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# Sexual Agglutination Factors From the Yeast Pichia amethionina

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Pichia amethionina is a heterothallic yeast isolated from necrotic cactus tissue. Haploid cells of opposite mating type, designated *a* and $\alpha$, agglutinate strongly when mixed. The agglutination factors of the two cell types have been solubilized from the cell walls by $\beta$-glucanase digestion and then partially purified by affinity adsorption to the opposite cell type and by gel filtration. From $\alpha$-cells was obtained a large, heat-stable glycoprotein with the ability to agglutinate *a*-cells. This $\alpha$-agglutinin was inactivated by mercaptoethanol, probably because the recognition sites are linked to the glycoprotein core by disulfide bonds. Digestion of *a*-cells with $\beta$-glucanase released a large heat-labile glycoprotein that did not agglutinate $\alpha$-cells but did inhibit agglutination of *a*-cells by $\alpha$-agglutinin. Subtilisin digestion of this *a*-factor released a carbohydrate-free protein of 27,000 daltons that retained the biological activity of the factor. These agglutination factors are sex- and species-specific and are not found on the surface of heterozygous diploid cells.

**Key words: glycoproteins, cell surface recognition, affinity adsorption, amino acid compositions**

Mating between haploid cells of certain yeasts (Hansenula wingei, Pichia amethionina, and Saccharomyces kluyveri) is facilitated by a sex- and species-specific agglutination reaction that is mediated by constitutive cell surface macromolecules [1, 2, 3], in contrast to Saccharomyces cerevisiae in which cell-cell interaction is inducible and relatively weak [4]. A striking characteristic of the first group of three yeasts is that one haploid cell type from each carries a heat-stable factor that is inactivated by treatment with mercaptoethanol owing to the release of disulfide-linked recognition sites, and the other haploid cell type of each carries a heat-labile factor that on controlled proteolysis releases a small recognizer protein of about 27,000 daltons [1]. This asymmetric system of cell-cell recognition has obvious similarities to the fertilization reactions of higher organisms [5, 6] and is different from the bilateral recognition involved in cellular adhesion [7-10].

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In the present paper, we report on the isolation and partial characterization of the sexual agglutination factors from the yeast P amethionina. Although this yeast shows no agglutinative cross-reactivity with H wingei, the isolated factors from its $\alpha$ and *a* haploid cell types have properties that parallel closely those of H wingei 5- and 21-cells, respectively. From a comparative biochemical study of these factors, it is our hope that the structural basis of cell recognition in yeast can be elucidated.

## MATERIALS AND METHODS

Pichia amethionina cultures of *a* and $\alpha$ mating types [11] were provided by H.J. Phaff, University of California, Davis, and they were grown on a medium consisting of 0.7% yeast extract, 0.5% $KH_2PO_4$, and 3% D-glucose. Chromatographic and ion exchange resins were from Bio-Rad and Pharmacia; Zymolyase 60,000 ($\beta 1 \rightarrow 3$-glucanase) was obtained from Kirin Brewery, Tokyo, Japan, Subtilisin BPN′ from Sigma; and pancreatic trypsin (grade A) was from CalBiochem. The low molecular weight protein standard mixture was from Bio-Rad.

Methods for carbohydrate, protein, and phosphate determination and for polyacrylamide gel electrophoresis are reported elsewhere [1]. Bovine serum albumin was used as a protein standard. Agglutination assays were done in microtiter plates (Dynatech Laboratories, Alexandria, Va) by mixing 25 $\mu$l each of the two cell types suspended at $2 \times 10^8$ cells/ml in 0.9% NaCl. A positive reaction was indicated by the formation of cell clumps within 15 min of mixing. Solubilized $\alpha$-factor was assayed by its ability to agglutinate *a*-cells, and *a*-factor was assayed by its ability to inhibit the agglutination of *a*-cells by $\alpha$-cells or purified $\alpha$-factor. One unit of $\alpha$-agglutinin causes agglutination of $5 \times 10^6$ *a*-cells in 50 $\mu$l of 0.9% NaCl, whereas one unit of *a*-factor inhibits the agglutination between $5 \times 10^6$ *a*-cells and $5 \times 10^6$ $\alpha$-cells in 50 $\mu$l of 0.9% NaCl.

