122. Isolation of a Peptidyl Factor Controlling Mating Tube Formation in Rhodosporidium toruloides

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The yeast, Rhodosporidium toruloides, has a sexual generation in its life cycle, and haploid strains in this yeast are classified into two mating types (type A and type a). When haploid cells of the opposite types are mixed with each other, each cell forms a mating tube, which recognizes the tube of mating partner to bring about sexual conjugation, followed by cell-to-cell fusion. In consequence, the ovoid form of haploid cells becomes filamentous as diploid cells. Through the investigation on the morphological change in the early stages of the mating process, Abe et al. demonstrated the involvement of the following events: 1) A type cells (M-919 strain) secrete constitutively a substance named A factor; 2) in response to A factor, a type cells (M-1057 strain) form mating tubes and, at the same time, secrete another substance named a factor; 3) A type cells receive a factor and form mating tubes; 4) each mating tube elongates to the cell or tube of mating partner resulting in cell fusion.

With an intention to elucidate the mating process in Rhodosporidium toruloides from a chemical standpoint, we attempted the isolation of A factor which plays an important role as a trigger to induce sequential events mentioned above. As the result, the substance was obtained successfully as a peptide capable of inducing mating tube formation in a type cells even at a concentration of 0.06 µg/ml. In this paper, we describe preliminarily our experimental result leading to the isolation of A factor from culture filtrates of A type cells.

The isolation procedure is illustrated in Fig. 1. At each step of purification, biological activity was assayed in the following way. Cells of a type strain (M-1057) were cultured in a 50-ml Erlenmeyer flask containing 8 ml of YS medium (1% yeast extract, 1% sucrose and 0.25% sodium chloride) at 27°C for 16 hr with reciprocal shaking. The cultured fluid was diluted with YS medium to adjust cell population to 2×10^6 cells/ml. Five ml of this cell suspension was mixed with the same volume of YS medium containing 3% agar, and

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the mixture was poured into a Petri dish (10 cm diameter). The solid agar film thus obtained was cut into pieces of 5 mm squares, and each two pieces were put into a small glass tube containing a test sample dissolved in 1 ml of YS medium. After incubation at 27°C for 5 hr, the response of the a type cells in each agar piece was examined under a microscope (x 300). Distinct formation of filamentous mating tubes from the cells was observed, if the test sample contained A factor. Further, the concentration of the factor was estimated semi-quantitatively by serial dilution of the sample solution.

Prior to the isolation, the stability of A factor at various pHs as well as in various solvents was examined by the measurement of biological activity (Table I). The factor was found to be extremely labile under acidic conditions: it lost completely the biological activity when kept in acetic acid or ethyl acetate for 2 hr at 40°C. The instability in ethyl acetate may be due to the presence of a trace amount of acetic acid liberated spontaneously.

For the large scale preparation of A factor, M-919 cells were used.
cultured in a 30-liter jar fermentor charged with 18 liter of YS medium under aeration (18 liter/min) and rotation (400 rpm) at 28°C for 20 hr. After repeated four runs, culture filtrates were combined, adjusted to pH 7 and extracted with two 25-liter portions of n-butanol. The combined extracts were evaporated to 2 liter and were washed with a small volume of water. On evaporation, the butanol solution yielded 9.0 g of crude powder, which was partitioned between chloroform and water. The aqueous phase showing biological activity was lyophilized and subjected to gel filtration using Sephadex LH-20 and methanol. The front fractions were combined and partitioned with benzene-n-hexane-methanol-water (10:10:8:2). The lower phase was subjected to column chromatography on Avicel by the successive use of the solvent systems of n-butanol-water (20:1, 10:1, and butanol saturated with water). The combined active fractions were charged onto a CM Sephadex column, and the column was developed with methanol-0.01 M ammonium acetate (1:1) (Fig. 2). The middle fractions showing activity were combined and subjected to gel filtration on Sephadex LH-20 with methanol (Fig. 3).

Thus, 6 mg of pure A factor was obtained as an almost colorless powder, whose homogeneity was confirmed by thin layer chromatography (TLC): Avicel (Funakoshi, Avicel SF) with sec-butanol saturated with water; silica gel (Merck GF254) with n-butanol-acetic acid-water (4:1:5, upper phase). Further, thin layer electrophoresis of the powder on an Avicel plate at pH 6.5 revealed only one spot. Unexpectedly, the biological activity was undetected in any
fractions, when the powder was subjected to gel filtration on Sephadex LH-20 with methanol-water (1:1). Then, all the fractions were
combined and applied to TLC on an Avicel plate, which revealed new spots below that of A factor, suggesting the occurrence of decomposition in the factor. This change might be due to the action of the free carboxyl functions on Sephadex LH-20 in aqueous methanol without a buffer solution. The assumption was confirmed by the treatment of A factor with 1 M acetic acid, followed by TLC on an Avicel plate, which showed the same pattern as in the case of the Sephadex (Fig. 4).

Pure A factor thus obtained induced the mating tube formation in M-1057 cells at a concentration of 0.06 μg/ml. The factor was considered to be a peptide, since it gave, on thin layer chromatograms, faint coloration with ninhydrin reagent and deep coloration with chlorine-o-tolidine reagent. It showed a ultra violet absorption peak at 278 nm (E\text{1%} 27.9, methanol) with two shoulders at 275 and 300 nm, suggesting the existence of tyrosine and tryptophan in its molecule. On hydrolysis with 6 N hydrochloric acid at 105°C for 20 hr, the factor yielded tyrosine, serine, proline, glycine, alanine, valine, isoleucine, leucine, phenylalanine, arginine, lysine, threonine, aspartic acid and glutamic acid.

As for sex hormones in the yeast, Duntze et al.\textsuperscript{2} isolated, from \(\alpha\) type cells of \textit{Saccharomyces cerevisiae}, a cupric ion-containing pep-
tide named α factor, which enhances cell elongation and inhibits DNA synthesis in the opposite α type cells. Furthermore, Sakurai et al. succeeded quite recently in the isolation, from α type cells of the same kind of yeast, of a factor named α substance-I, which induces sexual agglutinability in α type cells at the initial stage of the mating reaction, and they concluded that the substance should be a peptide. It is very interesting that peptidyl nature is a characteristic common to the sex factors hitherto isolated from yeasts including *Rhodosporidium toruloides*.

In conclusion, from culture filtrates of A type cells of *Rhodosporidium toruloides* was isolated, through our experiment, a peptidyl substance (A factor), which induced distinctly the formation of mating tubes in the opposite α type cells at very low concentrations. It is worthy of note that the factor is extremely unstable, as compared with usual peptides, under acidic conditions and is easily decomposed resulting in the complete loss of biological activity. This suggests the existence of unusual component or linkage in the molecule of the factor. Investigation on the decomposition products from A factor with acetic acid is in progress. The result will give us useful informations on the stability as well as on the structure of the original compound.

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