ISOLATION AND PARTIAL CHARACTERIZATION OF CYTOPLASMIC α AGGLUTINATION SUBSTANCE IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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1. Introduction

Sexual agglutination, which is indispensable for zygote formation [1], occurs through the complementary binding of sex-specific glycoproteins (agglutination substances) on the cell surface of opposite mating types in *Saccharomyces cerevisiae* [2] as well as in *Hansenula wingei* [3,4]. The cell wall agglutination substances were purified and partially chemically characterized in both yeasts [2,5–7]. However cytoplasmic agglutination substances have been neither isolated nor characterized so far, although the presence of the substances was reported [8,9]. The isolation and chemical characterization of cytoplasmic agglutination substances are necessary to know the regulatory mechanism of the production of cell wall agglutination substances.

Here, we describe purification and partial characterization of agglutination substance in the cytoplasm of α-cells in *S. cerevisiae*.

2. Materials and methods

The prototrophic haploid strains of *S. cerevisiae*, T26(α) and T27(*a*) were used, as α and *a* testers, respectively. T26 was used also for the extraction of cytoplasmic α agglutination substance. Cells were grown at 28°C till the late logarithmic phase in YHG with air bubbling as in [2].

2.1. *Assay of biological activity of agglutination substances*

Bioassay was carried out, based on the masking action of α agglutination substance on sexual agglutinability of *a* mating type tester cells having high sexual agglutinability, as in [2] with the following modification. The *a* tester cells treated with a sample to be tested were washed with 10 mM phosphate buffer (pH 5.5) (PBS) twice before mixing with tester α cells, to remove the non-specific substances attaching to the cell surface. To determine mating-type specificity of a sample in the masking action, we treated α tester cells with α agglutination substance. One unit of α agglutination substance was defined as the minimum amount of the substance which masked sexual agglutinability of 1×10^7 cells of the *a* tester cells. Agglutinability was expressed by agglutination index (*AI*) measured after mixing with opposite mating-type tester cells as in [2]. Hence, the lower index indicates higher activity and/or higher contents of agglutination substances in a sample. *AI* is roughly proportional to mean diameter of aggregates [10].

2.2. *Preparation of cytoplasmic extracts*

The 1.5 kg (wet wt) α cells of which wall agglutination substance had been removed by the autoclave method [2,9,11] were washed with PBS buffer 3 times then subjected to freeze–thawing. The thawed cells were suspended in 1.5 l PBS buffer, followed by twice homogenization with glass beads in a Dyno-mill (KDL type, Willy A. Bachofen, Basel) at 2 l/h flow rate. The homogenate was centrifuged at 12 000 × *g* for 30 min and the pellets were washed with 500 ml PBS buffer. The resultant supernatants were pooled. After adjusting the supernatants to pH 2 with conc. HCl, the pellets were removed by centrifugation at 21 000 × *g* for 30 min. The supernatants were neutralized with KOH, then dialyzed against water overnight. All of the above treatments were done at 4°C.

Cells were boiled for 3 min before the above treatments. The boiling which had little effect on sexual agglutinability not only removed non-specific inhibitory substances, but also prevented cells from autolytic changes.

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2.3. *DEAE-cellulose column chromatography*

The dialyzed samples were applied onto a DEAE-cellulose column (7 × 20 cm, OH-type, Brown, NH) pre-equilibrated with 10 mM Tris–HCl buffer (pH 7.0). The adsorbed substances were eluted by addition of 500 ml 0.25 M NaCl in 10 mM Tris–HCl buffer (pH 7.0). After the eluate was dialyzed against water at 4°C, 200 ml of it was applied onto a DEAE-cellulose column (2.5 × 30 cm) and eluted with 400 ml NaCl linear gradient (0–0.25 M) in the Tris–HCl buffer as in [2]. Each 4 ml fraction was collected and the biologically active fractions were combined and dialyzed against water at 4°C, followed by rechromatography on a DEAE-column of the same size. A NaCl linear gradient (800 ml, 0–0.2 M) was applied onto the column. Each 4 ml fraction was collected.

