Amino Acid Sequence of Rhodotorucine A, a Peptidyl Factor Controlling Mating Tube Formation in *Rhodospiridium toruloides*

Yuji KAMIYA, Akira SAKURAI,
Saburo TAMURA, Nobutaka TAKAHASHI,
Keiko ABE,* Eiko TSUCHIYA*
and Sakuzo FUKUI*

The Institute of Physical and Chemical Research,
Wako-shi, Saitama-ken

*The Institute of Applied Microbiology,
The University of Tokyo,
Bunkyo-ku, Tokyo

Received November 10, 1977

In the yeast, *Rhodospiridium toruloides*, the mating reaction between two haploid cells designated *A* and *a* type¹⁾ is controlled by substances secreted from each mating type cells.²⁾ In the previous paper, we reported the isolation of a peptide named *A* factor which was produced by the *A* type cells and induced the mating tube formation in the opposite *a* type cells at a concentration of 8 ng/ml. The amino acid composition of the factor has been determined to be Asp 1, Thr 1, Ser 1, Glu 1, Pro 1, Gly 1, Ile 1, Tyr 1, Arg 1, Trp 1 based on the amino acid analysis of an acid hydrolyzate and the measurement of UV absorption.³⁾ In this paper, we wish to report the results leading to propose the amino acid sequence of *A* factor which we designate anew rhodotorucine *A*.

N-terminal amino acid was determined by dansyl (DNS) method with some modifications. The peptide (2 μ g) was treated with 2 μ l of 0.2 M NaHCO₃ and 2 μ l of 0.3% DNS-chloride in acetone at 45°C for 30 min and the product was hydrolyzed with 6 N HCl at 100°C for 16 hr. DNS-amino acids were detected by thin-layer chromatography (TLC) on polyamide sheets (5×5 cm, Cheng Ching Trading Co.) with two-dimensional development by the following solvent systems; A)

H₂O-28% NH₃(H₂O) (100:1) for the first direction and *n*-heptane-*n*-BuOH-HCOOH (10:10:1) for the second direction; B) H₂O-HCOOH (200:3) for the first direction, benzene-AcOH (9:1) for the second direction and AcOEt-MeOH-AcOH (20:1:1) for the third run in the same direction as the second. The solvent system B was found suitable for the mutual separation of DNS-Glu and DNS-Asp. As a result, *O,N*-di-DNS-Tyr, *N*-DNS-Tyr and *O*-DNS-Tyr were identified as the DNS-amino acids. Consequently it was concluded that the N-terminal amino acid of the peptide was tyrosine.

Because of very small amounts of the materials available for the structural study (yield of the peptide was 1.6 mg from 300 liter culture filtrate), we applied micro scale Edman-dansyl procedure for the sequence analysis. Edman degradation was performed according to the procedure described by Hartley⁴⁾ by use of 40 μ g of the peptide with scaling down method.⁵⁾ At each step of degradation about one tenth of the resulting peptide was taken for dansylation and the DNS-peptide was hydrolyzed with 6 N HCl at 100°C for 16 hr. DNS-amino acid in each hydrolyzate was identified by TLC on polyamide sheets as described earlier. At the fifth step of degradation, no DNS-amino acid was detected. This suggested the occurrence of tryptophan residue in this position. Thus the DNS-amino acids up to the eighth step were identified as shown in Table I. Based on the amino acid composition of rhodotorucine *A*, C-terminal amino acid was presumed to be glycine.

In order to differentiate glutamic acid and glutamine or aspartic acid and asparagine, conventional Edman degradation, in which the resulting PTH-amino acids are identified directly, was carried out by use of 200 μ g of rhodotorucine *A*. The PTH-amino acids were detected by TLC on polyamide sheets (5×5 cm) with two-dimensional development by successive use of toluene-*n*-hexane-AcOH (60:30:16) containing 0.025% butyl PBD as a fluorescence indicator and H₂O-AcOH (3:1).

