Purification and partial characterization of α-pheromone-binding protein from *a* mating-type cells in the yeast *Saccharomyces cerevisiae*

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Two kinds of proteinacious substances which bind to α pheromone were isolated from heat-shock extract of *a* mating type cells of *Saccharomyces cerevisiae*. One has M_r ~300000 and the other M_r 58000. We purified the smaller binding protein by successive application of DEAE and CM-cellulose chromatography, gel filtration, and isoelectrofocusing. The purified binding protein showed a single band by polyacrylamide gel electrophoresis. This binding protein was detected in *a* cells but not in α cells.

Binding protein *α-Pheromone* *Sexual agglutinability* *Receptor* Saccharomyces cerevisiae *Yeast*

1. INTRODUCTION

In the yeast *Saccharomyces cerevisiae*, *a* and α haploid cells secrete diffusible sex-specific peptidyl *a* and α pheromones, respectively [1,2]. The sex pheromones act on the target cells of respective opposite mating type to induce the production of agglutination substance (mating-type-specific glycoprotein responsible for sexual agglutination) [1–3] and to arrest the cell cycle at G1 phase accompanied by the inhibition of DNA synthesis [4–6].

Although the action mechanism of peptide hormones in animal cells, especially insulin, has been considerably elucidated at molecular level [7], that in eukaryotic microbes, including the yeast, is still unclear. For example, even receptors of the sex pheromones in the yeast have not been identified yet, although it was demonstrated that the first step of the biological action of α pheromone of *S. cerevisiae* is binding to the surface of cells of the opposite mating type *a* [8].

Here, we describe the purification of the α-pheromone-binding protein and the biological significance of the protein as the candidate for α-pheromone receptor.

2. MATERIALS AND METHODS

2.1. *Organisms and culture conditions*

The prototrophic haploid strains of *Saccharomyces cerevisiae*, T55(*a*) and T56(α) having the ability to agglutinate with cells of opposite mating type, sexual agglutinability, constitutively, and T55s-41(*a*) which acquires sexual agglutinability in response to α pheromone [9] were used throughout this study. *a* and α are complementary mating types. T55 cells were cultured in each 5 l of the complete medium YHG [1] for 24 h with air bubbling for extraction of α-pheromone-binding proteins. T56 and T55s-41 cells were cultured in 50 ml YHG medium for 14 h at 28°C on a reciprocating shaker for the measurement of α pheromone activity. T56 cells were also used for the preparation of α pheromone. Cells were washed twice with PBS (10 mM phosphate buffer, pH 5.5) before experimental use.

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Abbreviation: SDS, sodium dodecyl sulfate

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2.2. *Assay of biological activity of α-pheromone-binding proteins*

The biological activity of the binding proteins was measured by their inhibitory action on the α pheromone activity to induce sexual agglutinability of T55s-41 cells [10,11]. Although the inhibition of α pheromone activity can be brought about through both degrading and binding actions, we have confirmed that at least the binding protein with a lower M_r-value, which we will treat mainly in the following, inactivates α pheromone not by degradation but by binding (in preparation). Each 1 ml sample was mixed with 1 ml PBS containing α pheromone (50 units/ml) and incubated for 30 min at 28°C. The mixture, then, was enriched with 2% glucose and 0.2% peptone, followed by inoculation with T55s-41 cells at a cell density of absorbance at 530 nm, 0.5 (2×10^7 cells/ml). After further incubation for 1.5 h at 28°C, induced sexual agglutinability of T55s-41 cells was measured by adding T56 cells having high sexual agglutinability, after heat treatment and expressed by agglutination index (*AI*) [12,13]. The lower the *AI* value, the higher the biological activity of α-pheromone-binding proteins. One unit of the binding proteins is defined as the minimum amount of the binding proteins which masked the agglutinability-inducing action of 50 units α pheromone.

