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Metabolites of Mating Pheromone, Rhodotorucine A, by a Cells of Rhodosporidium toruloides

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SUMMARY Rhodotorucine A which induces mating tube formation of a cells in Rhodosporidium toruloides is metabolized rapidly by a cells. By use of labeled rhodotorucine A, the degradation was found to be proteolytic. Two peptide fragments Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg and Asn-Gly-Cys(S-farnesyl) were identified as the metabolites. Proteolysis of the pheromone mainly occurred on the cell surface. Culture filtrate of a cells at log phase did not metabolize rhodotorucine A.

Haploid cells of the basidiomycete yeast, Rhodosporidium toruloides, exhibit one of two mating types designated A and a (1). At the initial stage of the mating process, A cells secrete a mating pheromone, rhodotorucine A, which induces mating tube formation of a cells (2). Rhodotorucine A inhibits the budding growth of a cells and lead them from vegetative growth to sexual growth (3). The chemical structure of rhodotorucine A was determined by us as H-Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg-Asn-Gly-Cys(S-farnesyl)-OH (4-7).

When rhodotorucine A was incubated with a cells for several minutes in YS medium, the supernatant of the culture did not have any biological activity of rhodotorucine A (8). This result suggested that rhodotorucine A was completely adsorbed on a cells or metabolized into inactive substances by a cells. To seek the fate of rhodotorucine A, labeled pheromones were prepared *in vivo* using [^{35}S]cystine, [^{3}H]tyrosine and [^{14}C]arginine. In this paper, we wish to report the chemical structures of the metabolites of rhodotorucine A metabolized by a cells.

MATERIALS AND METHODS

Preparation of labeled rhodotorucine A Mating type A cells (IFO M919) were grown for 15 hrs at 27°C in 50 ml of YS medium (9) containing 100 μCi of [^{35}S] cystine (94 mCi/mmol), [U-^{14}C]arginine (250 mCi/mmol) or [^{3}H]tyrosine (53 mCi/mmol). Following removal of the cells by centrifugation, the culture fluid

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was extracted with n-BuOH (30 mlx3). The combined extract was washed three times with water and evaporated to dryness. The residue was dissolved in 100 µl of MeOH and purified by cellulose TLC* (Merck, 20x20 cm) developed with ethylacetate-n-PrOH-water (2:1:2, v/v upper layer). Radioactivity on TLC was located by autoradiography using X ray film (Fuji Kx) or a Betacamera (Schmidt). The radioactive zone was cut out and eluted with MeOH containing 1% acetic acid. The main zone of radioactivity represented radioactive rhodotorucine A and its S-oxide derivative which were comigrate with authentic specimens on TLC and HPLC*. Radioactivity quantitated in a Tricarb 3330 (Packard) scintillation counter following the addition of 5 ml of dioxane scintillator.

Metabolisum of rhodotorucine A by a cells Mating type a cells (IFO M1057) were grown 18 hrs at 27°C in 10 ml YS medium. About $2x10^6$ cells were exposed to labeled rhodotorucine A (2000 units) (8) and allowed to incubate at 25°C. all reactions were carried out in plastic tubes. The reaction was terminated by addition of 100 µl of n-BuOH. Following removal of the cells by centrifugation, culture supernatant was partitioned with n-BuOH. Aqueous and n-BuOH layers were concentrated respectively and subjected to TLC and HPLC.

The culture filtrate of a cells at log phase was obtained by passing through a Milipore filter. The filtrate was exposed to labeled rhodotorucine A as described above.

Identification of metabolites of rhodotorucine A To identify metabolites of rhodotorucine A, TLC and HPLC were performed under the following conditions. TLC;Silica gel (Wako gel plate, 5x10 cm) developed with n-BuOH-acetic acid-water (4:1:5. v/v upper layer) HPLC;LiChrosorb RP-8 (Merck) φ4.6x250 mm solvent systems (acetonitril-0.01M ammonium acetate pH 7.0, 40:60 and 20:80) flow rate 1.0 ml/min.

