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Isolation and Characterization of Protease Modified Ribonucleases from *Rhizopus* sp.

SACHIKO MINE,^a EIJI WAKABAYASHI,^a AKIHIRO SANDA,^b
YOSHIO TAKIZAWA,^b KAZUKO OHGI^a
and MASACHIKA IRIE^{*,a}

*Department of Microbiology, Hoshi College of Pharmacy,^a Ebara 2-4-41, Shinagawa-ku,
Tokyo 142 and Faculty of Public Health, Azabu University,^b
Fuchinobe, Sagami-hara, Kanagawa 229, Japan*

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In order to clarify the reason for the variation in specific activities of ribonuclease preparations from *Rhizopus* sp. ribonuclease (RNase Rh), low specific activity species (RNase Rh') were separated from native RNase Rh by DEAE Toyopearl 650 column chromatography and characterized. When RNase Rh' was subjected to gel electrophoresis in the absence of 2-mercaptoethanol, it gave a 24 kilodalton (kDa) protein band, but in the presence of the reducing agent it gave 17 and 7 kDa bands. These two peptides were separated by gel filtration and their NH₂-terminal amino acid sequences were determined. The results indicated that RNase Rh' was an enzyme species cleaved at about the 50th residue of native RNase Rh by proteases during the course of purification, but the two fragments were still covalently joined by S-S bridges. RNase Rh' retained about 70% of the native activity and has a similar conformation to the native enzyme.

Keywords—*Rhizopus*; ribonuclease; proteolytic modification; NH₂-terminal sequence

A base-nonspecific and adenylic acid-preferential ribonuclease (RNase Rh) has been purified by Tomoyeda *et al.*¹⁾ and Komiyama and Irie²⁾ from *Rhizopus* sp. During the course of purification, we very often observed the presence of crystalline RNase Rh species having relatively low specific activity, about 70% of the highest one (327 unit/mg protein) (RNase Rh'). In this paper, we report that the RNase Rh preparation contained enzyme species modified by proteases at around the 50th residue from the N-terminal end of RNase Rh.

Materials and Methods

Reagents—Ribonucleic acid (RNA) used as a substrate was obtained from Kojin Co. Diethylaminoethyl (DEAE)-Toyopearl 650M was purchased from Toyo Soda (Tokyo). Marker proteins, ovalbumin, α -chymotrypsinogen, cytochrome c and insulin, were purchased from Sigma Chem. Co.

Enzyme Preparation—RNase Rh preparations from *Rhizopus* sp. were purified according to the method reported previously,²⁾ and further subjected to column chromatography on DEAE-Toyopearl 650M at pH 7.5.

Enzyme Assay—The standard assay used during purification was performed according to the previous paper²⁾ using RNA as a substrate. Enzyme solution (5–10 μ l) was added to 2 ml of reaction mixture consisting of 50 mM acetate buffer (pH 6.0) and 0.25% RNA, and the reaction was performed at 37 °C for 1–5 min, then terminated by adding 1 ml of MacFadyen reagent.³⁾ An aliquot (0.3 ml) was diluted with 2 ml of deionized water, and the absorbancy at 260 nm was measured. The amount of RNase which produced an increase in absorbancy of 1.0 at 260 nm after a 5 min incubation was defined as one unit.

Protein Concentration—Protein concentration was estimated by measuring the absorbancy at 280 nm, taking that of a 0.1% solution as 1.0.

Polyacrylamide Gel Electrophoresis—Disc electrophoresis on polyacrylamide gel was performed by the method of Ornstein⁴⁾ and Davis⁵⁾ using pH 8.5 gel. Gel electrophoresis was performed at the current of 2.5 mA per tube for 1.5 h. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was performed according to the method of Shapiro *et al.*⁶⁾ using 7% polyacrylamide gel. Electrophoresis was carried out

for 4 h at 7 mA per tube. For both electrophoreses, approximately 20 μ g of protein was applied per tube. The gels were stained with 0.025% Coomassie brilliant blue. Slab-gel electrophoresis was conducted using 12% polyacrylamide gel according to the method of Laemmli.⁷⁾

Molecular Weight Determination—The molecular weights of RNase Rh', and the peptides were estimated by gel filtration on Sephadex G-50 column (1.5 $\times$ 170 cm) equilibrated with 50 mM trimethylamine-acetate buffer (pH 8.0) containing 0.1 M NaCl according to Andrews.⁸⁾ Fractions of 1.3 ml each were collected. Marker proteins used were ovalbumin (MW 45000), α -chymotrypsinogen (MW 25700), cytochrome c (MW 12400) and insulin (MW 6000).

