

## Immunochemical Studies on Ribonuclease F1

(ribonuclease F1 / rabbit antibody / ribonuclease T1 family)

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Antibody against ribonuclease (RNase) F1 was raised in a rabbit by intramuscular injection of the protein with Freund's complete adjuvant. Excess amount of the antibody totally inhibited the enzyme activity on RNA substrate. Quantitative precipitin analysis showed the presence of at least 4 or 5 epitopes (antigenic determinants) on the RNase F1 molecule. Cross-reactivity of the antibody with RNases T1, Ms, U2, St and T2 was examined. Although the double diffusion test detected no cross-reactivity with any of these RNases, Western blot analysis with anti RNase F1 antibody revealed weak cross-reactivity with RNases Ms and T1.

### INTRODUCTION

A family of ribonucleases (RNases) homologous to RNase T1, the guanine specific RNase from *Aspergillus oryzae*, have been known. Although enzymatic and structural studies on these enzymes are abundant, information on immunochemical relationships among these RNases is insufficient. The only study so far reported is by Uchida<sup>1)</sup>. She prepared rabbit antiserum against RNase T1 and found that the antiserum did not cross-react with RNases T2, U2 and N1. She also suggested cross-reactivity of the antiserum with RNase U1.

We have long been studying one of the family members, RNase F1 from a phytopathogenic fungus *Fusarium moniliforme*. Here, we have prepared rabbit antiserum against RNase F1, characterized basic properties of the antigen-antibody system and investigated cross-reactivity of the antibody with several other RNases.

### MATERIALS AND METHODS

*Materials and RNase Assay* — RNase F1 was prepared as described previously<sup>2)</sup>. RNase T1 was prepared from Taka-diastase (Sankyo) applying the same method as for RNase F1. RNase T2 was obtained according to Uchida

and Egami<sup>3)</sup>. RNases Ms, U2, and St were gifts as described in "Acknowledgement". RNase assay was carried out as described previously<sup>4)</sup>.

*Preparation of Anti RNase F1 Antibody* — RNase F1 (5 mg) was dissolved in 1 ml of distilled water and mixed with 1 ml of Freund's complete adjuvant (Nacalai). The mixture was injected intramuscularly into a male rabbit (about 2 kg body weight) at various spots. Six weeks later, 2.3 mg of the antigen was injected in the same way. Four weeks after the second injection, 1.9 mg of the antigen in 0.4 ml of 0.016 M phosphate buffer-saline, pH 7.3 (phosphate buffered saline or PBS) was injected intravenously. Two weeks after the booster injection, the whole blood of the animal was collected, and was processed in a standard way to make about 40 ml of the antiserum. Usual ammonium sulfate fractionation of the antiserum yielded immunoglobulin G (IgG) fraction as one third saturation precipitate. The precipitate was taken up in 10 ml of distilled water to yield about 5% (w/v) IgG solution, which will be referred to as the anti RNase F1 antibody solution.

*Inhibition of RNase Activity by the Antibody* — RNase F1 solution (8.8 µg / 20 µl) was mixed with various volumes of the anti RNase F1 antibody solution. The mixture was made up to 0.4 ml with saline, kept at 4 °C for 2 days with stirring twice a day and assayed for enzyme activity after appropriate dilution with saline. A control was run without the antibody. Essentially the same procedure was employed for investigation of the effect of the antibody on other RNases.

*Quantitative Precipitin Analysis* — The anti RNase

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F1 antibody solution (0.2 ml) was mixed with varying amounts of RNase F1. The mixture was made up to 0.5 ml with PBS, incubated at 37 °C for 1 h, then kept at 4 °C for 2 days with stirring twice a day. The precipitate formed was centrifuged off, washed twice with 0.2 ml of cold saline, and dissolved in 0.1 ml of 1 M NaOH. The solution was diluted with 0.9 ml of PBS and assayed for protein by measuring  $A_{280}$ . The molar ratio antibody/antigen was calculated as described by Uchida<sup>1)</sup>. It was assumed that RNase F1 gave  $A_{280}$  of 1.5 at 1 mg/ml in 0.1M NaOH. The enzyme activity in the supernatant was measured after appropriate dilution.

