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Sexual Agglutination in *Saccharomyces cerevisiae*

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Treatment of either mating type of *Saccharomyces cerevisiae* with the appropriate sex pheromone increased cell-cell binding in a modified cocentrifugation assay. Constitutive agglutination of haploids was qualitatively similar to pheromone-induced agglutination. Regardless of exposure to pheromone, agglutinable combinations of cells exhibited maximal binding across similar ranges of ionic strength, pH, and temperature. Binding of all combinations was inhibited by 8 M urea, 1 M pyridine, or 0.05% sodium dodecyl sulfate. From α-cells we solubilized and partially purifed an inhibitor of a-cell agglutinability. This inhibitor reversibly masked all a-cell adhesion sites and inactivated pheromone-treated and control cells with similar kinetics. The inhibitor behaved as a homogeneous species in heat inactivation experiments. Based on these results, we proposed a model for pheromone effects on agglutination in *S. cerevisiae*.

In *Saccharomyces cerevisiae*, sexual agglutinability is enhanced by sex pheromones. Each mating type synthesizes and secretes a peptide pheromone that affects the other mating type. Mating type a-cells produce the pheromone a-factor; α-cells produce the peptide α-factor.

When the two mating types are mixed in medium and incubated on a rotating platform, some strains begin agglutinating immediately (1, 9, 10). Pheromone pretreatment of such strains increases the rate of agglutination. Manney and Meade (9) called these strains "constitutive agglutinators." Other strains, such as X2180-1A (a) and X2180-1B (α), agglutinate only after a lag of 30 min. The lag can be eliminated by treatment of either mating type with the appropriate pheromone (1, 12). Such strains are called "inducible agglutinators" (9). Since cycloheximide added with the pheromone prevents induction of increased agglutinability in all strains, it is likely that protein synthesis is required for pheromone effects on agglutination.

In an alternate assay, the two mating types are mixed and centrifuged. The pellet is gently resuspended, transferred to fresh medium, and plated (2, 5). Formation of diploids is monitored. Hartwell (6) has improved the assay by estimating agglutination from the turbidity of the cell suspension. Such assays have yielded information on optimal conditions and have confirmed the constitutive agglutinability of some strains as well as the effects of the pheromones. However, these assays are tedious and imprecise due to variability in resuspension. By adding a controllable resuspension step we have made the cocentrifugation assay precise enough to distinguish agglutination systems of diverse properties. We were therefore able to determine that agglutination is qualitatively similar in pheromone-treated cells and in untreated cells.

MATERIALS AND METHODS

Strains and growth conditions. *S. cerevisiae* wild-type haploid strains X2180-1A (a) and X2180-1B (α), obtained from the Yeast Genetics Stock Center (Berkeley, Calif.), were used for most experiments. Cells were cultured in a glucose and ammonia minimal medium that has been previously described (8). The rapid agglutinators XP300-26c (α) and XP300-29b (a) were the generous gift of T. R. Manney, Department of Physics, Kansas State University, Manhattan. These latter strains were grown in defined minimal medium supplemented with (per liter): 20 mg of histidine, 50 mg of lysine, 30 mg of tryptophan, 90 mg of threonine, and 30 mg of adenine. Cultures were incubated on a rotary shaker at 30°C. Cell density was monitored as optical density at 660 nm, calibrated by hemocytometer counts.

Cycloheximide, glass beads, bovine serum albumin, Amberlite CG-50, and Amberlite XAD-2 were purchased from Sigma Chemical Co., St. Louis, Mo., and UltroGel AcA-34 was purchased from LKB Instruments, Inc., Hicksville, N.Y. Other materials were of reagent grade from commercial suppliers.

Protein was determined by binding of Coomassie brilliant blue G-250 (Bio-Rad Laboratories, Richmond, Calif.), and carbohydrate was determined by the phenol-sulfuric acid method (3). The assay for β-fructofuranosidase (EC 3.2.1.26, invertase) was that of Smith and Ballou (11).

Partial purification and assay of sex pheromones. α-Factor was prepared by column chromatography on Amberlite CG-50 as previously described (7).