Amino Acid Analysis—Proteins and peptides were hydrolyzed in evacuated, sealed tubes with 6 N HCl at 110 °C for 24 h. Analyses were performed by the method of Spackman *et al.*⁹⁾ with an amino acid analyzer (Nihon Denshi JLC-200A). Estimation of tryptophan content was performed by the method of Pajot.¹⁰⁾

Automated Edman Degradation—A protein sample (about 0.3 μ mol) was reduced and carboxymethylated in the presence of 8 M urea according to the method of Crestfield *et al.*¹¹⁾ The reduced and carboxymethylated protein and peptides were subjected to automated Edman degradation¹²⁾ with a JEOL JAS 47K sequencer. Phenylthiohydantoin (PTH)-amino acids were determined by high performance liquid chromatography (HPLC). HPLC was performed with JASCO Fine Sil C₁₈₋₅ column (4.6 $\times$ 12.5 cm) equilibrated with solvent A (0.05% H₃PO₄ : acetonitrile = 90 : 10, v/v). PTH-amino acids were eluted with a concave gradient of solvent A and solvent B (0.1% H₃PO₄ : acetonitrile = 60 : 40, v/v) for 16 min at a flow rate of 2.0 ml/min, followed by elution with solvent B. An aliquot of the PTH-amino acid was subjected to isocratic elution on a column of Zorbax ODS (4.6 $\times$ 25 cm) with 12.5 mM acetate buffer (pH 4.5) containing 33% acetonitrile. PTH-amino acids were detected by measuring the absorbance at 254 or 315 nm (for dehydrothreonine and dehydroserine).

Circular Dichroism (CD) Spectrum—CD spectra were measured with a JASCO J-40 spectropolarimeter at 25 °C. The light path of the cell used was 0.5 or 0.05 cm. All data are expressed as molar ellipticity. Protein concentration was 10 μ M.

Results

SDS Slab Electrophoresis of RNase Rh Preparation Having Low Specific Activity

The Specific activity of RNase Rh purified according to the previous paper²⁾ is about 237 unit/mg. However, we often observed RNase Rh preparations having low specific activity, such as 170—200 unit/mg. On disc electrophoresis at pH 8.5, such a preparation gave a single protein band at essentially the same location as RNase Rh having high specific activity. However, on 7% SDS disc electrophoresis in the presence of 2-mercaptoethanol, the low specific activity preparation gave two major bands and a very faint band having higher mobility (not seen in Fig. 1); one of the major bands had the same mobility as the high specific activity band and the other band showed slightly higher mobility. In order to estimate the molecular weight of the protein species involved in low specific activity preparation, it was subjected to 12% SDS slab electrophoresis in the presence of 2-mercaptoethanol. Two additional bands were seen, as well as a protein band having the same mobility as RNase Rh.

Fig. 1. Gel Electrophoreses of an RNase Rh Preparation Having Low Specific Activity (216 unit/mg)

Left column, disc electrophoresis at pH 8.5, in the absence of 2-mercaptoethanol; middle column, 7% SDS disc electrophoresis; right column, 12% SDS slab electrophoresis. Heavy arrows indicate the mobility of the native RNase Rh. The electrophoreses were performed as described in Materials and Methods.

