of b contained: urease dimer and monomer; and lane 6, bovine albumin trimer, dimer, and monomer.
In addition to the compounds shown in Table II, the following sugars or derivatives were found to be inactive at 0.1 M concentration: fucose, galactose, glucose, mannose of the L and D series, D-galactosamine, D-glucosamine, D-mannosamine, D-galactose 6-phosphate, D-glucose 6-phosphate, D-mannose 6-phosphate, and α- and β-p-nitrophenyl derivatives of D-galactose and D-mannose.

**Hemagglutination Inhibition Test of the D-Gal Specific Lectin**—Inhibition studies of the D-Gal specific lectin on rabbit erythrocytes are reported in Table III. Identical results were obtained with both excreted and cell-associated lectins. Among the monosaccharide derivatives tested, o- and p-nitrophenyl galactosides exhibited the strongest inhibitory activity, which appears to be independent of their anomeric configuration. The presence of the hydrophilic aglycone also increases the inhibitory efficiency. The hydroxyl group in position 6 of galactose is not involved in the binding, since D-fucose is as efficient as D-galactose. Sugars listed below were found to be inactive even when concentrations were over 0.1 M: L-fucose, L-galactose, D- and L-glucose, D- and L-mannose, D-galactosamine, D-glucosamine and D-mannosamine, 6-phosphate derivatives of D-galactose, D-glucose, and D-mannose, p-nitrophenyl-α- and β-D-galactosides, and p-nitrophenyl-β-D-mannoside.

**Flocculation and Flocculation Inhibition Test**—The common technique for measurement of flocculation consists of sedimentation speed evaluation combined with light extinction determination. In the present work, important variations in the viscosity of the Helm's buffer were introduced by the presence of sugars in the deflocculation test. Under such conditions, it was necessary to apply the method used by Burke et al. (16) in testing the inhibition of *Hansenula wingei* sexual agglutination factor. A typical assay of deflocculation is presented in Fig. 3 and the most efficient sugar derivatives for inhibition of flocculation are listed in Table IV. These inhibitors are similar to those used to inhibit the agglutination of rabbit erythrocytes at pH 7.4 and at pH 4.5, but the minimum amount completely inhibiting flocculation was slightly different: D-fucose, D-galactose, and Gal-β-1-4-Man were more efficient in flocculation inhibition than in agglutination inhibition, while the other sugars were more efficient in agglutination inhibition than in flocculation inhibition.

**Discussion**

The flocculation of *K. bulgaricus* was reversed by addition of D-Gal (11), although this hexose was absent from isolated cell wall polysaccharides of this yeast (21). The specificity of the interaction suggested a lectin-like mechanism, but isolation of the functional mediator was still lacking. The purification of two lectins from the calcium-enriched culture medium by affinity chromatography corroborated the previous hypothesis. The lectins are excreted in the medium, but a part of the activity remains associated with the outer layer of the cell wall, and upon deflocculation with D-Gal, a further quantity of lectins was released in the medium. According to the osidase activity investigations, the isolated lectins did not belong to the category of glycosidases that “act as lectin” at neutral pH, described by Shannon (22).

The D-GlcNAc specific lectin agglutinated sheep erythrocytes at pH 7.4 and at pH 4.5, but had no effect on deflocculated yeasts at pH 4.5. This result is in agreement with previous observations indicating the important decrease of D-GlcNAc residues in cell wall mannans for flocculent cultures of this yeast (20), and so, the absence of flocculating effect could be the consequence of the absence of the relevant receptor on the wall. The physiological role of this D-GlcNAc-binding protein remains to be studied. Investigations of the specificity of this lectin showed that the presence of acetamido groups on C-2 probably promoted the recognition because D-glucosamine is not an inhibitor, and the corresponding D-glucosides are about 10 times less effective than the N-acetyl-D-glucosaminides. The substitution of C-1 by a hydrophobic aromatic group considerably increased the inhibitory effect of D-GlcNAc residues. In addition, the strong inhibitory effect

