Notes

[Chem. Pharm. Bull. 26(2) 627—630 (1978)]

UDC 577.155.04.07 : 581.192 : 582.282.123.04

The Effect of Elimination of Amino Acids from the Carboxyl-terminal of the Minor Ribonuclease from *Aspergillus saitoi* by Carboxypeptidase A on the Enzymatic Activity

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(Received June 3, 1977)

In order to investigate whether carboxyl-terminal amino acid is involved in the active site of a base non-specific ribonuclease from *Asp. saitoi* (RNase Ms), removal of carboxyl-terminal amino acid residues by digestion with carboxypeptidase A was investigated.

By 24 hours digestion, about 3 serine residues and 1.0 alanine residue were removed from RNase Ms and its activity decreased to about 70% of the native enzyme so far as measured with ribonucleic acid (RNA) as a substrate. The pH optimum and K_m of carboxypeptidase treated RNase Ms (CP-RNase Ms) were very similar to those of native RNase Ms so far as measured with RNA as a substrate. However, K_m of CP-RNase Ms using ApC as a substrate seemed to be larger than that of native RNase Ms. The large increase in K_m value was not observed in the early stage of digestion where about 3 serine residues were removed.

The gross structure of CP-RNase Ms was quite similar to that of the native RNase Ms by judging from circular dichroism spectrum at wavelength between 230—205 nm. From the results described above, it was concluded that carboxyl-terminal amino acid was not involved directly in the active site of RNase Ms.

Keywords——*Aspergillus saitoi*; ribonuclease; active site; carboxypeptidase A digestion; structure-activity relationship; conformation

In the previous paper, it has been reported that *Aspergillus saitoi* produces two ribonucleases that are base non-specific, with molecular weights of 35000 and 12500.[2,3] The minor ribonuclease having molecular weight of 12500 (RNase Ms) cleaves all 16 possible dinucleoside phosphates consisting of common bases of ribonucleic acid (RNA), A, G, U and C,[4] but it shows a preference for those having guanosine at the 5′-end.

The modification of α-amino and one ε-amino groups of this enzyme with trinitrobenzenesulfonate caused about 30% decrease in enzymatic activity. By further introduction of one trinitrophenyl-group, RNase Ms lost about 81% of the enzymatic activity. These results indicated that these amino-groups were not directly involved in the active site of the enzyme.[5]

It is interesting to investigate whether the carboxyl-terminal amino acid residue is involved directly in the active site of RNase Ms. This report deals with the effect of removal of the carboxyl-terminal amino acid residue by digestion with carboxypeptidase A.

Results and Discussion

In order to know the role of carboxyl-terminal amino acid residue on the enzymatic activity, RNase Ms was digested with bovine carboxypeptidase A at pH 8.0. By digesting

1) Location: *2–4–41, Ebara, Shinagawa-ku, Tokyo, 142, Japan.*
2) M. Irie, *J. Biochem.*, **62**, 509 (1967).
3) K. Ohgi and M. Irie, *J. Biochem.*, **77**, 1085 (1975).
4) K. Ohgi and M. Irie, *J. Biochem.*, **80**, 39 (1976).
5) K. Ohgi, M. Kiryu, Y. Torano, Y. Hori, H. Watanabe, M. Iwama, and M. Irie, *J. Biochem.*, **82**, 939 (1977).

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with this enzyme for 24 hr, about 3 serine residues and 1.0 alanine residue were released from RNase Ms(Fig. 1). At this stage, the enzymatic activity of the digested RNase Ms was about 70% so far as checked by the enzyme assay using RNA as a substrate. Therefore, the removal of 4—5 amino acid residues from carboxyl-terminal did not cause serious inactivation. Carboxypeptidase A was removed from the reaction mixture by passing through a Sephadex G-75 column(Fig. 2). By using carboxypeptidase-free RNase Ms fraction, enzymatic properties of carboxypeptidase treated RNase Ms(CP–RNase Ms) were investigated. The optimum pH of CP–RNase Ms was at pH 4.5 and very similar to that of native RNase Ms. The Lineweaver-Burk's plot[6] of CP–RNase Ms using RNA as a substrate is shown in Fig. 3. The Michaelis