Oxidation of rhodotorucine A and its derivative S-Farnesylcysteine residues of rhodotorucine A and its derivative were oxidized with a methanolic solution of sodium periodate (1 mg/ml) within 1 hr at room temp. Rhodotorucine A oxide and Asn-Gly-Cys(S-farnesyl) oxide were purified by preparative HPLC. Rhodotorucine A oxide was reduced with 10% mercaptoethanol in 0.02M ammonium acetate pH 8.5 at room temp. for 10 hrs.

RESULTS AND DISCUSSIONS

To prepare radioactive rhodotorucine A, A cells were grown in YS medium with labeled amino acids. Purification of labeled rhodotorucine A was quite simple because it was easily extracted with n-butanol from the culture filtrate and separated from excess labeled amino acids. The main radioactive substance in n-butanol extracts was rhodotorucine A. Preparative TLC gave a mixture of labeled rhodotorucine A and its oxide. As S-farnesylcysteine residue of rhodotorucine A is apt to be oxidized to S-farnesylcysteine oxide during the storage or purification process, labeled rhodotorucine A prepared by this method contained some amounts of rhodotorucine A oxide. Rhodotorucine A and its oxide

*Abbreviations: TLC; thin layer chromatography, HPLC; high performance liquid chromatography.

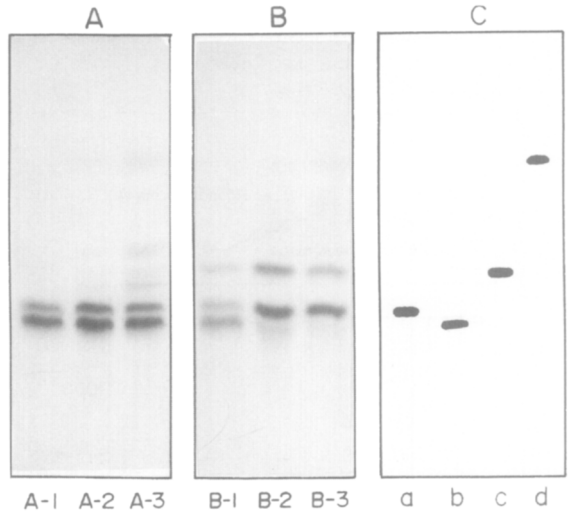

Fig. 1. Metabolism of rhodotorucine A by A and a cells
[^{35}S]Rhodotorucine A (30 nm; 60,000 cpm) was allowed to incubate in YS medium in the presence of (A) A cells and (B) a cells. The incubation times were as follows, 1; 15 min, 2; 60 min, 3; 300 min. (C) reference samples; (a) rhodotorucine A, (b) rhodotorucine A oxide, (c) Tr-2: Asn-Gly-Cys(S-farnesyl), (d) S-farnesylcysteine. Silica gel; BuOH-AcOH-water (4:1:5)

could be separated by HPLC using the reverse phase column, LiChrosorb RP-8. Rhodotorucine A oxide did not have biological activity but it was easily reduced with mercaptoethanol to give biological active rhodotorucine A.

When [^{35}S]rhodotorucine A was incubated with a cells, biological activity in the supernatant was lost within several minutes (8). As shown in Fig. 1, [^{35}S]rhodotorucine A was metabolized by a cells, resulting in the appearance of a new spot on TLC. This conversion was a mating type specific, as the mobility of the pheromone remained unaltered after incubation with A mating type cells. The metabolite, showing the new spot on TLC, was less polar than rhodotorucine A and more polar than S-farnesylcysteine (5). It must be a small peptide fragment which has S-farnesylcysteine at the C-terminus.