The hormone was stored and used in the pyridine acetate buffer in which it was eluted. Activity was assayed by ability to induce morphogenesis of **a**-cells (4). One unit of α-factor was defined as the minimum amount that will induce morphogenesis. This is equivalent to 4 U by the method of Duntze et al. (4).

a-Factor was obtained by the method of Strazdis and MacKay (personal communication, V. MacKay, Waksman Institute for Microbiology, Rutgers University, Piscataway, N.J.). Two to five liters of X2180-1A (**a**) cells was grown to late log or early stationary phase. The cells were harvested by centrifugation and discarded. Amberlite XAD-2 beads were washed sequentially in water, methanol, and again in water. Wet beads (10 ml/100 ml of culture supernatant) were added, and the suspension was shaken at room temperature for 24 h. The beads were poured into a column and washed with 3 volumes of 4°C water and 1 volume of 4°C 20% (vol/vol) methanol. a-Factor was eluted with 4°C absolute methanol. The yellow fractions were pooled, evaporated under reduced pressure, and redissolved in a small volume of dimethyl sulfoxide.

The activity of a-factor was determined by its ability to induce morphogenesis of X2180-1B (α) cells. Samples of a-factor were added to suspensions of α-cells at 1×10^4 to 2×10^4 cells per ml in 375 μl of minimal medium containing 0.2 mg of bovine serum albumin per ml. After 4 h of incubation at 30°C, the cells were examined by microscopy. One unit per milliliter was defined as the minimum concentration inducing morphogenesis.

Agglutination assay. Haploid α-cells and **a**-cells were grown separately in minimal medium to between 2.2×10^6 and 1.4×10^7 cells per ml. The cells were harvested and washed at 4°C in saline-phosphate buffer (0.14 M NaCl, 0.01 M sodium phosphate, pH 6.0). The cells were then resuspended in a mixture of one part saline phosphate buffer and two parts fresh minimal medium at a density of 2×10^7 cells per ml. When α-cells were to be induced, bovine serum albumin was added to 0.2 mg/ml. For pheromone induction of agglutinability, cells were incubated with the appropriate pheromone (α-factor, 1 U/ml, 25 min; a-factor, 0.050 to 0.1 U/ml, 70 min) on a rotary shaker at 30°C. Cells that were not induced were maintained on ice during the incubation period. After induction, all cells were centrifuged and resuspended in an equal volume of 0.1 M sodium acetate buffer (pH 5.0) containing 10 μg of cycloheximide per ml, which prevented further induction of agglutinability.

Samples (1 ml) of each cell type were added to 13- by 100-mm test tubes. Control tubes received 2 ml of a single cell type. The volume of each tube was brought to 3 ml with 0.1 M sodium acetate (pH 5.0) containing 10 μg of cycloheximide per ml. Cells in all tubes were gently compacted by centrifugation ($200 \times g$ for 5 min). Hartwell's assay (6) was modified by resuspension of the pellet by stirring with a 7- by 90-mm paddle at 1,000 rpm for 4 s. A stop maintained the paddle at 6 mm above the bottom of the tube. A constant speed control unit (Cole-Parmer Instrument Co., model 4420) assured reproducible results. The suspensions were left undisturbed for 20 min to allow settling of the aggregates, and the test tubes were then used as cuvettes in a Bausch & Lomb Spectronic 20 to determine optical density at 660 nm. All samples were run in duplicate, and most in triplicate. The agglutination index (AI) was defined as: $AI = 1 - (2 \times A^{a+\alpha})/(A^a + A^\alpha)$ where A is the mean optimal density of the tubes containing the cell types or type indicated by the superscript. The AI varies from 0 to 1, with higher indices denoting a greater degree of agglutination.

Extraction and partial purification of α-agglutinin. Midlog cultures of X2180-1B (α) were harvested and washed once in 0.1 M sodium acetate buffer (pH 5.0). The cells were resuspended at an optical density at 660 nm of 30 to 100, and the suspension was brought to 5 mM EDTA and dithiothreitol. The suspension was cooled to 0°C, and 3 g of glass beads (450 to 500 μm) per ml was added. Homogenization in a Sorvall Omnimixer set to the highest speed broke 80 to 90% the cells in 4 to 6 min. The extract was cleared by centrifugation at $120 \times g$ for 5 min and then at 28,000 $\times g$ for 30 min in a Sorvall SS-34 rotor. The supernatant solution was applied to a 4- by 80-cm column of Ultrogel AcA-34 at 4°C. The column was eluted with 0.1 M sodium acetate buffer (pH 5.0).