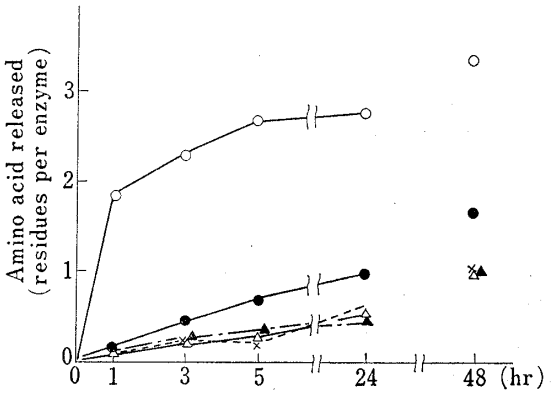

Fig. 1. Carboxypeptidase A Digestion of RNase Ms at pH 8.0

The experimental conditions were described in the text. —○—, Ser; —●—, Ala; ×, Tyr; △, Asp and ▲, Val.

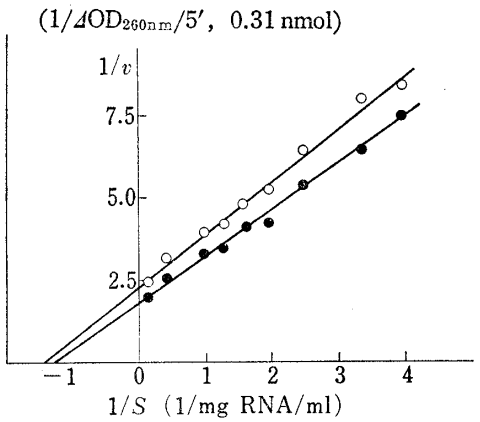

Fig. 3. Lineweaver-Burk's plot of RNase Ms and CP-RNase Ms using RNA as a Substrate at pH 5.0 and 37°

The experimental conditions were as described in the text. The enzyme used for this experiment was 0.31 nmol. ●, native RNase Ms; ○, CP-RNase Ms.

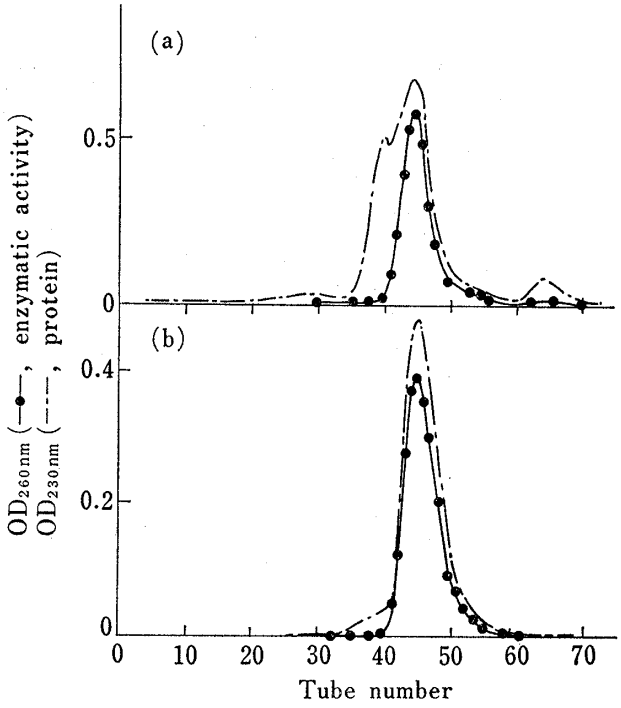

Fig. 2. (a)Sephadex G-75 Column Chromatography of Carboxypeptidase A digested RNase Ms