2.4. *Gel filtration*

The biologically active fractions obtained by the DEAE-cellulose column chromatography were combined and concentrated with an Amicon ultrafiltration cell (Amicon, Lexington MA) by using a membrane UM 20 [2]. The concentrated sample dissolved in 3 ml 10 mM Tris–HCl buffer (pH 7.0) containing 8 M urea, 5 mM EDTA and 0.02% sodium azide was applied onto a Sephacryl S-300 column (2.5 × 75 cm, Pharmacia Fine Chemicals, Uppsala) pre-equilibrated with 10 mM Tris–HCl buffer (pH 7.0) containing 6 M urea, 5 mM EDTA and 0.02% sodium azide (elution buffer). The biologically active fractions (each 3.5 ml) were concentrated by the ultrafiltration as above, then 3 ml 10 mM Tris–HCl buffer (pH 7.0) containing 8 M urea, 5 mM EDTA and 0.02% sodium azide was added to the concentrated sample. The sample was applied onto a Sepharose CL-6B column (2.5 × 77 cm, Pharmacia Fine Chemicals, Uppsala) pre-equilibrated with the above elution buffer. Each 3.5 ml fraction was collected. The active fractions concentrated by the ultrafiltration after combining were dissolved in 3 ml 8 M urea buffer. After 1 h incubation at 40°C, the sample was subjected to the second cycle of Sepharose CL-6B gel filtration. Each 3.5 ml fraction was collected.

2.5. *Electrophoresis*

Polyacrylamide gel electrophoresis was done as in [12] with a slight modification on 6 × 0.5 cm disc gel containing 5.6% polyacrylamide. Urea (8 M) was used instead of sodium lauryl sulfate and 2-mercaptoethanol was omitted.

2.6. *Isoelectrofocusing*

The lyophilized active samples were dissolved and electrofocused in a pH 3.5–5 linear gradient made by Ampholine carrier ampholyte (LKB, Bromma) in a 0–50% discontinuous sucrose gradient column (2.2 × 28 cm) for 40 h constant 900 V.

2.7. *Carbohydrate and protein contents*

Carbohydrate and protein contents were determined by the phenol/sulfuric acid method [13] and the Lowry method [14], respectively.

2.8. *Treatment with enzymes and chemicals*

Glycosidase 'mixed' (Seikagaku Kogyo, Tokyo) (50 μg/ml final conc.), zymolyase 60 000 (Seikagaku Kogyo, Tokyo) of pronase (Kaken Kagaku, Tokyo) were added to PBS solutions (~2 units/ml) of the cytoplasmic α agglutination substance purified by the Sepharose CL-6B gel filtration, followed by incubation at 37°C for 60 min. The glycosidase 'mixed' is a mixture of mannosidase, galactosidase and *N*-actylglucosaminidase as major components and 4 other glycosidases as minor components. The enzyme reaction was terminated by heating the mixtures in boiling water for 5 min. 2-Mercaptoethanol (final conc. 5% (v/v)) was added to the purified substance (~2 units/ml) to determine whether 2-mercaptoethanol inactivated the substance. The mixture was boiled for 1 min, and then dialyzed against water at 4°C. The agglutination substances treated as above were subjected to the measurement of their biological activity to mask sexual agglutinability of *a* tester cells.

3. Results and discussion

The process of the purification with the changes of specific activity and recovery is shown in table 1. Specific activity of the purified cytoplasmic α agglutination substance was 10^3-times higher than that of the starting material, the supernatant of the cell homogenate. To check the purity of the active fraction obtained through the final step of Sepharose CL-6B column chromatography, analysis by polyacrylamide gel electrophoresis was done. Laemmli's method [15] was not applicable because the applied substance was stacked on the gel top. Electrophoreses