Agglutinin activity was assayed by inhibition of **a**-cell agglutinability. Fractions were incubated with 2×10^7 pheromone-treated **a**-cells in a total of 2 ml of sodium acetate buffer containing 10 μg of cycloheximide per ml for 90 min at 30°C. The incubated **a**-cells were resuspended in fresh buffer and agglutinated with α-cells as in the agglutination assay. If the agglutinin was active, it inhibited agglutination, and the mixture had a lower AI than the controls. The change in AI was linearly dependent on the amount of extract added at low extract concentrations (correlation coefficient, 0.995).

Reversal of binding. Agglutinated mixtures were de-agglutinated by a series of washes. The cells were washed three times in 0.05 M Tris buffer (pH 9.0) containing 10 μg of cycloheximide per ml. The washed cells were then rinsed twice in 0.1 M sodium acetate (pH 5.0) containing 10 μg of cycloheximide per ml. When urea was the reversal agent, the initial three washes were in 8 M urea in sodium acetate buffer (pH 5.0) containing 10 μg of cycloheximide per ml. Such washes were also used to reverse the effects of α-agglutinin on **a**-cells.

RESULTS

S. cerevisiae haploid cells were constitutively agglutinable when both mating types were present. Agglutination was enhanced by treatment of **a**-cells with α-factor or α-cells with a-factor (Table 1). Treatment of either mating type with its own pheromone had no effect on agglutinability. Neither mating type was ever selfagglutinable, nor did either mating type agglutinate with diploids under any combination of pheromone treatments.

Control and pheromone-treated agglutination mixtures had parallel responses to variations of agglutination and resuspension conditions. AIs increased as the centrifugation time was increased from 1 to 5 min. There was little change

TABLE 1. *Effect of pheromone treatment on agglutination of S. cerevisiae cells*

a-Cells (X2180-1A)	α-Cells (X2180-1B)	Diploid cells (X2180)	AI
Control	Control		0.35 to 0.42
Induced with α-factor	Control		0.61 to 0.82
Control	Induced with a-factor		0.67
Control		Control	0.01
Control		Induced with α-factor	0.04
Induced with α-factor	Induced with a-factor		0.85
	Control + induced with a-factor		0.07
Induced with α-factor		Control	0.04
	Control	Control	0.06
	Control	Induced with α-factor	0.04

at longer times. AIs were dependent on the shear force applied (Fig. 1). The AIs were independent of the time of stirring between 2 and 16 s. Under standard conditions, analysis of the supernatant after settling revealed that the suspended particles consisted of single cells and a few small aggregates (2 to 3 cells). The fraction of agglutinable cells could be obtained by difference, and was proportional to the AI.

Optimal agglutination of X2180-1A (**a**) with X2180-1B (α) occurred between pH 5.0 and 7.0 (Fig. 2) and at an ionic strength between 0.030 and 0.300. There was little difference in the effect of different ions, including sodium acetate, NaCl, and Tris-hydrochloride. Added Ca^{2+} or EDTA had no effect on agglutination. There was little temperature dependence between 12 and 37°C, but a decrease in agglutination was observed below 10°C (data not shown). The effects of pH, ionic strength, and temperature were similar for mixtures with untreated **a**-cells and mixtures in which the **a**-cells had been treated with α-factor.

Standard assay conditions were chosen to maximize the difference between control and induced cells for X2180-1A (**a**) and X2180-1B (α). Under standard conditions, replicate assays had a mean AI of 0.39 ± 0.11 (standard deviation) for mixtures of untreated **a**- and α-cells and 0.72 ± 0.06 for pairs in which the **a**-cells were pretreated with α-factor. These results were obtained from 16 experiments carried out over 3 months. For a single experiment, the standard error was 0.03 AI (triplicate tubes).

