ISOLATION AND PURIFICATION OF THE SEXUAL AGGLUTINATION SUBSTANCE OF MATING TYPE a CELLS IN SACCHAROMYCES CEREVISIAE

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The substance responsible for the sexual agglutinability was successfully solubilized by a newly established autoclaving method from the surface of mating type a cells of Saccharomyces cerevisiae and purified by DEAE cellulose chromatography, gel filtration, affinity chromatography and electrophoresis. The substance was found to consist of at least two different glycoprotein subunits. The molecular weight of the substance was estimated to be about 23,000 daltons by gel filtration. The substance was univalent in its biological activity and specifically masked the sexual agglutinability of the mating type α cells. The substance formed a complementary complex with the agglutination substance from α cells in vitro.

In animal as well as in plant cells, and microoganisms, cell surface glycoproteins have been shown to play important roles in the intercellular recognition process (1). The sexual intercellular recognition which brings about sexual agglutination between a and α cells is considered to be indispensable for the subsequent process of the mating reaction, cell fusion and karyogamy in Saccharomyces cerevisiae (2-5). The agglutination has been believed to take place through a complex formation between the sex-specific substance responsible for sexual agglutination (agglutination substance or mating factor) on the cell surface of each mating type, as well as in the yeast Hansenula wingei (6-12). In H. wingei, multivalent and univalent agglutination substances were enzymatically solubilized from the cell walls of 5 and 21 type cells respectively, and purified to be glycoproteins (6-12). The formation of a molecular complex between 5 and 21 agglutination substances in vitro has been detected by gel filtration (12).

Attempts at solubilization of the substances from cell walls by snail gut enzymes have been tried in S. cerevisiae (13). The released substances were univalent and unfortunately had extremely high molecular weights (more than 1×10^6 daltons) that was probably due to the contamination of non-functional wall components (13). This caused a difficulty in the purification of the substances

and the detection of the molecular complex between the opposite mating types. The detection of the molecular complex is indispensable for the agglutination substances, especially in the case of univalent substances responsible for sexual agglutination.

In this paper, we describe the isolation and purification of the sexual agglutination substance of a cells of S. cerevisiae from the crude substances released by the newly established autoclaving method. The formation of a molecular complex between agglutination substances from a and α cells in vitro is also included in this paper.

MATERIALS AND METHODS

Organisms: Two prototrophic heterothallic haploid strains of Saccharomyces cerevisi T26(α) and T27(a) were used throughout the experiment. Homothallic diploid strain, D8 was also used.

Culture condition: 10ml of a 2-day-old preculture of a or α cells in YHG medium(2) was inoculated into each 5 liter of YHG medium. The inoculated cells were cultured for 20 hr at 28° C with air bubbling. At the end of the culture, about 500g wet weight cells were harvested from 25 liter culture medium.

Solubilization of agglutination substances: After washing with water, about 500g wet weight cells were resuspended in 500ml of 10mM phosphate buffer, pH 5.5 (PBS) and then boiled for 5min. The cells were immediately chilled in crushed ice and then washed with 4 liters of PBS. The washed cells were then suspended in 500ml of 30mM Tris-HCl buffer solution (pH 7.0) containing 5mM EDTA and 1M urea, and the cell suspension was autoclaved in an Autoclave HL36 (Hirayama, Tokyo, Japan) at a pressure of 1kg/cm^2 at 120° C for 5min. After autoclaving, the cell suspension was immediately chilled in crushed ice and then centrifuged. The cell pellets were wash with 100ml of the above Tris-EDTA-Urea buffer. The resultant combined supernatant was used as starting material for the purification and characterization of the agglutination substances.