Table 1
Purification of cytoplasmic agglutination substance of α mating type

Step	Vol. (ml)	Protein (mg)	Act. (units)[a]	Spec. act. (units/mg protein)	Purification (-fold)
Crude[b]	1000	2×10^4	2×10^5	10[c]	1
pH 2.0	1000	10^4	2×10^5	20[c]	2
DEAE-cellulose (3 cycles)	100	23	4×10^4	1740	174
Gel filtration (Sephacryl S-300)	10	2	8000	4000	400
Gel filtration (Sepharose CL-6B) (2 cycles)	10	0.5	5000	10 000	1000

[a] For unit see section 2
[b] Supernatants of cell homogenate from 500 g wet cell paste
[c] Background non-sex-specific activity to inhibit agglutinability was subtracted

according to other recipes including [12] gave only diffusible bands stained with Coomassie brilliant blue and Schiff's reagent, which migrated little. The poor migration and diffusible band were probably due to the high carbohydrate content of the substance (~75%). Furthermore, the substance had a strong tendency to form aggregates. To overcome these problems, we carried out electrophoresis by a modified method of [12] in the presence of 8 M urea as in section 2. Under this condition, BPB marker dye migrated from the top to the bottom within 1.5 h at 5 mA/gel while most proteins found after the first DEAE-column chromatography still remained in the top region (0–2.5 cm from the top) of the gel as shown in fig.1(1). The purified substance migrated a little after 7 h run (fig.1(2–4)). However, the profile clearly showed a single band which was stained with both Coomassie brilliant blue (fig.1(2)) and Schiff's reagent (fig.1(3)). The sample extracted from the glycoprotein position of the gel in a parallel run showed the activity to mask sexual agglutinability of *a* cells as shown in fig.1(4). These results indicate that the purified substance was a glycoprotein. 2-Mercaptoethanol gave little effect on the profile and activity of the substance on the gel electrophoresis, indicating that the purified substance was not cleaved by 2-mercaptoethanol.

The properties of cytoplasmic agglutination are essentially the same as those of wall agglutination substance except carbohydrate content and M_r-value as shown in table 2. The contamination of the wall substance in the cytoplasmic substance was virtually excluded because most wall substance was removed by autoclave method before the extraction of the cytoplasmic agglutination substance. Furthermore, the purified cytoplasmic substance was different from the wall substance in molecular weight and carbohydrate content (see table 2).

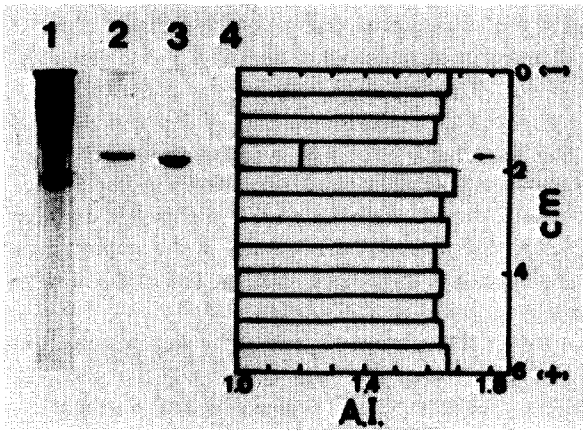

Fig.1. Polyacrylamide gel electrophoresis of the purified cytoplasmic α agglutination substance. The purified substance (~20 μg/gel in the case of sugar staining and ~50 μg/gel in the case of protein staining) was applied onto 5.6% gel columns. After the run, gels were stained with dyes, or cut into segments and homogenized in PBS buffer to assay biological activity of each homogenate. [(1), the first DEAE-cellulose fraction (section 2); (2–4), the purified sample] (1) Gel stained with Coomassie brilliant blue, run for 1.5 h at 5 mA/gel; (2) gel stained with Coomassie brilliant blue, run for 7 h at 5 mA/gel; (3) gel stained with Schiff's reagent, run for 7 h at 5 mA/gel; (4) Biological activity in each gel segment. The arrow indicates the segment with biological activity.