2.3. *Preparation of crude α pheromone*

T56(α) cells were cultured in minimal medium (MM) containing 50 g glucose, 5 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 2 g $MgSO_4 \cdot 7\,H_2O$ and traces of vitamins in 1 l deionized water for 48 h at 28°C. α Pheromone was partially purified by using a column of Amberlite CG-50 according to [14]. One unit of α pheromone was the minimum amount of α pheromone which induces visually-detectable sexual agglutinability of 2×10^7 T55s-41(*a*) cells.

2.4. *Heat-shock extraction of α-pheromone-binding proteins*

1.0 kg T55(*a*) cells from 40 l culture, suspended in 700 ml PBS were heated for 5 min at 100°C [10,11], followed by immediate chilling in crushed ice. After centrifugation, the resultant supernatant was used as starting material for purification.

2.5. *DEAE-cellulose chromatography*

The heat-shock extract (1.5 l) was subjected to 60–90% $(NH_4)_2SO_4$ precipitation and then the precipitate was extensively dialyzed against 10 mM Tris–HCl buffer (pH 7.0). The dialyzed sample was applied to a column of DEAE-cellulose (2.5 × 30 cm) equilibrated with the above buffer. A 450 ml linear gradient of NaCl (0–0.6 M) in 10 mM Tris–HCl buffer (pH 7.0) was applied to the column.

2.6. *CM-cellulose chromatography*

The biological active fractions of the DEAE-cellulose chromatography were pooled, dialyzed against 10 mM citrate buffer (pH 4.5) and applied to a column of CM-cellulose (2.5 × 15 cm) equilibrated with the above buffer. The unadsorbed fractions having biological activity were pooled and concentrated by ultrafiltration using a UM-10 membrane filter (Amicon Corp.).

2.7. *Gel filtration*

The sample concentrated by ultrafiltration was applied to a gel filtration column of AcA 34 (2.5 × 90 cm) (LKB) equilibrated with 10 mM Tris–HCl buffer (pH 7.0) containing 0.1 M NaCl, 0.5% Brij 35 and 0.025% sodium azide. Each 4 ml fraction was collected.

2.8. *DEAE-cellulose rechromatography*

The active fractions with a lower M_r in the gel filtration were pooled, dialyzed against 10 mM Tris–HCl buffer (pH 7.0), then applied to a column of DEAE-cellulose equilibrated with the above buffer. After the chromatography, the active fractions were collected, dialyzed against water and then lyophilized.

2.9. *Isoelectrofocusing*

The lyophilized sample was subjected to isoelectrofocusing with 1% Ampholine (pH 3.5–5.0) (LKB) in a discontinuous sucrose gradient (0–60%) column (2.2 × 28 cm). The electrofocusing was carried out for 48 h at a constant 900 V below 4°C. After the fractionation, absorbance at 280 nm and pH of each fraction were measured at 4°C.

Table 1

Purification of α-pheromone-binding protein II

Step	Volume (ml)	Protein (mg/ml)	Activity (U/ml)	Spec.act. (U/mg)	Purification (-fold)
1. Heat-shock extract	15000	2.5	200	80	1
2. 60–90% $(NH_4)_2SO_4$ fraction	700	9.0	3000	330	4.13
3. DEAE-cellulose	2100	0.714	600	840	10.5
4. CM-cellulose	3000	0.382	300	790	9.9
5. Gel filtration	400	0.51	1000	2000	25
6. 2nd DEAE-cellulose	50	0.106	2000	19000	238
7. 1st Isoelectrofocusing	4	0.987	20000	20000	250
8. 2nd Isoelectrofocusing	–	–	–	–	–
9. 3rd Isoelectrofocusing	2	0.062	15000	240000	3000

U, units; see section 2

2.10. *Protein determination*

Protein content was estimated as in [15] using bovine serum albumin as standard.

2.11. *Gel electrophoresis*

Electrophoresis was carried out in 5.6% polyacrylamide gel without SDS according to the formula in [16]. A densitogram was obtained with a densitometer (Shimadzu CS-910). SDS–polyacrylamide gel electrophoresis was carried out according to [17] in the disc gel of 10% separating gel and 3% stacking gel. Samples or marker proteins were denatured in the presence of 2% SDS and 5% 2-mercaptoethanol at 100°C for 2 min before the electrophoretic run. Gels were stained with 0.25% Coomassie brilliant blue R-250.