Assay of biological activity of agglutination substances: The biological activity of the agglutination substance is given by the masking action on the sexual agglutinability of the respective opposite mating type cells, since a and α substances are univalent in the binding action (see Table 1, 13-14). The sexual agglutinability is expressed in terms of the agglutination index which was measured based on the decrease in optical density due to the formation of cell aggregates (2, 13-14). The mixture for the agglutinability test contained 1 ml of each sample, 0.2ml (optical density at 530nm, OD_{530}=5/ml) of the tester cells of the opposite mating type and 2.6ml PBS. After shaking for 1 hr, 0.2ml (OD_{530}=5/ml) of tester cells of the opposite mating type was added and shaken for 2 hr to measure agglutination index. The agglutination index was the ratio of OD_{530} after sonication of the reaction mixture to that before sonication as described previously (13-14). When the index was above 1.2, large cell aggregates were detectable with the unaided eye. On the other hand, no cell aggregates was microscopically observed when the index was below 1.10 or the mixture was sonicated. The lower the agglutination index the higher the activity and/or content of agglutination substances in the sample, when agglutination index of tester cells treated with the sample containing the agglutination substances of the opposite type cells was measured. One unit of the substance was defined as the minimum amount of the substance which completely masked the agglutinability of 1x10^7 cells of the opposite mating type.

DEAE cellulose column chromatography: After adjusting pH of the material solubilized from a or α cells by autoclaving to 1.5 with HCl, the engendered pellets were

removed by centrifugation. The resultant supernatant was neutralized with NaOH and then dialyzed against water. An aliquot of the neutralized supernatant or solubilized sample without the acid treatment was applied onto a DEAE cellulose column (30x2.5cm, OH type, Brown, New Hampshire, USA) which had previously been equilibrated with 10mM Tris-HCl, pH 7.0. A NaCl gradient in 10mM Tris-HCl (pH 7.0) was applied onto the column. Biological activity, protein, and carbohydrate contents of each fraction (4ml) were estimated by the agglutination index, absorbance at 280nm and phenol sulfuric method (15), respectively.

Ultrafiltration: Biologically active fractions separated by DEAE cellulose column chromatography were pooled and concentrated by an Amicon ultrafiltration cell (Amicon, Lexington, USA) by using the membrane UM20 which cut off molecules lower than 10,000 to 20,000 daltons.

Gel filtration: Concentrated samples by the ultrafiltration or marker proteins (Schwartz-Mann, New York, USA) were applied onto a Ultrogel column (73x2.5cm, AcA 34, LKB, Bromma, Sweden) which had been previously equilibrated with 10mM phosphate buffer (pH 5.5) containing 0.1M NaCl and 0.025% sodium azide as described previously (16). Each 4ml fraction was collected.

Affinity chromatography on Con-A Sepharose: Biologically active fractions separated by gel filtration were dialyzed against water and made up to 10mM Tris-HCl buffer (pH 7.0) containing 0.1M NaCl, 1mM $MnCl_2$ and $CaCl_2$, and then applied onto a Con-A Sepharose column (Concanavalin A covalently bound to Sepharose, 4x3.5cm, Pharmacia, Uppsala, Sweden). Adsorbed fraction was eluted with 0.1M α-methyl-D-mannoside (Sigma, St. Louis, USA), dialyzed against water and then lyophilized or concentrated by ultrafiltration. The sample for gel filtration or polyacrylamide gel electrophoresis was taken from the above fraction.

Electrophoresis: The lyophilized or concentrated samples were made up to 0.04M Tris, 0.02M sodium acetate, 0.01M EDTA and 1M urea (pH 7.2) and then applied onto 3 separate 5.6% polyacrylamide gel columns (6x0.5cm) which had been previously polymerized in the presence of 0.04M Tris, 0.02M sodium acetate, 0.025% TEMED, 0.15 % ammonium persulfate, 0.21% Bis and 1M urea. Electrophoresis was carried out in a buffer 6g Tris, 28.8g glycine in 1 liter water (pH 8.6) at 5mA/column for 1 hr. After the run, the first gel was stained with Coomassie Brillant Blue (16). After destaining, the gel was scanned with a Toyo densitometer (Toyokagaku, Tokyo, Japan) (17). The second one was cut with razor blades. The cut gel segments were homogenized with water to estimate the biological activity. The third one was fixed with 10% TCA and stained with Schiff's reagent after periodate treatment. SDS polyacrylamide gel electrophoresis was carried out according to a slightly modified method (16) of Laemmili (18).