(b)Rechromatography of the RNase Ms treated with Carboxypeptidase A

(a)About 6 mg of RNase Ms treated with carboxypeptidase A was applied on the column of Sephadex G-75 equilibrated with 0.1 M acetate buffer (pH 5.5). Column size: 2×45 cm. Each 4 g fractions were collected. The column was eluted with the same buffer. The conditions for carboxypeptidase digestion were described in the text. The enzymatic activity was determined with RNA as a substrate by the increase in absorbancy at 260 nm.
(b)Enzyme fractions from tubes No. 44—77 (Fig. 2a) were combined and applied on column of Sephadex G-75. The other experimental conditions were the same as those in Fig. 2(a). The fractions 43—55 were combined and used for further studies.

constant of CP–RNase Ms was very similar to that of the native RNase Ms. However, as shown in Table I, when ApC was used as a substrate, the decrease in the enzymatic activity of CP–RNase Ms was more marked than that towards RNA.

6) M. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

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Table I. Enzymatic Activity of RNase Ms during Course of Digestion with Carboxypeptidase A using ApC and RNA as Substrates at pH 5.0

Substrate	Digestion (hr)	Substrate concentration (μM)	Relative activity[a] (%)
ApC[b]	5	100	72
		50	72
	24	100	66
		50	55
RNA[c]	5		86
	24		77

a) The enzymatic activity was expressed as the percentage of the activity of RNase Ms at the same concentration of substrate.
b) The experimental conditions were the same as described in the text.
c) The standard assay using RNA as a substrate. Enzyme concentration was 0.31 nmol.

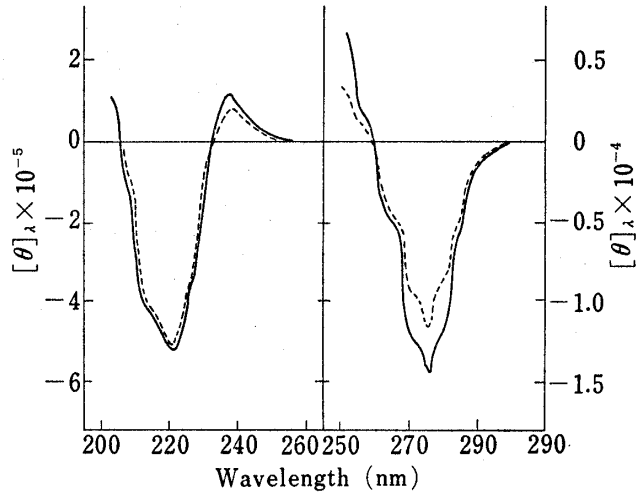

Fig. 4. CD spectra of RNase Ms and CP-RNase Ms at pH 5.0

——, RNase Ms; ------, CP-RNase Ms. The experimental conditions were described in the text.

The results shown in Table I showed that at 5 hr digestion where about 3 serine residues were released, the enzymatic activity for 50 and 100 μM ApC was in the range of 70%, but by further digestion, decrease in enzymatic activity at 50 μM became more marked than that at 100 μM. At this stage of digestion, about 3 serine residues and one alanine residue were released from the enzyme. Therefore, it could be concluded that the release of the third or fourth amino acid residues from the carboxyl-terminal of RNase Ms induced a marked decrease of Michaelis constant towards lower molecular weight substrate, such as ApC.

CD spectrum of CP–RNase Ms was very similar to that of the native enzyme(Fig. 4). The results indicated that the gross structure of CP–RNase Ms might be very similar to that of the RNase Ms.

From the results described above, it was concluded that carboxyl-terminal amino acid was not invovled directly in the active site of RNase Ms.

Experimental

Enzyme——RNase Ms was purified according to the previous method[3] from a commercial digestive "Molsin," (*Aspergillus saitoi*).

Reagents——Yeast RNA used as a substrate was obtained from Kojin Co., Ltd. 2′,3′-Cyclic CMP and adenosyl(3′—5′)cytidine (ApC) were purchased from Sigma. Carboxypeptidase A treated with diisopropyl phosphorofluoridate was obtained from Worthington Biochem. Corp.