**Table III**

| Sugars                      | Minimum amount completely inhibiting hemagglutination (mM) |
|-----------------------------|----------------------------------------------------------|
|                            | Culture medium lectin | Cell wall bound lectin |
| D-Fucose                    | 64.0                   | 64.0                   |
| p-Galactose                 | 62.0                   | 62.0                   |
| p-Nitrophenyl-α-D-galactoside| 0.2                    | 0.2                    |
| p-Nitrophenyl-β-D-galactoside| 0.3                    | 0.3                    |
| o-Nitrophenyl-α-D-galactoside| 0.2                    | 0.2                    |
| o-Nitrophenyl-β-D-galactoside| 0.3                    | 0.3                    |
| p-Nitrophenyl-α-D-mannoside | 5.0                    | 5.0                    |
| Lactose (Gal-β-1-4-Glc)     | 5.0                    | 5.0                    |
| p-Nitrophenyl-β-D-lactoside | 0.5                    | 0.5                    |
| Melibiase (Gal-α-1-6-Glc)   | 1.0                    | 1.0                    |
| Gal-β-1-4-Man               | 31.0                   | 31.0                   |

**Table IV**

| Sugars                      | Minimum amount completely inhibiting flocculation (mM) |
|-----------------------------|------------------------------------------------------|
|                            | Culture medium lectin | Cell wall bound lectin |
| D-Fucose                    | 16.0                   | 16.0                   |
| D-Galactose                 | 15.5                   | 15.5                   |
| p-Nitrophenyl-α-D-galactoside| 0.4                    | 0.4                    |
| p-Nitrophenyl-β-D-galactoside| 0.6                    | 0.6                    |
| o-Nitrophenyl-α-D-galactoside| 0.4                    | 0.4                    |
| o-Nitrophenyl-β-D-galactoside| 0.6                    | 0.6                    |
| p-Nitrophenyl-α-D-mannoside | 5.3                    | 5.3                    |
| Lactose (Gal-β-1-4-Glc)     | 10.0                   | 10.0                   |
| p-Nitrophenyl-β-D-lactoside | 1.1                    | 1.1                    |
| Melibiase (Gal-α-1-6-Glc)   | 2.1                    | 2.1                    |
| Gal-β-1-4-Man               | 15.5                   | 15.5                   |
of \( p \)-nitrophenyl-\( \text{N}, \text{N}' \)-diacetyl-\( \beta \)-d-chitobioside suggests the binding site of the lectin is extended.

Evidence is also given for the excretion of a D-Gal specific lectin and for the fixation of this protein at pH 4.5 on rabbit erythrocytes and yeasts. Both lectins agglutinated glutaraldehyde-treated sheep erythrocytes at pH 4.5, but did not at pH 7.4. This result indicates that both lectins are more active in acidic than in neutral medium and may explain the observation that the extent of yeast flocculation which occurs at pH 4.5 decreased upon neutralization (22).

Polycracylamide gel electrophoresis showed the monomeric nature of the two lectins, and this result implied that at least two binding sites should be present on each lectin, in order to create a bridge between adjacent red blood cells. The molecular weight (60,000) of the isolated lectins is close to that of S. kluyveri 17 cell sexual agglutinin which has been shown to be a proteolytic product of a larger macromolecule of the cell wall (8). Further works are required to determine whether the K. bulgaricus lectins have a larger molecular weight in situ and whether the 60,000 lectins are released by an endogenous protease, directly or indirectly activated by the incubation of the cells in the presence of D-Gal.

To our knowledge, this is the first report on the excretion of a lectin implicated in the flocculation mechanism of K. bulgaricus. However, excretion of lectins by other yeast species is recently shown by Basu et al. (5) for Saccharomyces mutants and by Critchley and Douglas (23, 24) for Candida albicans. In the first report, a D-Gal specific lectin was isolated, whereas in the second example a D-GlcNAc specific adhesion was evidenced. The nature of the receptors of the D-Gal specific lectin on K. bulgaricus cell wall is not yet known; we are currently investigating the inhibitory effect of oligosaccharides produced by acetylation of cell wall polysaccharides.

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