When rhodotorucine A is digested with trypsin, it gives two peptide fragments Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg (Tr-1) and Asn-Gly-Cys(S-farnesyl) (Tr-2) (4). Rf values of the new spot on TLC in two different solvent systems were completely identical with those of Tr-2. To confirm this, the fraction B-2 (Fig. 1) was subjected to HPLC. As shown in Fig. 2, one of the radioactive

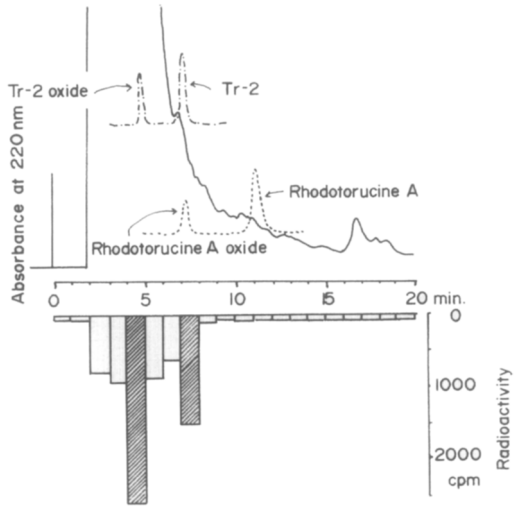

Fig. 2. HPLC of B-2 fraction
LiChrosorb RP-8, φ 4.6x250 mm, Solvent system; Acetonitril:0.01M ammonium acetate pH 7.0, 40:60, Flow rate 1.0 ml/min.

peaks is identical with the Tr-2 peak. The B-2 fraction showed another radioactive spot with the same Rf value as rhodotorucine A on TLC, but it did not show biological activity, suggesting this spot is not rhodotorucine A. HPLC of the B-2 fraction did not show radioactivity at the retention time of rhodotorucine A but showed a strong peak before the Tr-2 peak. As rhodotorucine A oxide eluted before rhodotorucine A in HPLC, the strong radioactive substance was predicted to be Tr-2 oxide. Tr-2 oxide obtained by treatment of Tr-2 with a methanolic solution of sodium periodate was shown to be identical with strong radioactive metabolite on TLC and HPLC.

Since [^{35}S]rhodotorucine A did not give information about the chemical nature of the fragment corresponding to Tr-1, [^{3}H, Tyr]rhodotorucine A and [^{14}C Arg]rhodotorucine A were prepared and subjected to the feeding experiments. The radioactivity of metabolites of [^{3}H]rhodotorucine A and [^{14}C]rhodotorucine A was not extracted with n-butanol. The radioactive metabolite in aqueous phase shows the same Rf value as Tr-1 on TLC. The radioactive metabolite of [^{14}C] rhodotorucine A is confirmed to be identical with Tr-1 in HPLC as shown in Fig. 3

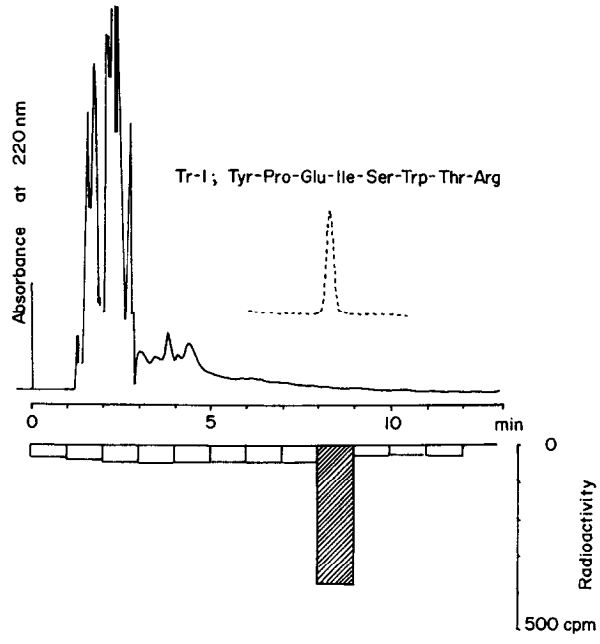

Fig. 3. HPLC of the metabolite of [^{14}C]rhodotorucine A by a cells. LiChrosorb RP-8, φ 4.6x250 mm, Solvent system; Acetonitril:0.01M ammonium acetate pH 7.0, 20:80, Flow rate 1.0 ml/min.