Comparison of weakly and strongly adhering cells. Haploids X2180-1A (**a**) and X2180-1B (α) have been classified as inducible agglutinators (9) because they show no constitutive agglutinability in rotation assays. We compared their agglutinability to that of XP300-29b (**a**) and XP300-26c (α), strains which Manney and Meade isolated as excellent inducible agglutinators (9). Substitution of either XP300 haploid for the appropriate X2180 strain yielded a mixture with increased constitutive agglutinability (Table 2). There were less marked differences in agglutination after treatment of the **a**-cells with α-factor. Dependence of agglutination on pH and ionic strength were similar for both sets of cells (data not shown). In our assay, inducible and constitutive agglutinators were qualitatively similar, differing only in the strength of intercellular bonding.

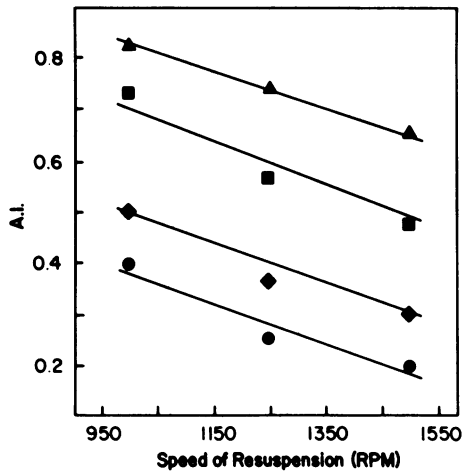

FIG. 1. *Resistance of aggregates to shear. Agglutination mixtures were centrifuged at 200 × g for 5 min and then suspended for 4 s at several rotation speeds. The mixtures are:* (▲) *both mating types induced,* (■) *only a-cells induced,* (◆) *only α-cells induced, and* (●) *neither mating type induced.*

Pheromone induction of agglutinability. **a**-Cells responded to α-factor after a 10-min lag. Maximal induction was reached in 18 min (Fig. 3). α-Factor was maximally effective at doses above 1 U/ml (5×10^{-8} U/cell). a-Factor induced increased agglutinability almost immediately, but 60 to 90 min was needed for full induction (Fig. 3). The α-cells started to recover from the effects of the pheromone after 90 min. a-Factor induced maximal agglutinability in α-cells at concentrations above 0.05 U/ml.

Inhibition of agglutination. The similarity

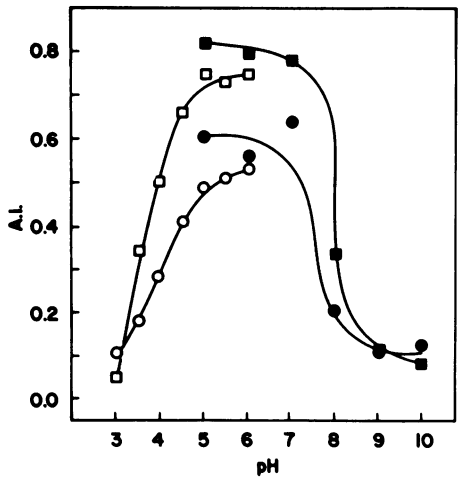

FIG. 2. *Dependence of agglutination on pH. Haploid cells were agglutinated under standard conditions except for differences in the agglutination buffer:* (□, ○) *agglutination in 0.1 M sodium acetate;* (■, ●) *0.1 M Tris-hydrochloride;* (□, ■) *mixtures in which the a-cells were pretreated with α-factor;* (○, ●) *assays with uninduced cells.*

TABLE 2. *Assays of strong agglutinators*[a]

a-Cells	α-Factor treatment	α-Cells	AI
X2180-1A	−	X2180-1B	0.39
X2180-1A	+	X2180-1B	0.72
X2180-1A	−	XP300-26c	0.63
X2180-1A	+	XP300-26c	0.84
XP300-29b	−	X2180-1B	0.61
XP300-29b	+	X2180-1B	0.79
XP300-29b	−	XP300-26c	0.57
XP300-29b	+	XP300-26c	0.80

[a] All experiments were performed at pH 5.0. The rapid agglutinators exhibit maximal agglutination at pH 6.0. Experiments carried out at pH 6.0 yielded similar results. Cells were incubated with or without pheromone, centrifuged, resuspended in buffer, mixed, and then agglutinated.

of the effects of pH, ionic strength, and temperature on agglutination of induced and uninduced a-cells suggested that only a single set of agglutinins was active. We subjected agglutination mixtures to treatment with a variety of perturbants to test whether there were different susceptibilities of pheromone-treated and untreated cells. Between the four agglutinable combinations of cells, however, there were only minor differences in the susceptibility to a variety of disruptive reagents (Table 3).