## RESULTS

### Isolation and Purification of $\alpha$-Agglutinin

The agglutination factor was released from $\alpha$-cells by digestion with Zymolase and it was purified by adsorption to *a*-cells at pH 7 followed by elution at pH 1.8 [1]. The recovered material was further purified by gel filtration.

The $\alpha$-cells were grown in a 200 liter fermenter and were harvested as they entered stationary phase, giving 2 kg of wet cells. About 200 g of $\alpha$-cells were incubated with shaking in 1 liter of 1.0 M ethylenediamine tetraacetate (EDTA) for 30 min at 30°C. The cells were centrifuged and washed once with 10% KCl. To the pellet were added 0.1 mg/ml of Zymolyase 60000, 2 mM phenylmethane sulfonylfluoride (PMSF), 20 ml of 0.1 M EDTA, 100 ml of 20% KCl, and 100 ml of 0.05 M potassium phosphate buffer, pH 7.5, and the volume was adjusted to 1 liter with 10% KCl. This suspension was incubated at 30°C for 1 hr with shaking at the lowest speed sufficient to prevent the cells from settling. Once the conversion to spheroplasts was greater than 90%, the suspension was centrifuged for 20 min at 27,000 g.

The supernatant from the above step was concentrated by an Amicon XM 300 filter membrane and lyophilized. For adsorption to *a*-cells, 700 mg of the crude preparation of $\alpha$-agglutinin was dissolved in 100 ml of 0.9% NaCl. This solution was added in four 25-ml portions to 60 g of saline-washed *a*-cells suspended in 60 ml of 0.9% NaCl. After

each 25-ml addition of crude α-agglutinin, a sample of the mixture was centrifuged and tested for agglutination activity in the supernatant to assure that the *a*-cells were in excess.

After 1 hr at 23°C, the cells were collected by centrifugation, washed once with 0.9% NaCl, and the α-agglutinin was eluted from the *a*-cells by shaking them for 1 hr in 100 ml of pH 1.8 HCl-KCl solution. The *a*-cells were removed, and the supernatant was concentrated by lyophilization and passed through a Bio-Gel A1.5m column (1 × 100 cm) in 0.1 M $KH_2PO_4$. The fractions with activity (tubes 50-62 in Fig. 1) were collected and lyophilized. The yield of pure α-agglutinin was about 50 mg from 200 g of α-cells.

## Properties of α-Agglutinin

The most highly purified preparation of α-agglutinin contained about 80% mannose, 12% protein, and 3% phosphate. Based on its elution position from the Bio-Gel A5m column, it appears to have a molecular weight of approximately $10^6$, in comparison with the elution volume of H wingei 5-agglutinin on the same column [3]. The amino acid composition (Table I) shows that at least 50% of the protein component is serine and threonine. Treatment with 0.1 N NaOH under conditions that cause β-elimination of sugar linked to serine and threonine released 75% of the mannose from the α-agglutinin but only 35% of the mannose from the bulk cell wall mannoprotein from α-cells. The agglutinin-derived carbohydrate was separated by gel filtration (Bio-Gel P-4) into oligosaccharide homologs that ranged from monosaccharide to pentadecasaccharide in size (data not shown).

**TABLE I. Amino Acid Compositions of Agglutination Factor Preparations**

| | Pichia amethionina factors[a] | | | Hansenula wingei factors[b] | |
|---|---|---|---|---|---|
| | α-Agglutinin | | | | |
| Amino acid | Prep #1 | Prep #2 | *a*-Factor-S | 5-Agglutinin | 21-Factor-T |
| Lysine | 0.5 | 1.9 | 1.7 | 0.2 | 1.7 |
| Histidine | 0.9 | 1.1 | 2.3 | 0.2 | 0.5 |
| Arginine | 0.1 | 0.3 | 0.6 | 1.2 | 0.8 |
| Aspartate | 4.6 | 6.2 | 11.6 | 2.8 | 15.9 |
| Threonine | 18.7 | 20.2 | 6.4 | 9.2 | 12.9 |
| Serine | 37.5 | 30.8 | 26.4 | 55.3 | 8.3 |
| Glutamate | 5.5 | 6.3 | 10.3 | 7.0 | 12.7 |
| Proline | ND | ND | 4.2 | 2.0 | 4.3 |
| Glycine | 6.2 | 6.4 | 15.7 | 1.6 | 7.9 |
| Alanine | 6.2 | 7.1 | 12.0 | 5.1 | 6.1 |
| Valine | 10.3 | 8.5 | 2.3 | 6.3 | 7.7 |
| Methionine | ND | ND | ND | ND | ND |
| Isoleucine | 3.7 | 3.3 | 1.4 | 3.5 | 6.3 |
| Leucine | 2.9 | 3.2 | 1.9 | 3.5 | 7.5 |
| Tyrosine | 0.6 | 0.8 | 1.7 | 0.9 | 4.3 |
| Phenylalanine | 0.9 | 1.1 | 1.6 | 0.4 | 3.1 |

[a]Residues per 100 amino acids.
[b]Taken from [1] and [3].
ND = Not determined.