3. RESULTS AND DISCUSSION

The purification steps of the α-pheromone-binding protein with lower M_r, designated as II, are summarized in table 1. Each step will be explained in the following. Two kinds of α-pheromone-binding proteins, designated as I and II, were separated by gel filtration (fig.1). The M_r of I was estimated to be ~300000 and that of II was ~56000. We concentrated on purification of II because of the possibility that I was aggregate of II or larger wall fragments were contained in I. Further purification of II was carried out by isoelectrofocusing after the removal of the detergent by DEAE-cellulose rechromatography. The resultant active fraction was subjected to the recycle of isoelectrofocusing. In the 2nd isoelectrofocusing, three A_{280} peaks with pI-values of 3.8, 4.0 and 4.2 were separated. Only the pI 4.0 peak was biologically active (not shown). The active fraction was further purified by the third isoelectro-

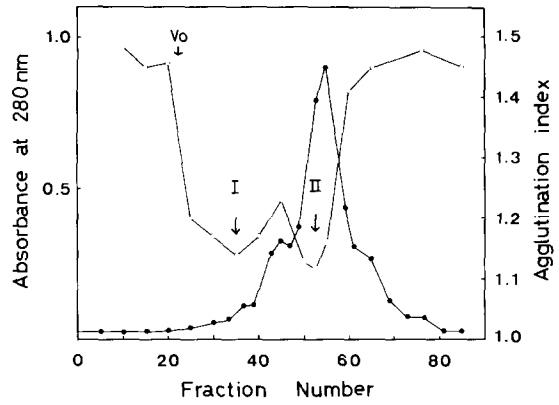

Fig.1. Gel filtration of α-pheromone-binding proteins. The heat-shock extract of *a* mating-type cells was successively purified by ammonium sulfate precipitation and DEAE- and CM-cellulose chromatography, then applied to the Ultrogel AcA 34 column. Biological activity was expressed in terms of agglutination index. The lowest agglutination indices indicate the highest activity of two binding proteins (α-pheromone-inactivating activity). Biological activity of each fraction was assayed after dilution of 20 μl of each fraction to 1 ml with PBS. (●—●) absorbance at 280 nm, (○—○) agglutination index.

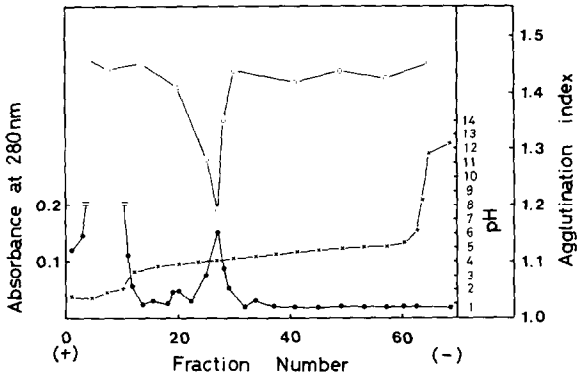

Fig.2. Isoelectrofocusing of α-pheromone-binding protein II. The active fraction II in fig.1 was subjected to isoelectrofocusing after removal of the detergent and lyophilization. The isoelectrofocusing was repeated further twice. The final profile is shown in this figure. Biological activity is expressed in terms of agglutination index. Biological activity of each fraction was assayed after dilution of 20 μl of each fraction to 1 ml with PBS. (●—●) absorbance at 280 nm, (○—○) agglutination index, (×—×) pH of each fraction.