*Qualitative Binding Assay with Related RNases —*

Double diffusion test was carried out on 1.2% agarose gel plate by the standard Ouchterlony method. RNase solutions (about 100 ng in 3  $\mu$ l) were put into wells around a center well containing 3  $\mu$ l of the antibody solution. Precipitin lines were observed after diffusion for 18 h at 37 °C. Western blot analysis was carried out as follows. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run according to the standard procedure using 8% polyacrylamide gel with a prestained molecular mass marker (Bio Rad). The experiment was carried out in duplicate with different amounts of samples. In experiment A, approximately 3  $\mu$ g of the samples was charged on each lane of a slab gel. After the electrophoresis, the gel was stained with coomassie brilliant blue. In experiment B with one tenth the amount of the samples, the gel was

electroblotted onto a Hybond N<sup>+</sup> membrane (Amersham), and the membrane was immunostained using the anti RNase F1 antibody and a second antibody, alkaline phosphatase-conjugated goat anti rabbit IgG, which was visualized by use of chromogenic substrates, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride. The second antibody and the substrates were obtained from Boheringer, and the staining was carried out according to a manual provided by the manufacturer. In short, the membrane was first blocked with skim milk protein, then it was treated with the anti RNase F1 antibody. After thorough washing with PBS containing a detergent, it was reacted with the

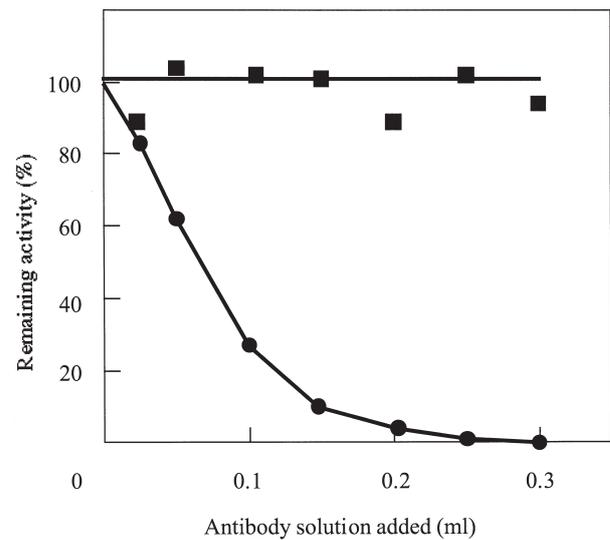


Fig. 1. Inhibition of RNase activity by anti RNase F1 antibody. Circles and squares represent data using the RNase F1 antibody and normal rabbit serum, respectively.

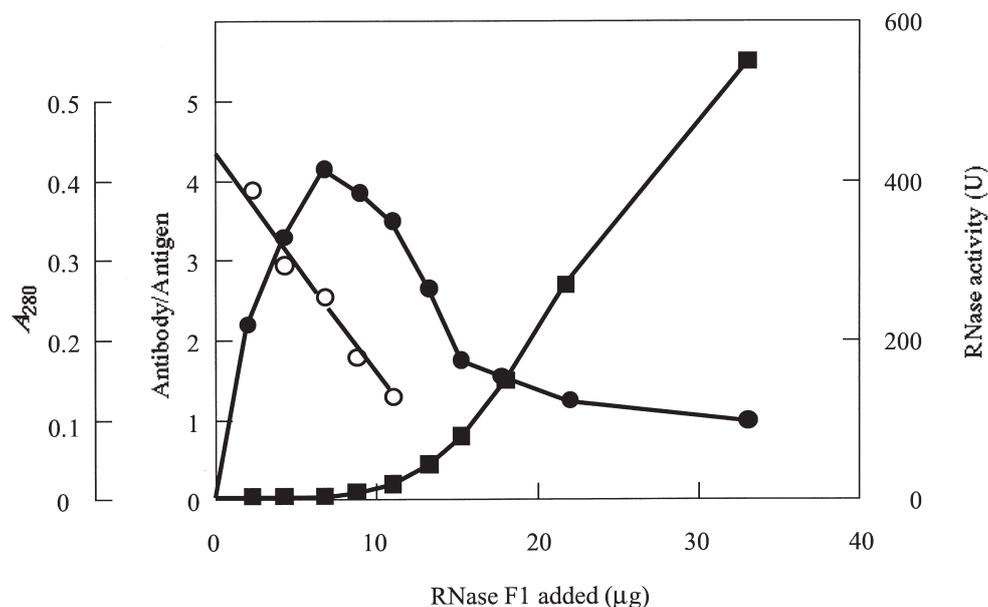


Fig. 2. The quantitative precipitin analysis for the RNase F1-antibody system. Filled circles and squares represent quantities of the immunoprecipitin in terms of  $A_{280}$  and RNase activities in the supernatant, respectively. Open circles show the molar ratio antibody/antigen in the precipitin.

second antibody, washed and left in a substrate mixture solution until appropriately intense color developed.

## RESULTS AND DISCUSSION

### *Inhibition of RNase Activity by Anti RNase F1 Antibody—*

Figure 1 shows that RNase F1 activity on RNA substrate was progressively inhibited by the addition of the increasing amount of the anti RNase F1 antibody.

An excess amount of the antibody totally inhibited the enzyme activity. Based on the titration curve, 1 ml of the original antiserum against RNase F1 will neutralize approximately 11  $\mu\text{g}$  of the antigen. Normal rabbit serum did not inhibit the RNase.

*Quantitative Precipitin Analysis for RNase F1–Antibody System*—A classic precipitin curve was obtained for the RNase F1–antibody system (Fig. 2).