TABLE I. AMINO ACID SEQUENCE ANALYSIS OF RHODOTORUCINE *A*
BY EDMAN-DANSYL METHOD

Step of degradation	<i>N</i> -terminus	1	2	3	4	5	6	7	8
DNS-amino acid detected	<i>O</i> -Tyr <i>O,N</i> -Tyr <i>N</i> -Tyr	Pro	Glu	Ile	Ser	—	Thr	Arg	Asp
Possible sequence	H-Tyr-Pro-Glx ^{a)} -Ile-Ser-Trp-Thr-Arg-Asx ^{a)} -Gly								

^{a)} By this method, differentiation of glutamine and glutamic acid or asparagine and aspartic acid in the peptide was not achieved.

TABLE II. PEPTIDE FRAGMENTS OBTAINED BY
ENZYMATIC HYDROLYSIS OF RHODOTORUCINE *A*

Peptide fragments ^{a)}	Tr-1		Tr-2
	Th-1	Th-2	
<i>N</i> -terminal amino acid ^{a)}	Tyr	Ile	Asp
Amino acid composition	Tyr, Pro, Glu	Ile, Ser, Trp, ^{b)} Thr, Arg	Asn, ^{b)} Gly

^{a)} See text.

^{b)} These amino acids were identified by cleavage with aminopeptidase M followed by dansylation.

At the third step of degradation, only PTH-Glu was detected and there was no trace of PTH-Gln. As a result, the sequence of H-Tyr-Pro-Glu- was proposed. Further degradation, however, was limited by the loss of the sample during the solvent extraction process.

Then rhodotorucine *A* was digested with several peptidase to confirm the peptide sequence. Rhodotorucine *A* (1 mg) was dissolved in 1 ml of 0.1 M AcONH₄ buffer (pH 7.0) and digested with TPCK-trypsin (20 μg) at 30°C for 4 hr. It gave two peptide fragments Tr-1 and Tr-2 which were purified on Sephadex LH-20. The amino acid composition and *N*-terminal amino acid of the fragments were identified by dansylation shown in Table II. Incubation of Tr-2 with aminopeptidase M in 0.1 M phosphate buffer (pH 7.0) at 40°C for 2 hr, liberated asparagine and glycine. Therefore, the *N*-terminus of Tr-2 was identified as asparagine.

When rhodotorucine *A* was incubated with carboxypeptidase A and Y, no free amino acid was liberated. This suggested the C-terminus of the peptide was blocked. Then authentic Asn-Gly-NH₂, Asn-Gly-OMe and Asn-Gly-

OEt were synthesized and compared with the fragment Tr-2 which contains C-terminal group of the peptide. Unexpectedly, Tr-2 was not identical with any of the authentic peptides and behaved as a less polar substance on TLC than any of them. Tr-2 was slightly soluble in water and easily extracted with *n*-BuOH. These facts suggested the attachment of a lipophilic group to the C-terminus. Tr-2 was acetylated with the mixture of (CH₃CO)₂O and (CD₃CO)₂O (1:1) and subjected to the chemical ionization mass spectrometry. The strong ion peak, *m/e* 303(306) was appeared beyond *m/e* 214(217) and 157(160) which were due to Ac-Asn-Gly. Thus 89 mass units (303–214) will be a part of an unknown lipophilic group.

Although the direct information on the structure of the lipophilic fragment has not obtained so far, the above results clearly indicate the unique structural feature of rhodotorucine *A* composed of the decapeptide and the unknown lipophilic moiety as shown I.

I

Recently, two analogues of the decapeptide whose C-terminal groups were -Gly-OH and -Gly-NH₂ were synthesized.* The enzymatic degradations of these peptides confirmed the correctness of the proposed amino acid sequence. The synthetic peptides, however, were much polar than rhodotorucine *A* and

* We are indebted to Dr. M. Fujino of Central Research Division, Takeda Chemical Industries Ltd. for the synthesis of the analogues. The details of the synthesis will be reported elsewhere.

showed no biological activity even in high concentrations. This suggests that the lipophilic group plays an important role to show the biological activity. The determination of the complete structure and chemical synthesis of rhodotorucine A are now in progress.

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