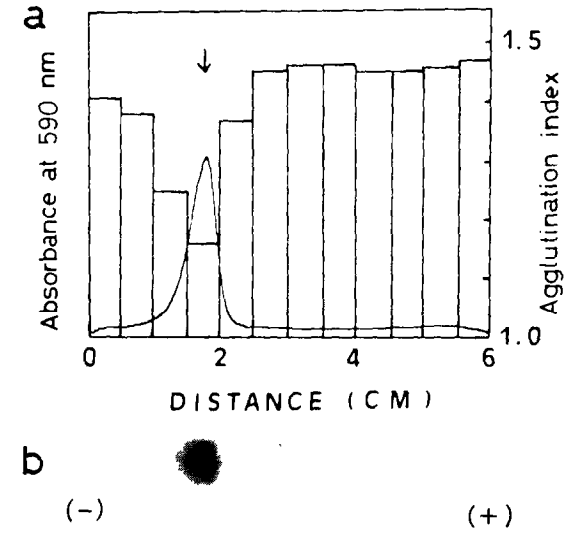

Fig.3. Polyacrylamide gel electrophoresis of α-pheromone-binding protein II in the absence of SDS: (a) densitogram obtained by scanning the gel after staining the protein with Coomassie brilliant blue, and the biological activity expressed in terms of agglutination index (open bars); (b) protein staining with 0.25% Coomassie brilliant blue. Each 5 mm gel disc was homogenized in 1 ml PBS. After centrifugation, the α-pheromone-binding activity of each resultant supernatant was assayed (see section 2). An arrow indicates the segment with the binding activity. Run for 1.5 h at 5 mA/gel.

focusing. Fig.2 shows that the A_{280} peak with pI 4.02 completely coincides with the biological activity. This indicates the successful achievement of the purification. In order to check the purity of a sample obtained by the third isoelectrofocusing, the sample was subjected to 5.6% polyacrylamide gel electrophoresis in the absence of SDS. As shown in fig.3, only one single protein band which had the biological activity was clearly recognized. The profile of SDS–polyacrylamide gel electrophoresis gave again a single protein band of M_r 58000 which is consistent with an M_r-value obtained by gel filtration (fig.4). The above electrophoretic data indicate not only the high purity of the sample but also no subunit structure of the binding protein.

The biological role of the α-pheromone-binding protein and its genetic background are under investigation. The protein is one of the *a* mating-type-specific proteins, thought to be encoded by *a*-

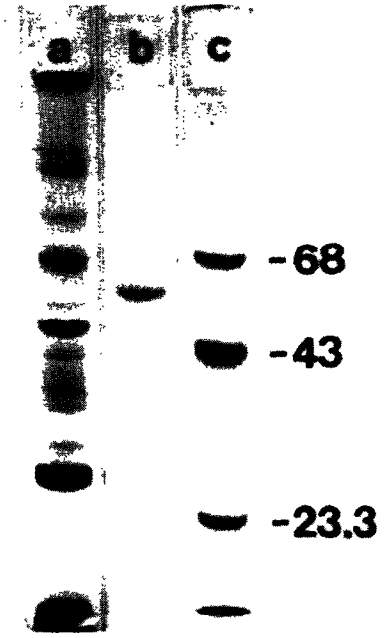

Fig.4. SDS–polyacrylamide gel electrophoresis of purified α-pheromone-binding protein II. Samples from steps 2 and 9, and marker proteins were subjected to electrophoresis as described in section 2: (a) sample from step 2; (b) 3 μg purified α-pheromone-binding protein II; (c) the marker proteins (3 μg), bovine serum albumin, ovalbumin and trypsin with M_r values ($\times 10^{-3}$) of 68, 43 and 23.3, respectively. Run for 2.5 h at 3 mA/gel.

specific genes which are under the control of the mating type locus [18], because we could not detect the protein in α or diploid (a/α) cells (in preparation). *a* Cells are known to show barrier action that is caused by the degradation of α pheromone by *a* mating-type-specific endopeptidase(s) [19–23]. There is no possibility that α-pheromone-binding protein II is the peptidase or its subunit because of the presence of the binding protein II even in *bar* 1-1 *a* mutant cells showing no barrier action (in preparation). We have not yet obtained direct evidence for that α-pheromone-binding protein II is involved in the α pheromone action to induce sexual agglutinability. However, it is highly probable that α-pheromone-binding protein II is one of the best candidates for α pheromone receptor.

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