Antigenic studies on flocculating brewer's yeast, *Saccharomyces cerevisiae* NCYC 227

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Batch cultures of a brewer's strain of *Saccharomyces cerevisiae*, NCYC 227, in a defined medium exhibited characteristic flocculation during the late exponential phase of growth. However, early exponential cells were non-flocculent and flocculation of such cells could not be induced even in the presence of Ca^{2+} . A specific glycoprotein, absent from the cell walls of non-flocculating exponentially growing cells of this yeast and those of non-flocculating strains of *S. cerevisiae*, was identified on the cell wall surface of flocculating exponentially growing cells of *S. cerevisiae* NCYC 227. Flocculent cells of *S. cerevisiae* NCYC 227 dispersed with EDTA and coated with monovalent Fab portions of the antibody showed reduced flocculation. Removal of the monovalent antibody portions from the cell surfaces induced cell flocculation in the presence of Ca^{2+} . These results suggest a role for this glycoprotein in yeast cell flocculation.

Introduction

Flocculation of yeast cells is a particular case of cell adhesion that is of considerable interest in brewing and may provide a model system of the cell aggregation process (Rose, 1984). Though early studies described wort components that induce yeast flocculation, recent investigations have established molecules of yeast cell walls as the key substances in causing flocculation. Several authors have claimed that adhesion is due to bridges between anionic groups of the cell walls. According to Lyons & Hough (1970, 1971), flocculation is due to the formation of calcium bridges through phosphodiester linkages in cell mannan, whereas Harris (1959), Mill (1964), Stewart *et al.* (1975), Jayatissa & Rose (1976) and Beavan *et al.* (1979) claimed that phosphodiesterases are not involved in flocculation and that bridge formation arises principally through carboxyl groups. Based on specific inhibition of flocculation by mannose, various authors (e.g. Taylor & Orton, 1978; Miki *et al.*, 1982) have suggested a lectin-like interaction between mannan and protein of adjoining cells.

Despite these reports suggesting wall-wall interactions in flocculation, studies on the gross composition of cell walls of flocculent and non-flocculent yeasts have not revealed meaningful differences (Cawley & Ballou, 1972; Stewart, 1975; Miki *et al.*, 1981). Flocculating yeasts when inoculated into growth medium have been shown to remain dispersed and non-flocculent during the

exponential phase of growth, with cells flocculating at the stationary growth phase (Amri *et al.*, 1982). In this paper we describe preliminary results on the antigenic variation in cell walls of a flocculent yeast strain during such a transition.

Methods

Yeast strains. The flocculating strain *S. cerevisiae* NCYC 227, and the non-flocculating strains *S. cerevisiae* NCYC 431, NCYC 619, NCYC 620, KD6, SD 164 Labatt and CS 101 were used. Strain CS 101 was a culture maintained in this laboratory.

Growth medium. This comprised: glucose, 200 g; $(\text{NH}_4)_2\text{SO}_4$, 3 g; KH_2PO_4 , 3 g; CaCl_2 , 0.25 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; yeast extract, 4 g; distilled water, 1000 ml; pH 4.5.

Measurement of growth and flocculation. Starter cultures of *S. cerevisiae* NCYC 227 were prepared by inoculating 50 ml medium in 250 ml conical flasks with cells from slope cultures and incubating at 30 °C for 12 h. A portion of the starter was then inoculated into fresh medium to obtain an initial OD_{660} of 0.1. After different periods of growth, cells were sedimented by centrifugation at 3600 g for 20 min at 4 °C and the OD_{660} of cells washed with 250 mM-EDTA solution and dispersed in deionized water was determined.

Flocculation was determined by the method described by Jayatissa & Rose (1976). Cells of different growth phases were washed with EDTA solution and dispersed in deionized water. Cell suspension (2.0×10^8 cells ml^{-1}) in deionized distilled water was distributed in tubes and flocculation was initiated by the addition of 50 mM- CaCl_2 solution. The degree of flocculation was expressed by applying a subjective gradation (Stewart & Goring, 1976).

Effect of mannose on flocculation. Flocculated cells (late stationary phase of growth, from 48 h cultures) were dispersed with 250 mM-EDTA and suspended in deionized water. Mannose was added at different concentrations and flocculation was determined at 660 nm in the presence of 50 mM-CaCl₂.