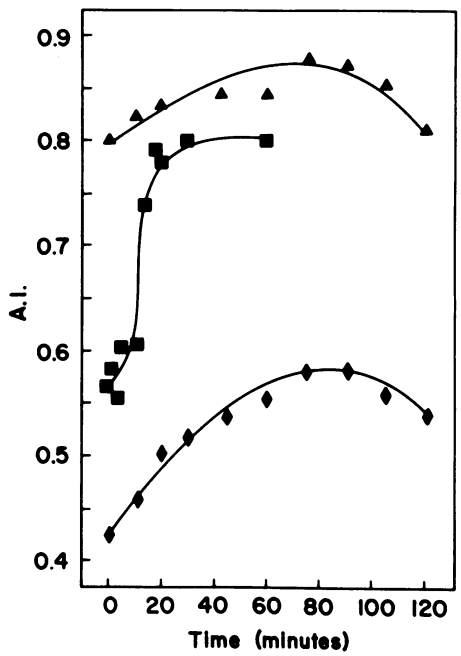

FIG. 3. *Time course of induction of haploids. a-Cells* (■) *were incubated with 1 U of α-factor per ml for the indicated time at 30° C. Induction was stopped by the addition of 10 μg of cycloheximide per ml. The cells were then washed, resuspended in agglutination buffer, mixed with uninduced α-cells, and agglutinated. α-Factor effects were determined by treating α-cells with 0.1 U of α-factor per ml for the indicated time. The cells were then cooled to 0° C, treated with cycloheximide (10 μg/ml), washed, and mixed with uninduced a-cells* (◆) *or a-cells pretreated with α-factor* (▲).

TABLE 3. *Effect of potential inhibitors on agglutination*

Inhibitor[a]	% of control A.I.[b]			
	Neither induced	α-Cells induced	a-Cells induced	Both induced
1 M NaCl	90	79	89	83
5 M NaCl	29	26	18	28
0.3 M KI	90	88	94	97
0.01% sodium dodecyl sulfate	61	87	90	93
1% Tween 80	95	97	99	96
1 M pyridine	44	57	56	71
20 mM EDTA[c]	106	101	104	99
50 mM dithiothreitol				
α-cells pretreated[d]	45	31	26	23
α-cells pretreated[d]	95	88	99	103

[a] Inhibitors were included in the agglutination buffer unless otherwise noted.

[b] Percent of control AI with an agglutination mixture in which the cells were treated with pheromone under standard conditions.

[c] Agglutination was at pH 7.0.

[d] The indicated cells were pretreated with dithiothreitol for 30 min at pH 7.0, washed once with buffer at pH 5.0, and agglutinated as usual.

Urea, pyridine, and sodium dodecyl sulfate were reversible in their effects on agglutination. When the cells were washed free of such reagents, the mixtures regained agglutinability, even in the presence of 10 μg of cycloheximide per ml. Therefore, it was likely that the affectors had prevented formation of intercellular bonds, but had not irreversibly disrupted molecular structure. These three disruptive reagents showed single thresholds for inactivation in titrations of their affects on agglutination (Fig. 4).

To demonstrate that other surface functions were differentially affected by such reagents, cellular invertase was assayed in the presence of two inhibitors (Fig. 4). We grew X2180-1A (**a**) and X2180-1B (α) in minimal medium containing 2% sucrose as a carbon source. The harvested cells were washed, and the two mating types were mixed in assay buffer in the presence of perturbant. We determined invertase activity, correcting for the effect of the perturbant on the reducing sugar determination. The susceptibilities were clearly different from those of agglutination. Invertase was more labile to urea and was not affected by sodium dodecyl sulfate. The increase in activity in the latter case was probably due to permeabilization of the cells and to the consequent assay of both intra- and extracellular enzyme.