Table 2
Comparison of cytoplasmic agglutination substance (a.s.) with wall a.s. from α cells

	Wall a.s.[a]	Cytoplasmic a.s.
M_r	130 000	200 000
Characteristic of proteins	Glycoprotein	Glycoprotein
Carbohydrate content	47%	75%
Binding activity	Univalent	Univalent
pI	4.3	4.3
Effects of enzymes and chemicals		
2-mercapto-ethanol	Stable	Stable
Glycosidase (50 μg/ml)	Stable	Stable
zymolyase (50 μg/ml)	Stable	Stable
pronase (50 μg/ml)	Unstable	Unstable

[a] Data from [16]

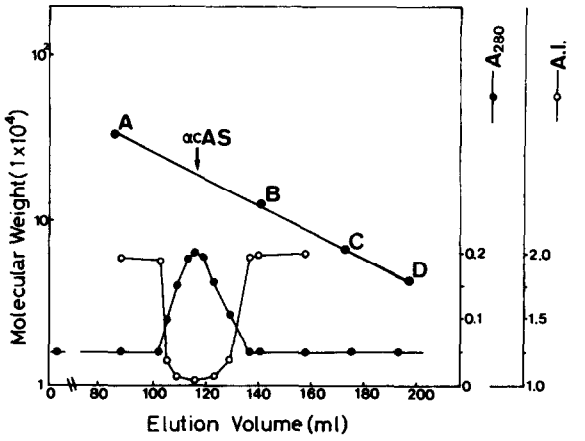

Fig.2. M_r estimation of cytoplasmic α agglutination substance on a Sepharose CL-6B column. The profile shows the second gel filtration on the Sepharose CL-6B of cytoplasmic α agglutination substance (~10 mg). 1/20 diluted sample of each fraction was used for bioassay (*AI*) (A–D) subunit of thyoglobulin, subunit of galactosidase, bovine serum albumin and ovalbumin, respectively. αcAS indicates cytoplasmic α agglutination substance.

The cytoplasmic agglutination substance was estimated to have M_r ~ 200 000 by gel filtration on a Sepharose CL-6B gel column (fig.2). The substance showed a single peak with pI of 4.3 by isoelectric focusing in carrier ampholyte (pH 3.5–5) (not shown). The treatment with pronase completely destroyed the biological activity of the substance. On the other hand, neither glycosidase 'mixed' nor zymolyase-60 000 caused any changes in the activity of the substance (see table 1). These suggest that the active site of the substance is not in the carbohydrate moiety but in the protein moiety. This is the same as in the case of the wall substance.

Since the cytoplasmic substance was isolated from autoclaved cells, there was a possibility that the cytoplasmic substance might change by heat during the autoclaving. To check this possibility, intact cells were homogenized directly in PBS buffer in the presence of the proteinase inhibitor, 2 mM phenylmethylsulfonyl fluoride (PMSF). After successive centrifugation, acid precipitation and DEAE-cellulose chromatography, the cytoplasmic agglutination substance was subjected to gel filtration on a Sepharose CL-6B column. The profile clearly showed the presence of an agglutination substance of 200 000 M_r at the position corresponding to the cytoplasmic substance from autoclaved cells (not shown). Without PMSF, agglutination substances of <200 000 M_r were observed, indicating enzymatic modification of the agglutination substance. This indicates that the autoclaving had little deterioration effect on the substance and, further, protected the substance from the digestive enzymes.

The product–precursor relationship between cytoplasmic and wall agglutination substance is unclear. The M_r of the cytoplasmic agglutination substance is higher than that of the wall substance. A large precursor may be processed before integration into the cell wall. The alternative explanation is that the carbohydrate moiety of the wall agglutination substance was shortened during the release process by autoclaving. Anyhow, the fact that the biologically active agglutination substance is present in cytoplasm suggests that the substance, at least the active site of the substance, is synthesized in cytoplasm and then incorporated into cell wall to give cells sexual agglutinability. The isolation and characterization of cytoplasmic agglutination substance from *a* cells are in progress.

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