Assay of RNase Activity——(1) RNA as a substrate: Enzymatic activity was usually measured as described previously[2] by the increase in optical density at 260 nm of the acid soluble nucleotides formed from RNA during 5 min incubation at 37°. Reaction mixture consisted of 5 mg of RNA, 2 ml of 0.05 M acetate buffer (pH 5.0) and 10 μl of enzyme solution. The substrate concentrations used for measurement of Michaelis constant were 5.0—0.25 mg per 2 ml reaction mixture. Michaelis constants were calculated from the double reciprocal plots according to Lineweaver-Burk.[6]

(2) ApC as a Substrate: The enzymatic activity using ApC as a substrate was measured by the increase in optical density at 268 nm at 25°.[7] The reaction mixture consisted of 50 or 100 μM of ApC in 2 ml of 0.1 M acetate buffer (pH 5.0) and 3.1 nmol of enzyme.

Determination of Protein Concentration——Protein concentration was measured from the absorbancy at 280 nm taking that of a 0.1% protein solution as 1.70 [3]

Amino Acid Analysis——Amino acid analysis was performed by the method of Spackman, *et al.*[8] with an amino acid analyzer Nihondenshi JEOL 6AH.

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8) D.H. Spackman, W.H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1192 (1958).

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Carboxypeptidase A Digestion of RNase Ms——Six mg of RNase Ms was dissolved in 6 ml of Tris–HCl buffer (0.1 M, pH 8.0) and to the reaction mixture 30 μl (0.8 mg) of carboxypeptidase A was added. The reaction mixture was shaken at 37° and the amino acids released in 0.3 ml aliquots were analyzed by an amino acid analyzer. Serine released was distinguished from glutamine and aspargine by its location on the chart of amino acid analysis and the ratio of color values at 570 nm and 440 nm. The ratios for serine, glutamine and aspargine were 5.8, 5.1 and 5.1, respectively in our amino acid analysis system.

CD Spectrum——Circular dichroism spectra of RNase Ms and carboxypeptidase treated RNase Ms were measured with a JASCO J-40 spectropolarimeter at room temperature. The cells having 0.5, 0.1 and 0.05 cm light path were used. The protein concentrations used for CD spectra measurement were 80—40 μM for 250—350 nm wavelength region and 20 μM for 200—250 nm region.

Acknowledgement The authors thank Dr. F. Yoshida of Kikkoman Syoyu and Seishin Pharm. Co. for the supply of "Molsin," the enzyme source.

[Chem. Pharm. Bull. 26(2) 630—632 (1978)]

UDC 547.759.04 : 547.585.04

Photochemical Synthesis of 5,10,11,12,12a,12b-Hexahydro-12b-hydroxyisoindolo[2,1-*a*]benz[*cd*]indol-5-one[1,2]

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(Received June 20, 1977)

Photocyclization of N-(5,6,7,8-tetrahydro-1-naphthyl)phthalimide (3) afforded 5,10,11,12,12a,12b-hexahydro-12b-hydroxyisoindolo[2,1-*a*]benz[*cd*]indol-5-one (4) in good yield. On acid treatment, 4 was readily dehydrated to give 5,10,11,12-tetrahydroisoindolo [2,1-*a*]benz[*cd*]indol-5-one (5).

Keywords——photochemical synthesis of heterocycles; Norrish type II reaction; benz[*cd*]indole system; photocyclization of N-substituted phthalimide; N-(5,6,7,8-tetrahydro-1-naphthyl)phthalimide

The photochemistry of aromatic cyclic imides, *e. g.*, phthalimides, has received intensive study[4] since its discovery in 1972,[5] and the most frequently reported transformations have been photocyclizations which are likely the Norrish type II reactions of the excited imide carbonyl group.[4,6] Earlier it was observed that N-*o*-tolylphthalimide (1) on irradiation undergoes cyclization to form a tetracyclic system (2) furnishing synthetically useful yield of cyclic pentanols[4] as shown in Chart. In a subsequent work[7] we examined the substituent effects either in the A or B ring of 1 on the photoreaction and, in a previous paper,[1] we described the results of a synthetic investigation with N-pyridylphthalimides as a variation

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