Solubilization of α-agglutinin. Clarified extracts of α-cells did not agglutinate **a**-cells, but did decrease the agglutinability of the **a**-cells. A species mediating cell-cell binding would exhibit such properties if it were monovalent. We designated such activity "α-agglutinin" to denote its origin and function in the intact cell. The properties of the extracted α-agglutinin matched those of the cell-bound agglutinin. The soluble material inactivated **a**-cells maximally at pH 5 to 6. Low concentrations of extract inactivated a fraction of the **a**-cells proportional to the amount of extract added, showing that the inactivation was a saturable process. The inactivation was partially reversed by washing these cells at pH 9 or in 8 M urea in the presence of cycloheximide.

Gel filtration yielded several active fractions (Fig. 5). The material eluting in region I was approximately 80-fold more active than the initial extract and exhibited the expected properties of the agglutinin. It was maximally active at pH 5 to 7 and affected only **a**-cells. **a**-Cells treated with this fraction completely recovered their agglutinability after washing in pH 9 buffer or in 8 M urea. The activity eluting in regions II and III permanently inactivated **a**-cells. Consequently, all subsequent experiments were carried out with material from region I.

Agglutinin-mediated inactivation of a-cells. The α-agglutinin inactivated **a**-cells in

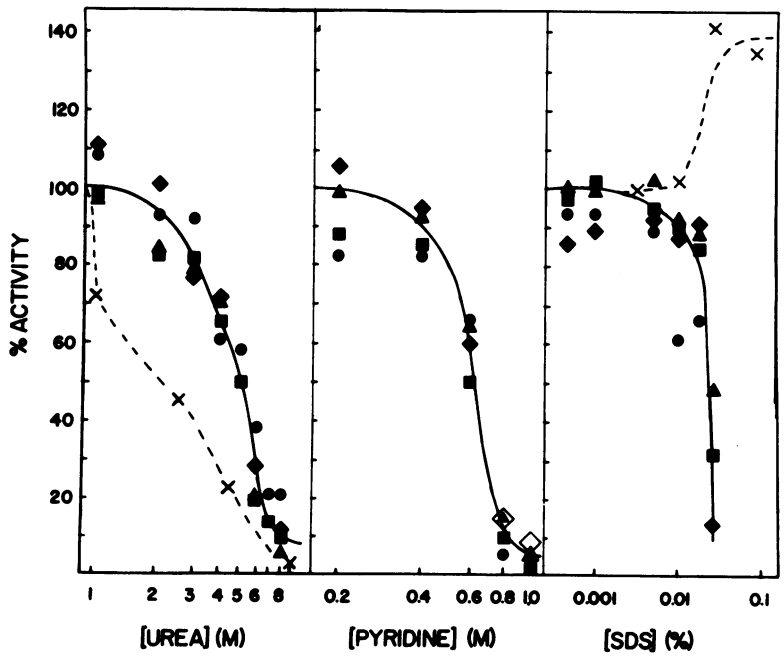

FIG. 4. *Titrations of agglutinations by three inhibitors. The processes assayed are agglutination of mixtures with:* (▲) *both mating types induced,* (■) *only a-cells induced,* (◆) *only alpha-cells induced, and* (●) *neither mating type induced. Percent activity was defined as (AI with inhibitor/AI of control) × 100. Cellular invertase assays* (×) *are also presented.*

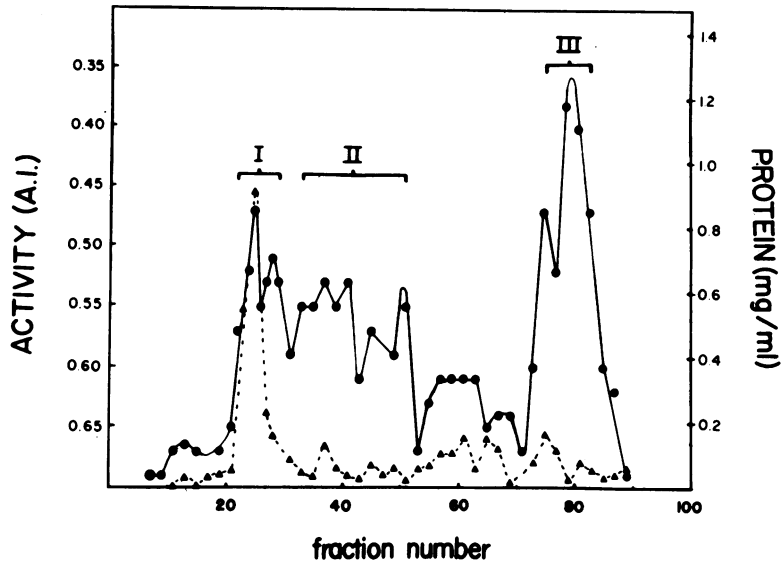

FIG. 5. *Gel filtration of α-cell extract on UltroGel AcA-34. The column was 4 by 80 cm, the eluant was 0.1 M sodium acetate (pH 5.0), and 10-ml fractions were taken: (●) activity, (▲) protein.*

