J. Biochem. 119, 548-552 (1996)

Enzymatic Properties of Mutant Forms of RNase Rh from *Rhizopus niveus* as to Asp51[1]

Kazuko Ohgi, Mitsuaki Takeuchi, Masanori Iwama, and Masachika Irie[2]

Department of Microbiology, Hoshi College of Pharmacy, Ebara 2-4-41, Shinagawa-ku, Tokyo 142

Received for publication, October 25, 1995

In order to determine the role of Asp51 of RNase Rh from *Rhizopus niveus*, enzymes with mutations at the 51st position, D51N, D51E, D51Q, D51S, D51T, D51A, and D51K, were prepared, and their enzymatic properties were investigated as to specific activity and base specificity. All the mutant enzymes showed relatively high activity toward poly I and poly C, and markedly reduced activity toward poly A and poly U. In particular, the enzymatic activities toward poly I of D51T and D51S were higher than that of RNase RNAP Rh. Among the mutant enzymes, D51N, D51S, and D51T showed more than *ca.* 30% of the activity of RNase Rh, when RNA, poly I and poly C were used as substrates, respectively. The substitution of Ala, Glu, or Lys at Asp51 is unfavorable for enzymatic activity. Among XpGs (X=A, G, U, or C), D51N, D51S, and D51T showed higher activity toward GpG then CpG. Therefore, Asp51 in RNase Rh plays a critical role in the adenylic acid preference of RNase T_2 family enzymes. Our results obtained with a protein engineering technique provide basic insights into the control of the base specificity of RNase Rh.

Key words: aspartic acid residue, base non-specific ribonuclease, mechanism of enzymatic action, *Rhizopus niveus*, ribonuclease.

Base non-specific RNases which cleave the 3'-5' nucleotidyl linkages of RNA forming 2',3'-cyclic phosphates at the 3'-terminal of the RNA are widely distributed in living creatures, such as viruses (*1*), bacteria (*2, 3*), fungi (*4-8*), plants (*9-15*), protozoans (*16*), and animals (*17-19*). These RNases (RNase T_2 family enzymes) each has a protein moiety, with a molecular mass of about 24 kDa. The mechanism of action of RNases in this group has been studied mostly for RNase Rh from *Rhizopus niveus* (*20-24*), RNase M from *A. saitoi* (*25, 26*), and RNase T_2 from *A. oryzae* (*27*).

Kinetic and protein engineering studies on RNase Rh revealed that its active site consists of His46, His109, His104 (*22*), Glu105 (*22*), and Lys108 (*24*). The former two residues function as general acid and base catalysts, respectively and His104 as a phosphate binding site, and the latter two facilitate catalysis, probably by stabilizing the intermediate or polarizing the P=O bond (*23, 24*).

As for the base recognition site, the contributions of Trp49, Tyr57, and Asp51 were demonstrated by Nakamura *et al.* (*28*) and Ohgi *et al.* (*29*). The three-dimensional structures of RNase Rh and its 2'-AMP complex were determined by X-ray crystallography by Kurihara *et al.* (*30*), and Nakamura *et al.* (*28*). In a previous paper (*22*), we reported that the substitution of Asn of RNase Rh from *R. niveus* for Asp51 decreased the base specificity towards the adenine base. The results indicated that the carboxyl group of the aspartic acid interacts with the amino group of adenine (*28*). The X-ray crystallographic data confirmed that the adenine base at the B_1 site (5'-side base of the scissile nucleotidic bond) interacts with Asp51 *via* hydrogen bonds. In the sequences of fungal RNases belonging to the RNase T_2 family, the Asp51 residue is conserved (Fig. 1). However, it was replaced in RNase Phy from *Physarum polycephalum* (*16*) and in a gene product of the self-incompatibility factor of petunia by Glu (*12*), in tomato RNase Le by Asn (*9*), in RNase MC_1 from *Momoridica charantia* (*11*) by Gln, in oyster RNase by Ser, and in *Drosophila melanogaster* RNase by Thr. In this study, in order to elucidate the role of Asp51 in the RNase Rh action, we prepared mutant enzymes at the 51st position including the three amino acid residues occurring in other RNase T_2 family enzymes by means of site-directed mutagenesis. They were Asn, Gln, Ala, Thr, Ser, and Lys. The enzymatic properties of these enzymes were investigated from the viewpoints of specific activity and base specificity.

MATERIALS AND METHODS

Substrates and Other Reagents—Yeast RNA was obtained from Kojin (Tokyo). Dinucleoside phosphates used as substrates were purchased from Sigma (St. Louis, MO).

Enzyme—Nucleic acid-modifying enzymes were obtained from Takara Shuzo (Kyoto) and used as recommended by the supplier. *Staphylococcus aureus* V8 protease was

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

[2] To whom correspondence should be addressed.