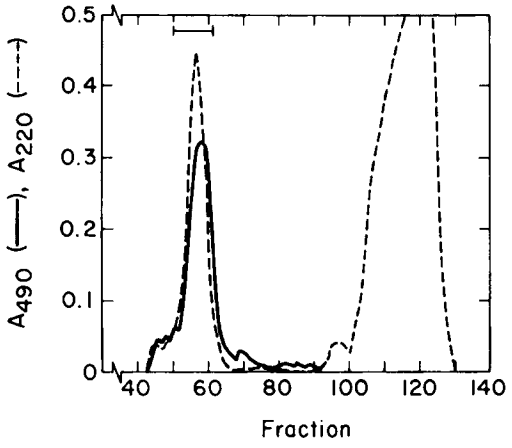


Fig. 1. Isolation of α-factor by gel filtration. The soluble extract from 200 g of α-cells, treated with 0.2 mg/ml of Zymolase at pH 7.5, was adsorbed to 200 g of *a*-cells at neutral pH and then eluted at pH 1.8. This material was fractionated on a Bio-Gel A5m column (1 × 100 cm) in 0.1 M $KH_2PO_4$, and the material that agglutinated *a*-cells is indicated by the bar. Fractions of 1.0 were collected, and carbohydrate ($A_{490}$) and protein ($A_{220}$) were monitored.

The amino acid composition of the Pichia α-agglutinin is compared in Table I with that of the Hansenula 5-agglutinin. The overall balance of acidic, hydroxy, and hydrophobic amino acids between the two agglutinins is very similar, although the ratio of serine to threonine in the Hansenula agglutinin is somewhat higher than that of the Pichia agglutinin. The striking fact remains that the sum of these two amino acids exceeds 50% in the two proteins and most of the hydroxy amino acids are glycosylated. As a consenquence of this high degree of glycosylation, the protein chains probably have little secondary structure.

The α-factor was multivalent as demonstrated by its ability to agglutinate *a*-cells. This agglutinative activity was lost on exposure to mercaptoethanol, presumably owing to the reduction of disulfide bonds and release of monovalent binding fragments. The agglutinative activity of α-factor was also lost above pH 7, but the material retained an ability to inhibit the agglutination of *a*-cells by α-cells, probably because the factor is dissociated at the high pH and the binding fragments neutralize the receptors on *a*-cells. Mercaptoethanol also inactivated whole α-cells, but their sensitivity was not as readily destroyed by high pH.

## Isolation and Purification of *a*-Factor

The *a*-factor activity was released from *a*-cells by digestion with Zymolyase *(a*-factor-Z) or subtilisin *(a*-factor-S). The intact factor was purified by adsorption to α-cells, whereas the *a*-factor-S could be purified by standard procedures applicable to proteins.

Cells of the *a*-mating type were grown in a 200-liter fermenter as described above for α-cells. The *a*-cells were pretreated with EDTA and then were digested with Zymolase to solubilize the cell wall and release spheroplasts. The spheroplasts were

removed by centrifugation at 27,000g, and the supernatant was concentrated by an Amicon XM 50 filter membrane and lyophilized to yield *a*-factor-Z. The *a*-factor-Z was purified by gel filtration (Fig. 2A), and it was eluted from Bio-Gel A5m in the position of a large mannoprotein. This material did not show any agglutinative activity with $\alpha$-cells, but it inhibited agglutination between *a*- and $\alpha$-cells. The carbohydrate to protein weight ratio was about 9.

Digestion of *a*-factor-Z with subtilisin released a small protein with the inhibitory activity of *a*-factor (Fig. 2B). For isolation of this *a*-factor-S, it was more convenient to treat *a*-cells directly with subtilisin and recover the active fraction by gel filtration (Fig.