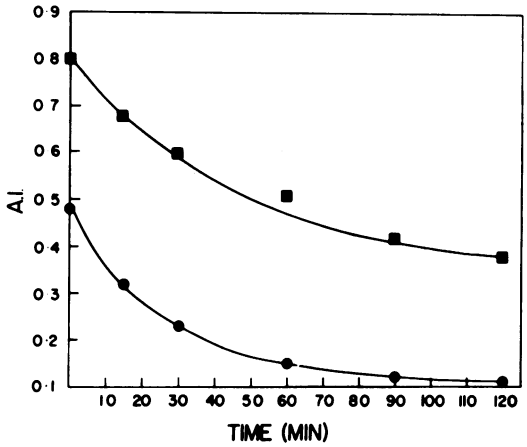

FIG. 6. *Kinetics of a-cell inactivation. The a-cells were incubated with AcA-34-purified α-agglutinin for the indicated times, washed, and agglutinated with uninduced α-cells. The inactivated cells were (●) uninduced a-cells, or (■) α-factor-treated cells.*

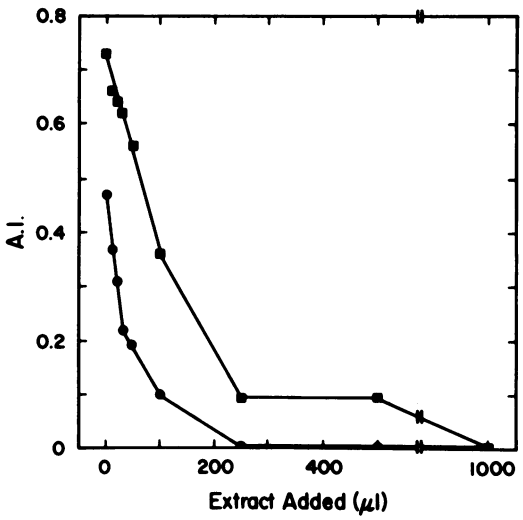

FIG. 7. *Inactivation of a-cells with α-agglutinin. α-Agglutinin was concentrated 13-fold by dialysis against polyethylene glycol. Pheromone-treated (■) or uninduced (●) a-cells were incubated with α-agglutinin for 90 min and then washed and agglutinated with untreated α-cells.*

90 to 120 min (Fig. 6). Both uninduced and α-factor-treated a-cells were inactivated with similar kinetics. For a 90-min binding period, the dose-response curves were linear for change in AI of 0.3 or less (Fig. 7). We also determined the response of untreated and pheromone-treated cells to various doses of concentrated alpha-agglutinin. The α-agglutinin contained species that masked all agglutination sites on a-cell surfaces (Fig. 7).

Heat sensitivity assays implied that the alpha-agglutinin preparations were functionally homogeneous. All activity was destroyed within 10 min at 55°C, but the activity was stable at 40°C (Fig. 8, inset). Inactivation kinetics at 50°C confirmed the homogeneity. Extract was heated for various times, cooled, and incubated with a-cells for 1 h. The a-cells were then assayed for agglutinability as usual. The inactivation had no significant difference in rate between the various combinations of tester cells (Fig. 8).

DISCUSSION

There are many possible models for sexual agglutination in *S. cerevisiae* and for the effects of sex pheromones on agglutination. Figure 9 shows three of these models. Consistent with observations on *S. cerevisiae* (1, 12), these models show pheromone induction of either mating type causing increased agglutination. The pheromones induce increased densities of cell surface agglutinins. Such increases would lead to increased probability of adhesion after collision in rotation assays (1, 12) as well as to the increased strength of cell-cell binding that we observed.