RESULTS AND DISCUSSION

Solubilization of agglutination substances from a and α cells by autoclaving - Cells suspended in the extraction buffer were autoclaved for various times. The autoclaved cell suspensions were immediately chilled in crushed ice and then centrifuged to remove cells. The biological activity of the resultant supernatant was tested. As shown in Fig. 1 and Table 1, the substances of a and α cells which specifically inhibited agglutination of the respective opposite mating type cells were successfully solubilized by the autoclaving. Each substance which was univalent and sex-specific, primarily came from cell surface (14). The inhibition of cell agglutinability by the substances is not due to their hydrolase activity because cells once masked by the substances almost completely restored their agglutinability by the incubation at pH 9.5 as shown in Table 1 (see also Fig. 6).

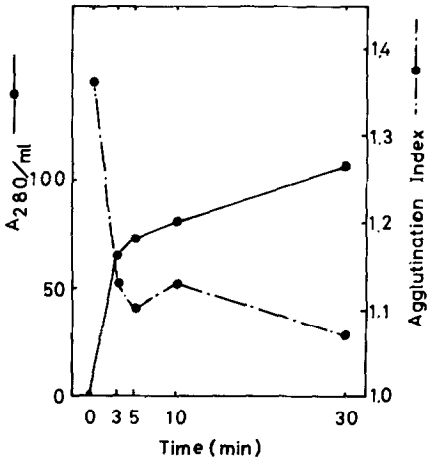

Fig. 1. Release of agglutination substance from a cells by autoclaving. An aliquot of a cells in 30mM Tris-HCl (pH 7.0) containing 5mM EDTA and 1M urea was autoclaved for various times. The autoclaved cell suspension was centrifuged. The biological activity and absorbance of the resultant supernatant were measured.

Table 1. Release of agglutination substances from a and α cells by autoclaving.

Source strains	Agglutination Index			
	Tester cells used for the agglutination			
	T26 (α)		T27 (a)	
	L	B	L	B
T27 (a)	1.08	1.05	1.34	1.35
T26 (α)	1.33	1.30	1.08	1.09
D8 (diploid)	1.36	1.41	1.40	1.42
T27 (a)*	1.40	1.38	1.37	1.41
T26 (α)*	1.39	1.42	1.38	1.37
Control (extraction buffer)	1.37	1.39	1.38	1.39

Extraction buffer and crude samples obtained from T26 (α), T27 (a) and D8 cells by autoclaving were mixed with tester cells and shaken for 1 hr. The treated cells were washed with PBS and tester cells of the opposite mating type were added. After shaking for 2 hr, the agglutination indices were measured. In the case of T27 (a)* and T26 (α)*, crude samples obtained from T27 (a) and T26 (α) were mixed with tester cells and shaken for 1 hr. The treated cells were incubated in 10 mM Tris-HCl buffer (pH 9.5) for 1 hr and washed with PBS. The agglutination indices measured as described above. L, Living cells; B, Boiled cells.

The optimum releasing conditions of the substances from a and α cells were described elsewhere (14). To prepare mannan, yeast cells were autoclaved at 140° C for 2 hr (19). In our autoclaving method, the same releasing mechanism is probably working.