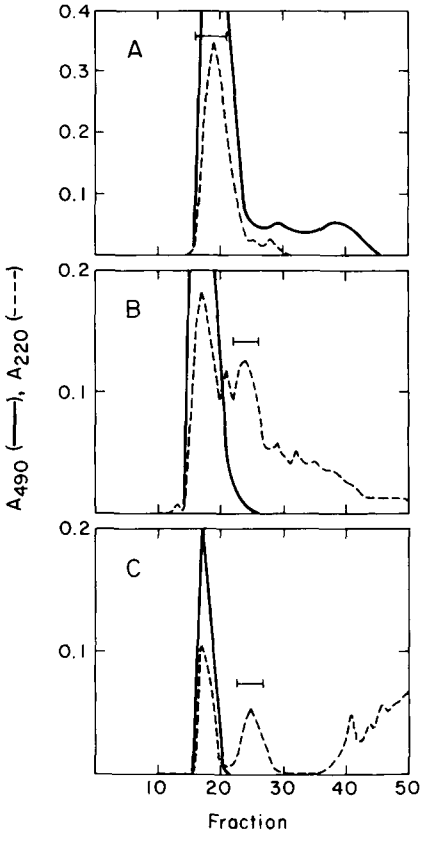


Fig. 2. Isolation of *a*-factor by gel filtration. A) *a*-cells were digested with Zymolyase, the soluble extract was chromatographed on a Bio-Gel P-60 column (1 × 50 cm) in 0.1 $KH_2PO_4$, and the 1-ml fractions with agglutination inhibitory activity (indicated by the bar) were combined. B) the *a*-factor-Z from A was treated with subtilisin (0.4 $\mu$g of enzyme per mg of factor in pH 7.4 Tris HCl buffer) and the digest was rechromatographed on the same column with the same buffer. The *a*-factor-Z-S activity appeared in fractions indicated by the bar. C) *a*-factor-S was prepared by treating *a*-cells with subtilisin for 1 hr at 37°C, 0.2 mg/ml of enzyme in pH 7.4 Tris HCl buffer. After removal of the cells, the digest was concentrated by an Amicon PM10 filter and fractionated on the Bio-Gel P-60 column. The *a*-factor-S activity is indicated by the bar, and its composition is given in Table I. Carbohydrate ($A_{490}$) and protein ($A_{220}$) were monitored.

2C). This material was purified 440-fold according to the steps in Table II, and it gave a major band on gel electrophoresis (Fig. 3). Trypsin did not release *a*-factor activity from cells or from *a*-factor-Z, in contrast to its action of H wingei 21-cells [1].

## Properties of *a*-Factor

The *a*-factor-Z is a large mannoprotein that is excluded on a Bio-Gel P-60 column from which a small active protein component could be released by subtilisin digestion. The purified *a*-factor-S protein was included on a Bio-Gel P-60 column, and it had a molecular weight of about 27,000 based on gel electrophoresis in sodium dodecyl sulfate (Fig. 3). The *a*-factor-S had a high content of acidic and hydroxy amino acids (Table I), and it was also rich in glycine, alanine, and valine. The activity of *a*-factor was not affected by reducing agents, but it was very labile to heat, both properties that are shown by intact *a*-cells [1].

The amino acid compositions of the subtilisin-released Pichia *a*-factor and the trypsin-released Hansenula 21-factor are compared in Table I. Although these two proteins are not as similar in composition as the agglutinins from the two yeasts, they both