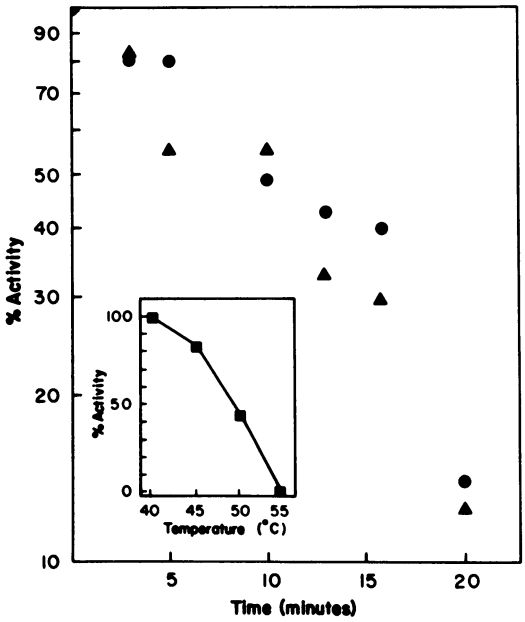

FIG. 8. *Thermal lability of α-agglutinin. The agglutinin was incubated at 50°C for the indicated times, cooled, and assayed. Assays were with both mating types induced (▲) or with neither type induced (●). Inset: inactivation threshold for α-agglutinin. Agglutinin was incubated for 10 min at the indicated temperature, cooled, and assayed with α-factor-treated a-cells and untreated α-cells. The percent activity remaining was calculated from the ratio of the remaining activity to that of the unheated agglutinin with the same tester cells.*

In model A there is only one kind of agglutinin on each mating type. Pheromone treatment increases the effective concentration of these agglutinins at the cell surface. In models B and C there are two pairs of complementary agglutinins. In model B, induction of either cell increases adhesions because the constitutive agglutinin is induced as well as a new type of agglutinin. In model C, pheromone treatment increases the concentration of one or both types of agglutinin, but both types are constitutively expressed. There are other models that fit the conditions listed above, but they have three or more complementary sets of agglutinins.

Whereas in the first model all intercellular bonds are the same, models B and C predict that induced pairs of cells have heterogeneous adhesive bonds. We found that under all tested conditions, the cells adhered as if there were only one type of adhesive bond. Untreated and induced cells adhered over the same range of pH, ionic strength, and temperature. Agglutination of treated and untreated pairs was prevented by the same perturbants. For three dissimilar perturbants, all combinations showed identical thresholds for disruption of intercellular bonds.

Experiments with the α-agglutinin were also consistent with model A. The α-agglutinin was extracted from untreated α-cells, but it com-

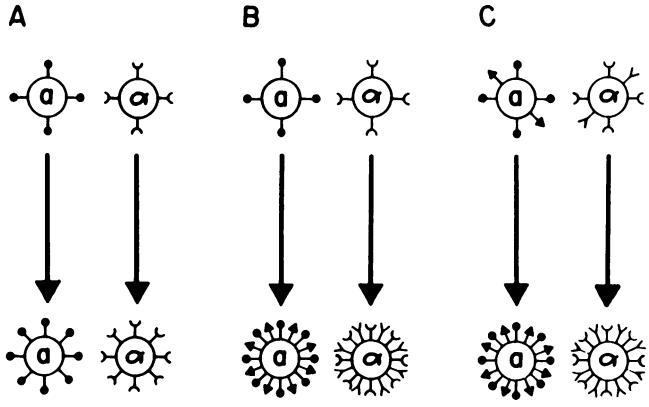

FIG. 9. *Models of sexual agglutination in S. cerevisiae: a, a-cells; α, α-cells. Arrows denote pheromone treatment of the cells. α-Agglutinins are represented by ⊣ and —≺, and a-agglutinins are represented by —● and →.*

pletely inactivated untreated and pheromone-induced a-cells. α-Agglutinin was therefore complementary to all expressed a-cell agglutinins. The thermal inactivation profile of this material was consistent with a single species of macromolecule being responsible for its activity.

All of our experiments therefore support the model of Fig. 9A for agglutination of *S. cerevisiae* haploids. The data imply that in yeast developmental induction of altered adhesive properties is dependent on quantitative rather than qualitative alterations of the cell surface.

ACKNOWLEDGMENTS

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