Preparation of antigens and immunization of rabbits. Whole yeast cells (late stationary phase of growth, from 48 h cultures) were autoclaved in 0.02 M-citrate buffer (trisodium citrate and citric acid; pH 7.0) at 121 °C for 90 min and the supernatant separated by centrifugation at 3600 g for 20 min at 4 °C. To the chilled supernatant, cold methanol (2 vols) was added. The precipitate was dissolved in phosphate-buffered saline (PBS; 0.1 M-sodium phosphate buffer containing 0.85% sodium chloride, pH 7.2) and was used as antigen. Rabbits were immunized with 1 mg protein per kg body weight for the production of antibodies. For immunization, intramuscular (with equal volume of Freund's complete adjuvant) and intraperitoneal injections were administered alternately at weekly intervals for 4 weeks. The antiserum separated from the blood 1 week after the fourth injection was subjected to ammonium sulphate precipitation for immunoglobulins. The precipitated immunoglobulins dissolved in 0.1 M-PBS were extensively dialysed against PBS before use.

Ouchterlony double diffusion. Agarose gels (2 mm thick) were made with 0.6% agarose in 0.05 M-PBS containing 0.1% sodium azide as preservative. Wells were punched using a template and cork borers. Double diffusion was carried out at 25 °C for 96 h. Whole yeast cells from different growth phases, washed in 250 mM-EDTA, suspended in 0.05 M-PBS and adjusted to identical concentrations (OD₆₆₀ = 2.0), were used as antigens.

Cross-absorption of the antibody. To a known volume of the antibody, freeze-dried cells of *S. cerevisiae* NCYC 227 (from an 8 h culture) were added in excess to absorb antibodies against antigens of these cells. The sediment obtained after centrifugation at 3600 g for 20 min at 4 °C was discarded and the supernatant used in some of the experiments.

Agglutination reactions. Whole yeast cells adjusted to OD₆₆₀ = 0.5 were used. Slide and tube agglutination tests were carried out with appropriately diluted antibody (whole antiserum titre as determined by tube agglutination 1:80). Controls consisted of cells treated with globulins of preimmune serum.

Native PAGE. Citrate buffer extracts of yeast cells precipitated with methanol and dissolved in PBS as described for antigen preparation were used. The protein concentration of the samples was determined by the Lowry method and 50 µl samples containing 1 mg protein ml⁻¹ were used for electrophoresis. The proteins in the samples were separated in 7.5% (w/v) acrylamide gels according to the method of Laemmli (1970). The gels were stained with Coomassie blue R-250 staining solution (0.2% Coomassie blue R-250; 40% methanol; 10% acetic acid) and destained in 5% methanol containing 7.5% acetic acid. Samples dialysed against the tank buffer (0.025 M-Tris base and 0.192 M-glycine; pH 8.3) were used for electrophoresis.

Indirect ELISA. EDTA-washed yeast cells suspended in absolute ethanol to OD₆₆₀ = 0.1 were used. One hundred microlitre volumes of the cell suspension in ethanol were applied to the wells of a polyvinyl microtitre plate. The wells were coated with the cells by fixing due to evaporation of the ethanol at 37 °C.

The coated wells were thoroughly rinsed with PBS containing 0.05% Tween 20, and 100 µl of the antibody (diluted 1:500) was added to each test well. After incubation for 1 h at 37 °C, the wells were repeatedly washed with PBS. The wells were then supplied with 100 µl peroxidase-conjugated anti-rabbit IgG (Sigma). After 30 min incubation, the wells were washed with PBS and the presence of bound peroxidase was determined using 0.005% H₂O₂ solution containing 3,3',5,5'-tetramethylbenzidine (Tijssen, 1985). Coated wells treated with preimmune

globulins and reacted with peroxidase conjugate as above served as control. The amount of antibody and conjugate added to each well was determined by chessboard titration.

Preparation of monovalent antigen-binding sites of immunoglobulins. Monovalent binding sites of immunoglobulins were made using the enzyme papain (Porter, 1959). Gamma globulins of cross-absorbed antibody preparation were lyophilized and used. Gamma globulins (150 mg) and papain (1.5 mg) were dissolved in 10 ml buffer (0.1 M-sodium phosphate, pH 7.0; 0.01 M-cysteine; 2 mM-EDTA). The solution was incubated at 37 °C for 18 h in the presence of toluene. It was then dialysed against distilled water with vigorous stirring and several changes of the outer liquid over 48 h. This procedure, which removes cysteine and EDTA and facilitates oxidation, inactivates the enzyme (Porter, 1959). The non-diffusible digestion product was treated with protein A (insoluble; Sigma; 200 mg) to absorb the Fc portions. The supernatant after centrifugation was freeze-dried and used. Presence of the active antigen-binding portion of the antibody in the preparation was tested by agglutination of monovalent-antibody-coated yeast cells in the presence of anti-rabbit goat IgG and by ELISA reactions using immobilized yeast cells coated with monovalent antibody and reacted with peroxidase-conjugated anti-rabbit goat IgG.