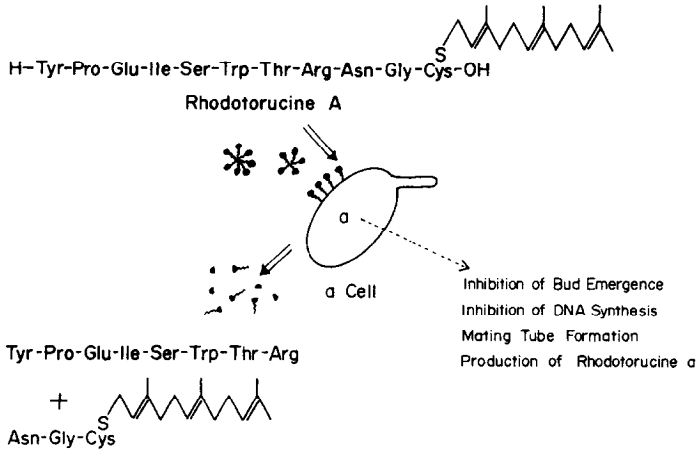

Fig. 4. Metabolisum of rhodotorucine A by a cells and early events of mating process.

Thus rhodotorucine A was shown to have been cleaved into inactive peptide fragments Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg and Asn-Gly-Cys(S-farnesyl). It is worth noting that these fragments were the same as those obtained by trypsin treatment of the pheromone.

When a culture filtrate of a cells at log phase was incubated with rhodotorucine A, rhodotorucine A remained unaltered. Therfore the inactivation of rhodotorucine A is due to a cell-associated protease of a type cells. The absence of any significant rhodotorucine A cleavage *in vivo* by A cells perhaps suggests a mating type specific location for this protease. (Fig. 4)

As we used a large amount of pheromone to a cells, inactivation of pheromone might be just removing an excess amount of pheromone. Recently, the metabolism of α factor in *Saccharomyces cerevisiae* was studied by two groups (10) and one of them proposed the mechanism of detoxification (11). The inactivation of rhodotorucine A might have the same meaning as *S. cerevisiae*.

In *R. toruloides*, a reciprocal exchange of the pheromone was revealed (2). After contact with rhodotorucine A, a cells produce another mating pheromone, rhodotorucine a, which induces mating tube formation of A cells (3). The production of rhodotorucine a is minute and the sensitivity of the bioassay for it is so low that we can not yet isolate it. But there might be a possibility that one of the metabolites of rhodotorucine A would be a precursor of rhodotorucine a.

REFERENCES

1. Banno, I. (1967) J. Gen. Appl. Microbiol. 13, 167-196
2. Abe, K., Kusaka, I. and Fukui, S. (1975) J. Bacteriol. 122, 710-718
3. Tsuchiya, E., Abe, K. and Fukui, S. (1978) J. Gen. Appl. Microbiol. 24, 291-294
4. Kamiya, Y., Sakurai, A., Tamura, S., Takahashi, N., Abe, K., Tsuchiya, E., Fukui, S., Kitada, C. and Fujino, M. (1978) Biochem. Biophys. Res. Comm. 83, 1077-1083
5. Kamiya. Y., Sakurai, A., Tamura, S., Takahashi, N., Abe, K., Tsuchiya, E. and Fukui, S. (1979) Agric. Biol. Chem. 43, 363-369
6. Kamiya, Y., Sakurai, A., Tamura, S. and Takahashi, N. (1979) Agric. Biol. Chem. 43, 1049-1053
7. Kitada, C., Fujino, M., Kamiya, Y., Sakurai. A., Tamura, S., Takahashi, N. Tsuchiya, E., Abe, K. and Fukui, S. (1979) Experientia 35, 1275-1276
8. Tsuchiya, E. and Fukui, S. (1978) Biochem. Biophys. Res. Comm. 85, 473-479
9. Kamiya, Y., Yamamoto, K., Sakurai, A., Tamura, S., Abe, K. and Fukui, S. (1975) Proc. Japn. Acad. 51, 571-576
10. Maness, P.F. and Edelman, G.M. (1978) Proc. Natl. Acad. Sci. USA 75, 1034-1308
11. Finkelsteine, D.B. and Strausberg, S. (1979) J. Biol. Chem. 254, 796-803