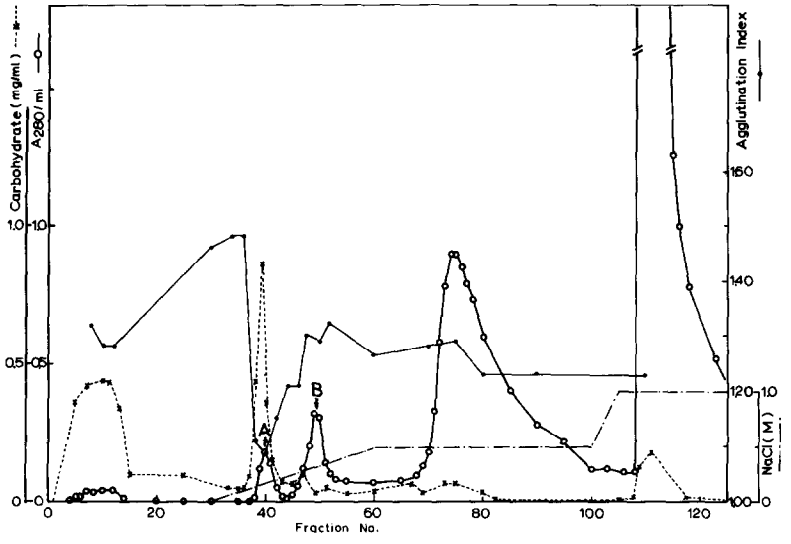

Fig. 2. DEAE cellulose column chromatography of released substances from a cells by autoclaving. The released substances from a cells by autoclaving were applied onto a DEAE cellulose column.

Purification of agglutination substance from a cells - (a) DEAE cellulose column chromatography: The sample released from a cells by autoclaving was directly applied onto DEAE cellulose column chromatography (Fig. 2). The first peak (peak A) which contained a considerable amount of carbohydrates, had biological activity while the other fractions had no activity. On the other hand, although the chromatographic profile of the sample from α cells was essentially the same as that from a cells, biological activity was found in the second peak (peak B) in this case (see also Fig. 6).

(b) Gel filtration: The pH value of the supernatant of the autoclaved suspension was adjusted to 1.5 with HCl and the resultant insoluble precipitate was removed by centrifugation. After neutralizing with NaOH, the supernatant was applied

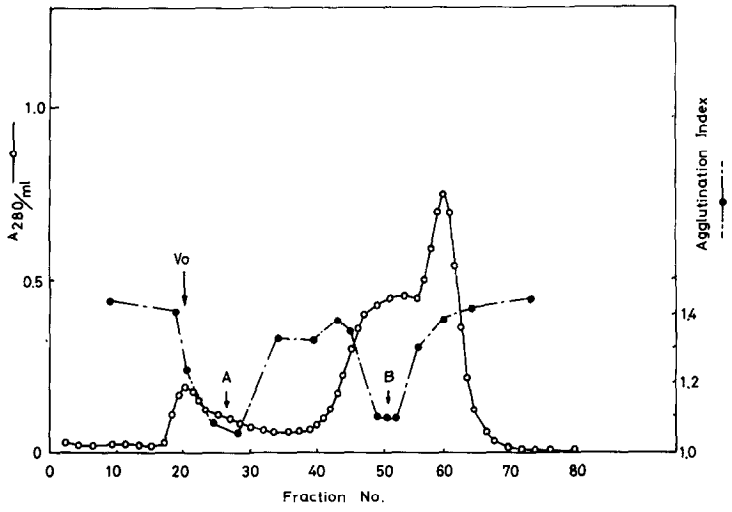

Fig. 3. Gel filtration of biologically active fraction from a cells separated by DEAE cellulose chromatography. The biologically active fractions (fraction A in Fig. 2) by DEAE cellulose chromatography were pooled and concentrated. The concentrated sample was applied onto a Ultrogel column.

onto DEAE cellulose chromatography. The biologically active fraction was concentrated by ultrafiltration and then applied onto a Ultrogel column as shown in Fig. 3. Two distinct biologically active fractions, A and B, separated. Fraction A, which eluted just behind the void volume, probably contained aggregates of agglutination substances and/or substances derived from non-functional wall components, because considerable amounts of the applied sample eluted at the same position as fraction A when fraction B was recycled onto the Ultrogel column.

(c) Affinity chromatography: Fig. 2 suggests that the biologically active substance contained carbohydrates. If so, affinity chromatography on Con-A Sepharose can be useful for further purification of the agglutination substance. Fraction B separated by the gel filtration as above was applied onto a Con-A Sepharose column. The adsorbed substance which had biological activity was successfully eluted with 0.1M α-methyl-D-mannoside as shown in Fig. 4. This indicates that the biologically active substance contains carbohydrates.