The molecular weights estimated by electrophoresis of these three bands were 24, 17 and 7 kilodalton (kDa). Since 24 kDa is the molecular weight of the native RNase Rh, the other two bands might be due to the low specific activity species. The sum of these two bands coincides with that of native RNase Rh (24 kDa), and the presence of these two extra bands indicated the cleavage of RNase Rh, probably by proteases. The fact that we were not able to observe these two bands, 17 and 7 kDa in the absence of 2-mercaptoethanol indicated that the enzyme preparation having low specific activity consists of the 7 and 17 kDa species connected by S-S bridges. Hereafter, we designate this modified enzyme preparation as RNase Rh'.

Fractionation of Low Specific Activity RNase Rh' by DEAE-Toyopearl Column Chromatography

The elution patterns on DEAE-Toyopearl 650M column chromatography of two enzyme preparations having specific activity 216 and 172 unit/mg are shown in Fig. 2a and 2b. In the former preparation, two RNase peaks appeared in addition to the major RNase peak corresponding to RNase Rh. In the latter preparation we observed a very small RNase Rh peak and two other enzyme peaks. SDS-slab electrophoresis of these two enzymes in the presence of 2-mercaptoethanol indicated the presence of the 17 kDa protein band and, thus they were RNase Rh'. By this step of purification, we could separate RNase Rh and RNase Rh' species very effectively. In this work, we tried to characterize the major RNase Rh'

Fig. 2. DEAE-Toyopearl 650M Column Chromatography

(a) The enzyme preparation (specific activity 216 unit/mg) was applied to a column of DEAE-Toyopearl 650M (1.8 × 80 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted with a linear gradient of NaCl from 0–0.2 M in the same buffer. Each 5.0 ml fraction was collected. ○, A_{280} (protein); ●, A_{260} (activity). The other experimental conditions were the same as described in Materials and Methods.

(b) The enzyme preparation (specific activity 172 unit/mg) was applied to the same column as in Fig. 2a. The other experimental conditions were the same as in Fig. 2a. The inset shows the results of slab electrophoresis of the fractions indicated by a–g.

Fig. 3. Crystals of RNase Rh'

Fig. 4. Effect of Temperature on the Activities of RNase Rh and RNase Rh'

(a) Effect of temperature on the activities of RNase Rh and RNase Rh'. The enzymatic activities of RNase Rh and RNase Rh' (specific activity 172 unit/mg) were measured at the temperature indicated by use of the standard assay system except for the temperature. The ordinate shows percent of the maximum activity.

(b) Stability of RNase Rh and RNase Rh' to heating. The enzyme (60 μ g) in acetate buffer (pH 5.0) was incubated at the indicated temperature for 5 min, then the enzymatic activity was measured at 37°C. ○, RNase Rh; ●, RNase Rh'.

component which was eluted just after RNase Rh (specific activity, 160 unit/mg).

Crystallization of RNase Rh'

RNase Rh' thus obtained was concentrated to a small volume (several mg/ml), then dialyzed against deionized water. The crystalline materials formed after exhaustive dialysis were collected by centrifugation. The photograph of typical crystals is shown in Fig. 3.

Effect of Temperature on the Enzymatic Activity and Heat Stability of RNase Rh'

The effect of temperature on the enzymatic activity of RNase Rh' was studied at pH 5.0 and compared with that of RNase Rh. Although the optimum temperature of RNase Rh' was about 10°C less than that of RNase Rh, the heat stabilities of the enzymes were comparable (Fig. 4).

CD Spectrum of RNase Rh'

The CD spectra of RNase Rh' and RNase Rh measured at pH 5.0 and room temperature were almost superimposable, indicating similarity in the gross conformation of both enzymes.

Amino Acid Sequences of 17 and 7kDa Proteins

In order to estimate the locations of the 17 and 7kDa proteins in RNase Rh, the two

Fig. 5. CD Spectra of RNase Rh and RNase Rh'

—, RNase Rh; ----, RNase Rh'. The experimental conditions were the same as described in Materials and Methods.