At the equivalence point where a maximum quantity of the precipitate was formed, the molar ratio antibody/antigen in the precipitate was approximately 2. Extrapolation to infinite excess of the antibody gave a maximum molar ratio of 4.3, suggesting the presence of at least 4 or 5 epitopes (antigenic determinants) on the RNase F1 molecule. Similar observations were reported by Uchida for RNase T1, where the number of epitopes on the molecule was estimated to be 4<sup>1)</sup>.

*Cross-Reactivity of Other RNases with Anti RNase F1 Antibody*—We examined whether the anti RNase F1 antibody exhibited cross-reactivity with other RNases. The followings were selected: RNases T1, Ms, U2, St and T2. The selection was by no means systematic but was made on the basis of availability. Among them, RNases T1, Ms and U2 belong to the RNase T1 family. Although RNase St has similarity in the action mechanism and the active site architecture, it belongs to a different RNase family<sup>5)</sup>. RNase T2, on the other hand, has nothing to do with the RNase T1 family both structurally and functionally<sup>6)</sup>. Therefore, we did not expect that RNases St and T2 would cross-react the anti RNase F1 antibody, but rather that they would serve as non-reactive controls.

First, we examined the effect of the anti RNase F1 antibody on enzyme activity of these RNases. When approximately 5  $\mu\text{g}$  of RNase and 0.4 ml of the antibody solution were used, remaining activities (%) were as follows: RNase F1 (0); T1 (106), Ms (99), U2 (101), St (105) and T2 (103). Clearly, enzyme activity of other RNases

was not affected by the antibody even under conditions where the activity of RNase F1 was totally suppressed. The result of the double diffusion test was also straightforward: only RNase F1 formed a single precipitin line with the antibody (data not shown). However, Western blot analysis revealed a subtler situation (Fig. 3).

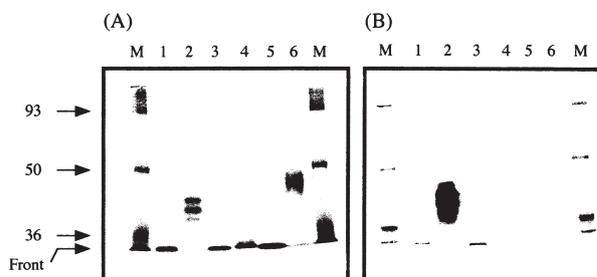


Fig. 3. SDS-PAGE of various RNases stained with coomassie brilliant blue (A) and anti RNase F1 antibody (B). M stands for molecular mass marker. Lane 1, RNase T1; 2, RNase F1; 3 RNase Ms; 4, RNase U2; 5, RNase St; and 6, RNase T2. Note that the markers are visible in experiment B because of prestaining.

The antibody cross-reacted weakly with RNase Ms and faintly with RNase T1. Interestingly, the phylogenetic tree constructed on the basis of the amino acid sequence<sup>2)</sup> by one of the authors (H. Y.) shows that closeness to RNase F1 is RNase Ms > RNase T1 > RNase U2, which is in substantial accord with the present results. It should be noted that the weak interaction demonstrated by the Western blot does not contradict the results described earlier. It is well established that formation of precipitin line in the double diffusion test requires multiple strong binding sites on the antigen molecule. Therefore, the anti RNase F1 antibody binds RNase Ms or T1 only weakly and/or at only one site. Probably, such a weak binding will not have any effect on the enzyme activity. In summary, RNase F1 is immunochemically unique in the RNase T1 family: its epitopes have little in common with those of other family members examined here. This is significant in view of close similarity of the overall protein fold and the active site architecture among the family members. (The tertiary structures of RNases F1 (1FUT), T1 (9RNT), Ms (1RMS) and U2 (1RTU) are available in the protein Data Bank.) In other words, these RNases essentially retain the same core structure but differ significantly in the surface structure.

Anomalous behavior of RNase F1 in SDS-PAGE is worth mentioning: it showed surprisingly low mobility. It is well established that the mobility of a protein in SDS-PAGE is closely correlated to its molecular mass<sup>7)</sup>. RNase F1

appeared at an area around 40 kDa although its molecular mass was approximately 1.1 kDa as those of other family members which migrated near the front (Fig. 3). It is known that RNase F1 has no post-transcriptional modification such as glycosylation<sup>8)</sup>. Therefore, this anomaly must be an intrinsic property of the protein. At present, we do not have any explanation for the observation. Unexpectedly also, RNase F1 was split into at least three bands in SDS-PAGE, all of which reacted strongly with the antibody. It was shown that RNase F1 was sometimes accompanied with a more acidic component, RNase F1', which was proven to be a derivative deamidated at C-terminal asparagine<sup>9)</sup>. If such deamidation took place spontaneously during storage, it might lead to band splitting in SDS-PAGE. This hypothesis, however, remains to be verified.

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