(d) Electrophoresis: In order to check the purity and subunit structure of the

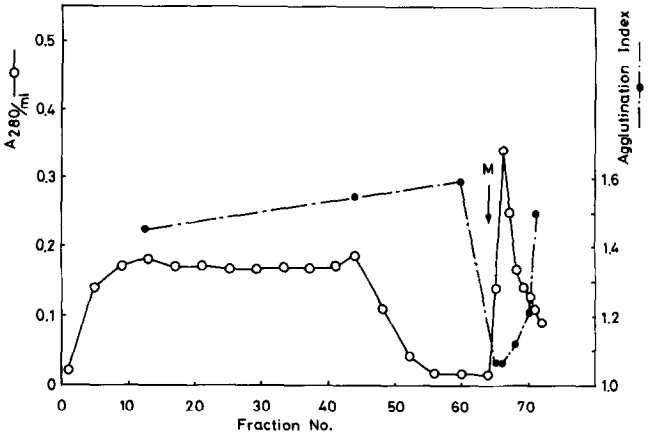

Fig. 4. Affinity column chromatography on Con-A Sepharose of biologically active fractions separated by gel filtration. The biologically active fractions (fraction A in Fig. 3) were pooled and then, applied onto a Con-A Sepharose column. The arrow M indicates the start of 0.1M α-methyl-D-mannoside addition.

substance, the biologically active substance purified as above was applied onto polyacrylamide gel electrophoresis. As shown in Fig. 5(a) and (b), the biological activity was completely consistant with a protein band stained with Schiff's reagent. Electrophoretic profile on SDS polyacrylamide gel is shown in Fig. 5(c). Two sharp protein bands were stained with Schiff's reagent (Fig. 5(d)). There were also non-movable carbohydrate moieties on the top of the gel (Fig. 5(d)).

Formation of complementary complex between a and α agglutination substances in vitro - In order to detect complex formation between a and α agglutination substances in vitro, equal units of a and α substances partially purified by lowering pH as described above were mixed and shaken for 2 hr. The mixture was successively shaken for 1 hr with a and α cells to remove the free substances by adsorption to the opposite mating type cells. After removing the cells by centrifugation, the lack of biological activity in the mixture was confirmed. Then, the pH of the mixture was shifted from 5.5 to 9.5 with NaOH and shaken for 1 hr in order to disrupt the binding linkage between a and α agglutination substances. The mixture was directly applied onto a DEAE cellulose column. As shown in Fig. 6, a and α substances were recovered on the corresponding positions at which independently

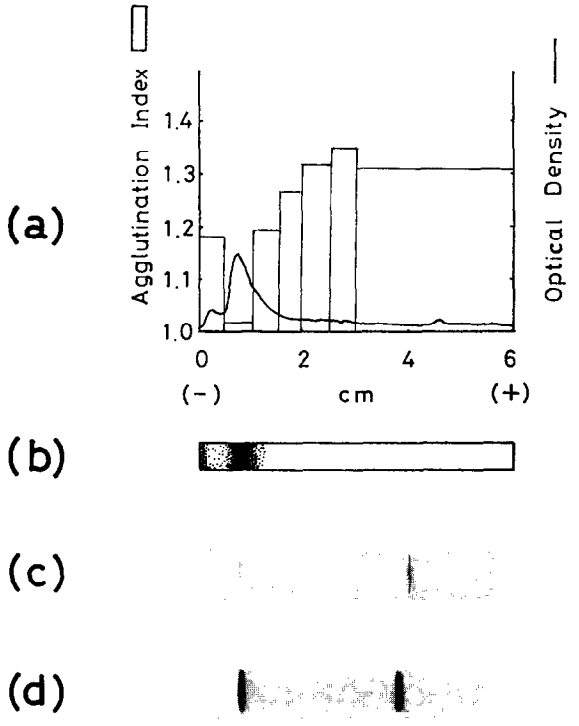

Fig. 5. Polyacrylamide gel electrophoresis of purified a agglutination substance. Purified a agglutination substance (about 50-100 μg/each gel) was applied onto 5.6% gel columns. After the electrophoretic run, gels were stained with dyes or cut into segments in order to assay the biological activity. (a) Superimposed biological activity of each gel segment into a densitometer scanning profile of gel stained with Coomassie Brillant Blue. 5.6% gels were used. (b) Gel stained with Schiff's reagent. 5.6% gel was used. (c) Gel stained with Coomassie Brillant Blue. 7.5% gel in the presence of SDS was used. (d) Gel stained with Schiff's reagent. 7.5% gel in the presence of SDS was used.