Fig. 6. Sephadex G-50 Column Chromatography of RCM RNase Rh'

RCM RNase Rh' (1.5 μ mol) was applied on a Sephadex G-50 column (1.8 $\times$ 210 cm) equilibrated with 50 mM trimethylamine-acetate buffer (pH 8.0). Each 2.0 ml fraction was collected. The column was eluted with the same buffer. $\circ$, A_{280} ; $\triangle$, A_{230} .

peptides were fractionated after reduced carboxymethylation of RNase Rh' by gel-filtration on a Sephadex G-50 column (Fig. 6). The high and low molecular weight components, component I and II, respectively, were pooled separately. The molecular weights of both components were estimated by gel filtration on Sephadex G-50 to be 17000 and 6–7000. The amino acid compositions of both components are shown in Table I. The sum of both components is very similar to the composition of the RNase Rh. The amino-terminal sequences of RCM RNase Rh, components I and II were determined by Edman degradation. The results are shown in Fig. 7. Since the N-terminal amino acid sequence of component II was the same as that of RNase Rh, it was concluded that component II corresponds to the NH_2 -terminal 50 residues or so of RNase Rh, and component I is the C-terminal 160–170 residues of RNase Rh. It was concluded that RNase Rh' consists of the enzyme species cleaved at about 50 amino acid residues from the N-terminal. However, the two peptides are connected by S–S bridges so that the enzyme retained *ca.* 70% of the activity of the native RNase Rh.

Fig. 7. N-Terminal Amino Acid Sequences of RNase Rh and Components I and II Derived from RNase Rh'

→ indicates each step of Edman degradation.

Discussion

RNase Rh', which has about 70% of the activity of native RNase Rh, has a very similar conformation to RNase Rh as judged from CD spectrum, and could be crystallized easily in spite of its proteolytic modification. The contribution of protease(s) to the formation of RNase Rh' is supported by the fact that when we prepared crude RNase Rh at room temperature instead of in a cold room, the yield of RNase Rh' increased markedly.

The evidence described here also indicated that the amino acid residues around the 50th position probably form a loop located at the surface of the molecule, being very susceptible to proteases during purification. This part of the molecule is probably far from the active site of RNase Rh. Component II of the minor RNase Rh' (the fraction between tube No. 420–450 in Fig. 2) gave the N-terminal sequence Gly-Gly-Cys-Asp---. Thus the proteases also attack between 3rd and 4th amino acid residues from the N-terminal of component I as well.

These enzyme species are very useful for chemical modification studies of RNase Rh, because the site of a chemically modified group at the N-terminal part of the molecule could be easily determined after separation of this part. The application of this enzyme for chemical modification studies will be reported in a separate paper.

TABLE I. Amino Acid Compositions of Components I and II
Obtained from RNase Rh'

Amino acid	RNase Rh (theoretical value)	RNase Rh'	
		Component I	Component II
		Residues/mol (nearest integer)	
CM-Cys	10	4.69 (5)	5.35 (5)
Asp	28	23.06 (23)	6.84 (7)
Thr	16	11.66 (12)	2.94 (3)
Ser	31	23.36 (23)	9.87 (10)
Glu	14	13.02 (13)	2.40 (2)
Pro	10	5.30 (5)	3.78 (4)
Gly	18	12.97 (13)	5.41 (5)
Ala	16	10.69 (11)	5.11 (5)
Val	11	8.68 (9)	1.82 (2)
Met	4	3.00 ^{a)} (3)	1.00 ^{a)} (1)
Ile	7	6.00 (6)	0.13
Leu	11	6.47 (6)	5.06 (5)
Tyr	17	15.11 (15)	2.13 (2)
Phe	6	4.80 (5)	1.06 (1)
His	3	2.22 (2)	1.31 (1)
Lys	9	8.04 (8)	1.37 (1)
Arg	4	3.85 (4)	0.07
Trp	5	2.86 (3)	1.77 (2)
Total	215	166	51

a) Assumed as Met 3.00 and 1.00 residues per mol for components I and II, respectively.

During the course of characterization, we determined the sequence of the NH₂-terminal 48 amino acid residues of RNase Rh. When this sequence is compared with that of the similar base non-specific and adenylic acid preferential RNase, RNase T₂ from *Aspergillus oryzae*,¹³⁾ several homologous sequences can be seen. They are underlined in Fig. 7.

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