Results and Discussion

Flocculation of *S. cerevisiae* NCYC 227

S. cerevisiae NCYC 227 grown in the synthetic medium showed characteristic flocculation from the late exponential phase of growth, remaining mainly as single cells during the early exponential phase (Table 1). Mannose inhibited flocculation (results not presented), as has been shown for some other flocculating yeasts (Miki *et al.*, 1982).

Antigenic variation in the yeast cell wall during the transition from the non-flocculent to the flocculent state

The results of the Ouchterlony double diffusion test using cells of different growth phases as antigens versus antibodies raised against citrate buffer extracts of late stationary phase cells indicated the appearance of a new antigen during the transition from the non-flocculent to

Table 1. Flocculation of *S. cerevisiae* NCYC 227 at different growth phases

Culture age (h)	Growth phase	Degree of flocculation*
8	Mid-exponential	—
10	Mid-exponential	—
12	Late-exponential	—
15	Early stationary	+++
18	Mid-stationary	++++
24	Late stationary	++++

* —, No flocculation; + + + +, extremely flocculent.

Fig. 1. Immunodiffusion reactions of the antibody raised against citrate buffer extracts of late stationary phase cells of *S. cerevisiae* NCYC 227 (400 μ l; centre well). Ammonium-sulphate-precipitated immunoglobulins dissolved in a minimum volume of 0.1 M-PBS after dialysis were used at a dilution of 1:6 in 0.1 M-PBS. The antigens were whole cells of *S. cerevisiae* NCYC 227 (200 μ l; $OD_{600} = 2.0$) harvested at various phases of growth: 1, early exponential phase (4 h); 2, mid-exponential phase (8 h); 3, late exponential phase (12 h); 4, early stationary phase (16 h); 5, mid-stationary phase (20 h); 6, late stationary phase (48 h).

Fig. 2. Native PAGE of citrate buffer extracts of flocculent cells (mid-stationary phase of growth; A) and non-flocculent cells (early exponential phase of growth; B). Fifty micrograms of protein was loaded in each lane. After electrophoresis in 7.5% acrylamide gel using Tris/glycine buffer (pH 8.3), protein bands were stained with Coomassie blue R-250.

the flocculent state (Fig. 1). Variation also occurred in the protein pattern of the citrate buffer extracts of the two types of cells as determined by PAGE (Fig. 2). Reactions of the cross-absorbed antibody (Fig. 3)

Fig. 3. Immunodiffusion reactions of cells from late stationary phase cultures (centre well; 200 μ l, $OD_{600} = 2.0$) with: 1, whole antibody (1:6 dilution in 0.1 M-PBS; 100 μ l); 2, antibody cross-absorbed with cells from an 8 h culture (1:4 dilution in 0.1 M-PBS; 100 μ l); 3, 0.1 M-PBS pH 7.2 (control); and 4, preimmune globulins (1:1 dilution in 0.1 M-PBS; 100 μ l).

Fig. 4. Immunodiffusion reactions of the antibody (centre well; 200 μ l; 1:6 dilution) against: 1, cells from a late stationary phase culture (100 μ l; $OD_{600} = 2.0$); 2, citrate buffer extracts of cells from a late stationary phase culture precipitated with methanol and dissolved in 0.05 M-PBS (100 μ l; protein concentration 1 mg ml^{-1}); 3, supernatant of concanavalin-A-precipitated citrate buffer extracts of cells from a late stationary phase culture (100 μ l) [methanol-precipitated citrate buffer extract dissolved in 0.1 M-PBS containing 1 mg protein ml^{-1} was treated with concanavalin A (1 mg ml^{-1}); the precipitate obtained was removed by centrifugation at 3600 g for 15 min at room temperature]; 4, pronase-treated cells from a late stationary phase culture (100 μ l) [whole cells in 1 M-sodium phosphate buffer, pH 7.5 ($OD_{600} = 2.0$) were treated with 1 mg pronase E ml^{-1} (Sigma) for 15 min at 37 °C; cells washed repeatedly and suspended in 0.05 M-PBS were used].