applied a or α substance was eluted (compare with Fig. 2), although the profiles of the absorbance at 280 nm was slightly disturbed possibly by contamination during the shaking process with cells. The fact that the biological activity of both a and α agglutination substances, which had once disappeared by mixing with each other, reappeared indicates that a molecular complex between a and α substance was actually formed in vitro. This finding strongly supports the notion that agglutination substances on a and α cell surface are directly involved in the sexual agglutination.

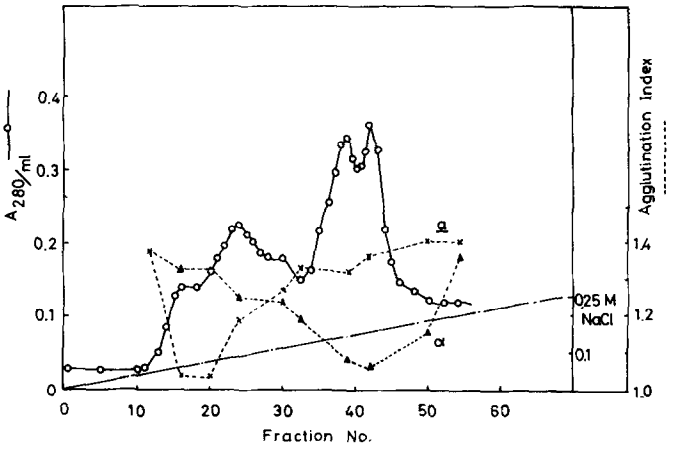

Fig. 6. DEAE cellulose chromatography of a and α agglutination substance which were recovered from a-α agglutination substance complex. The partially purified a and α agglutination substances were mixed and shaken to form complex. After shaking, the mixture was successively shaken with a and α cells to remove free substances. After confirmation of the lack of biological activity in the mixture, pH of the mixture was shifted to 9.5 in order to break linkages of a-α substance complex. Then, the mixture was applied onto DEAE cellulose chromatography. Biological activity of each fraction was assayed.

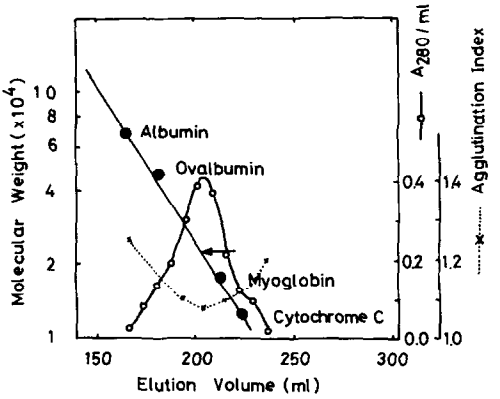

Fig. 7. Molecular weight estimation of a agglutination substance. Molecular weight marker proteins or a agglutination substance were applied onto a Ultrogel column. Calibration curve was constructed. The arrow indicates the position of a agglutination substance.

a and α agglutination substances are functionally and chemically different from each other (Fig. 2, 5 and 6).

Molecular weight of agglutination substance from a cells - The agglutination

substance a purified as above and marker proteins were applied onto the Ultrogel column. As shown in Fig. 7, the molecular weight of the agglutination substance a was about 23,000 daltons. SDS polyacrylamide gel electrophoresis (Fig. 5(c) and (d)) indicates that a agglutination substance consists of at least two glycoprotein subunits with a similar molecular weight. At present, we cannot resolve how many subunits are contained in the functional a substance because the molecular weights of the subunits have not yet been determined precisely.

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