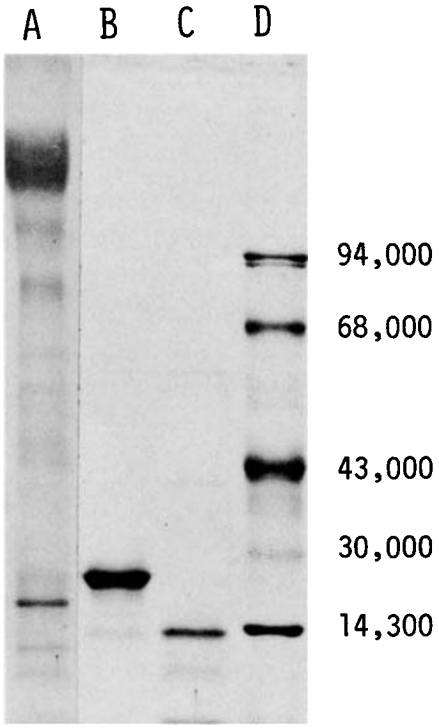


Fig. 3. Polyacrylamide gel electrophoresis of *a*-factor preparations. P amethionina *a*-cells were digested with Zymolyase to yield the *a*-factor activity, which was purified by passage over a Bio-Gel A-1.5m column to give the pattern in lane A. The active component is in the diffuse band near the top. Subtilisin digestion of this material, purification by passage over a Bio-Gel P-60 column, followed by adsorption to a DEAE-Sephadex A-25 column in 0.02 M phosphate buffer and elution with a NaCl gradient gave the material in lane B. Lane C shows the subtilisin used in the digestion, and lane D is a molecular weight standard consisting of phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (14,300).

**TABLE II. Purification of P amethionina *a*-Factor-S**

| Step | Total protein (mg) | Specific activity (units/mg protein)[a] | Purification (fold) |
|---|---|---|---|
| Subtilisin digest | 1,957 | 0.002 | 11 |
| Hollow fiber filtrate | 402 | 0.009 | 4 |
| Bio-Gel A1.5m column | 84 | 0.023 | 11 |
| DEAE-Sephadex column | 8.5 | 0.24 | 120 |
| Bio-Gel P-200 column | 2.3 | 0.88 | 440 |

[a]One unit inhibits agglutination between $5 \times 10^6$ *a*-cells and $5 \times 10^6$ $\alpha$-cells in 50 $\mu$l of 0.9% NaCl.

have high contents of acidic and hydroxy amino acids. Because these proteins are the active recognition parts released from larger molecules by selected proteolysis, some of the difference in composition may have resulted from differences in the sites of cleavage by the subtilisin and trypsin.

## DISCUSSION

P amethionina $\alpha$-cells yield a large, heat-stable agglutinin of *a*-cells after solubilization of the cell wall by the action of $\beta$-glucanase. The high content of mannose and serine and threonine suggest that this material, like the 5-agglutinin from H wingei 5-cells [3], is a glycoprotein with oligosaccharides of mannose attached O-glycosidically to serine and threonine in the protein. From its gel filtration properties, we conclude that the glycoprotein is very large. The agglutinin activity is destroyed by exposure to mercaptoethanol or to high pH, but the resulting material retains an ability to inhibit the agglutination between *a*- and $\alpha$-cells. This property is also shown by H wingei 5-agglutinin and reflects the conversion of the multivalent factor to monovalency as a result of the reductive release of the many small disulfide-linked recognition sites [3].

Treatment of P amethionina *a*-cells with the $\beta$-glucanase solubilizes a large heat-labile glycoprotein that does not agglutinate $\alpha$-cells but does inhibit agglutination of *a*-cells by $\alpha$-agglutinin. Controlled proteolysis of this intact *a*-factor releases a carbohydrate-free protein with a molecular weight of about 27,000 that possesses all of the inhibitory activity of intact *a*-factor. Similar material can be obtained directly from *a*-cells by subtilisin digestion. Both preparations, however, have a rather high content of acidic amino acids in common with subtilisin-released factor from H wingei 21-cells. These results suggest that the intact *a*-factor is held at the cell surface by a large glycoprotein portion that is embedded in the wall and that the recognizer function is carried by a nonglycosylated part of the molecule.

These studies on the sexual agglutination factors of P amethionina reveal a striking similarity to those already reported [1–3] for the corresponding factors from H wingei. The recognition system is characteristically asymmetric in nature, with one haploid cell type possessing a heat-labile recognizer molecule in which the activity resides in a small proteinaceous portion of a larger glycoprotein, whereas the other haploid cell type carries a heat-stable macromolecule with numerous recognition sites that consist of small glycopeptide units attached to the core glycoprotein by disulfide bonds. We have shown previously [1] that the heat-labile factor is on the haploid type

equivalent to Saccharomyces cerevisiae $\alpha$-cells and the heat-stabile factor is on the haploid type related to S cerevisiae *a*-cells. It should be emphasized, however, that the latter yeast does not exhibit the strong constitutive agglutinative property found on H wingei and P amethionina [4].

It has been postulated that the constitutive sexual agglutinins may promote a more resistant diploid state by facilitating the mating of haploid spores within the zygote [2], but regardless of the function, it is clear that very similar molecules have evolved in yeasts that carry exquisitely specific recognition properties that may have been conserved for recognition functions in higher eucaryotes [7].

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