confirmed the above results. Agglutination of cells from late stationary phase cultures in the presence of cross-absorbed antibody preparation suggested surface localization of these antigens on the cell wall. A number of reports have established mannoprotein as the surface layer of *S. cerevisiae* (Phaff, 1971; Rosenfeld & Ballou, 1972), and negative reactions of concanavalin-A-precipitated antigen extracts (Fig. 4) and stability of the

Fig. 5. ELISA reactions. Polyvinyl microtitre plates coated with cells of *S. cerevisiae* were reacted with 100 μ l of whole antibody or cross-absorbed antibody (1:500 dilution in 0.1 M-PBS). After washing, 100 μ l peroxidase-conjugated anti-rabbit goat IgG was added. Bound peroxidase activity in the washed wells was determined by using H_2O_2 solution containing tetramethylbenzidine. Wells 1 to 4: series A to D, reactions of cross-absorbed antibody with cells of strain NCYC 227 from a late stationary phase culture; series E to H, reactions of cross-absorbed antibody with cells of strain NCYC 227 from a late exponential phase culture. Wells 5 to 8: series A to D, reactions of cross-absorbed antibody with cells of strain NCYC 227 from a mid-exponential phase culture; series E and F, reactions of cross-absorbed antibody with cells of strain NCYC 227 from an early exponential phase culture; series G and H, reactions of cross-absorbed antibody with cells of NCYC 431 (wells 5 and 6) and NCYC 619 (wells 7 and 8). Wells 9 and 10: series A to H, reactions of the unabsorbed antibody with cells of *S. cerevisiae* NCYC 431 (well 9; A and B), CS 101 (well 9; C and D), NCYC 619 (wells 9; E and F), NCYC 620 (well 9; G and H), early exponential phase cells of NCYC 227 (well 10; A to D), and late exponential phase cells of NCYC 227 (well 10; E to H). Wells 11 and 12: series A to G, control reactions of unabsorbed antibody (A to D) and absorbed antibody (E to H) in uncoated wells.

antigens despite extraction after autoclaving with buffer indicate the glycoprotein nature of the antigens of strain NCYC 227. The results of pronase digestion of the antigens (Fig. 4) suggest involvement of protein groups of the glycoprotein in reactions with the antibody.

Non-flocculating and flocculating strains of *S. cerevisiae* could not be distinguished by ELISA using whole antibody preparation (Fig. 5). However, using cross-absorbed antibody for the ELISA, cells of *S. cerevisiae* NCYC 227 at the flocculating stage could be clearly distinguished from other strains of *S. cerevisiae* and from non-flocculating cells of *S. cerevisiae* NCYC 227 (Fig. 5). These results further confirm the formation of the identified glycoprotein only when the cells have entered the flocculent state. This finding could explain the results of Campbell *et al.* (1968) and Campbell & Brudzynski (1966), who found no antigenic differences between flocculent and non-flocculent strains of *S. cerevisiae*. They used aerobically grown cells from 3-d-old agar slants for their studies.

Involvement of a cell wall surface antigen in the flocculation of S. cerevisiae NCYC 227

The above results suggested synthesis of a specific antigen (a glycoprotein) on the cell wall surface during the mid-exponential phase of growth, when the cells characteristically flocculated. Increase in the concentration of this antigen concurrently with increased flocculation of cells (Fig. 5) suggested its involvement in flocculation. Results obtained using monovalent antibody confirmed the involvement of this glycoprotein antigen in flocculation. Flocculent cells dispersed with EDTA and coated with monovalent antibody specific for this antigen did not reflocculate, while removal of the coated antibody with 1 M-Tris/HCl (pH 10.2) caused reflocculation of the cells in the presence of 50 mM- $CaCl_2$. The effect described was confirmed to be due to the presence of monovalent antibody by the agglutination of such coated cells in the presence of anti-rabbit goat IgG (results not shown). The surface localization of

the antigen necessitated the use of monovalent antibody fragments, since divalent antibodies induced agglutination, which could not be differentiated from flocculation.

Based on the preliminary observations of Marfey *et al.* (1977), Beavan *et al.* (1979) suggested floc-forming ability in yeasts to be due to the appearance of a non-enzymic, acidic protein in the surface layers of the cell wall. Our studies described above indicate the appearance of an antigen (a glycoprotein) during the transition of the yeast cells from the non-flocculent to the flocculent state, causing flocculation to occur. Mannose inhibition of flocculation suggests this glycoprotein to have a lectin-like function as suggested by Miki *et al.* (1982). The thermostability of this glycoprotein and its localization on the cell wall surface substantiate earlier observations on flocculation of heat-killed cells (Mill, 1964) and isolated cell walls of flocculent yeasts (see Calleja, 1987).

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