Abbreviations and nomenclature: RNase Rh, base non-specific and purine nucleotide-preferential RNase from *Rhizopus niveus*; RNase RNAP Rh, RNase Rh with three extra amino acid residues, Ala-Ser-Gly, at its N-terminus. Nomenclature for mutant enzymes produced on site-directed mutagenesis of RNase RNAP Rh: Mutant enzymes in which the amino acid at the *i*th residue (X) was replaced by Y as a result of site-directed mutagenesis is abbreviated as X*i*Y. Thus, D51A is a mutant enzyme in which the aspartic acid residue at the 51st position has been replaced by alanine.

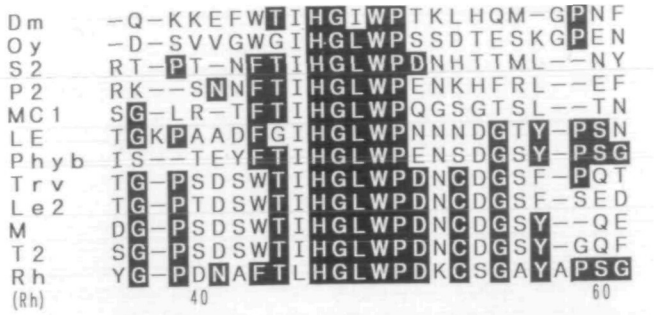

Fig 1. **Comparison of the sequences of one of two common segments containing active site amino acid residues in RNase T_2 family RNases.** Dm, RNase from *Drosophila melanogaster* (*19*), RNase Oy, oyster RNase (*17*), S2, a self-incompatibility factor with RNase activity from *Nicotiana alata* (*13*), P2, a self-incompatibility factor with RNase activity from *Petunia inflata* (*12*), MC1, an RNase from *Momordica charantia* (*11*), LE, an RNase from *Lycopersicon esculentum* (tomato) (*9*); Phyb, an RNase from *Physarum polycephalum* (*16*); Trv, an RNase from *Trichoderma viride* (*8*), Le2, an RNase from *Lentinus edodes* (shiitake, mushroom)(*7*); M, an RNase from *Aspergillus saitoi* (*6*); T2, an RNase from *Aspergillus oryzae* (*6*), Rh, *Rhizopus niveus* RNase (*4*). The amino acid residues identical to those of RNase Rh are indicated in white letters

obtained from Wako Pure Chem. (Osaka).

Site-Directed Mutagenesis—The mutant enzymes were prepared by site-directed mutagenesis according to the method of Kunkel *et al.* (*31*) with a Muta-Gene™ *in vitro* mutagenesis kit (Bio-Rad Japan, Tokyo). The synthetic oligonucleotides used are listed in Table I.

The structures of the mutant enzymes were confirmed by DNA sequence determination by the dideoxy method of Sanger and Coulson (*32*).

Expression of the Mutant Genes and Purification of Mutant RNase Rh—Site-specific mutants of pYE RNAP Rh, a plasmid for the expression of RNase Rh, were transformed into *Saccharomyces cerevisiae* R27-7C-1C, and the transformants were cultivated as previously reported (*20*). Purification of the mutant enzymes was performed as described in the previous reports (*20-22*). The mutant enzymes were verified by SDS-PAGE by staining with Coomassie Brilliant Blue.

Protein Concentration—The protein concentration was determined spectrophotometrically assuming the molar absorbance calculated from the amino acid composition (53,000).

Electrophoresis—SDS-PAGE was performed in 12% polyacrylamide gel by Laemmli's method (*33*) with protein molecular weight markers (Promega, Madison, WI, USA).

Enzyme Assay—(a) RNA as a substrate: (i) The enzymatic activity was measured by following the increase in acid-soluble nucleotides after digestion of yeast RNA at pH 5.0 and 37°C, as reported previously (*20*). (ii) The rate of hydrolysis of RNA was monitored at 22°C as the change in the absorbance at 300 nm according to Kunitz (*34*). Similarly, the depolymerization of four homopolynucleotides, poly A, poly I, poly C, and poly U, was monitored at 22°C, at 260, 240, 270, and 260 nm, respectively. The substrate concentration was 0.03 mg/ml. The buffer used was 0.05 M acetate buffer (pH 5.0). (b) Dinucleoside phosphates as substrates: the rates of hydrolysis of dinucleoside phosphates were measured according to the methods of Imazawa *et al.* (*35*) and Witzel and Barnard (*36*) by following the changes in absorbance during the course of the reaction at 22°C. Changes in absorbance were monitored with a Shimadzu UV200 spectrophotometer. The kinetic constants, K_m and V_{max}, of RNase RNAP Rh and its mutants were calculated from Lineweaver-Burk plots (*37*).

TABLE I. **Oligonucleotides for site-directed mutagenesis.**

Position in RNase Rh	Created mutation	Mutagenic oligonucleotide
Asp 51	Ser	5′-TTGGCCCTCTAAATGTTCT-3′
	Thr	5′-TTTGGCCCACTAAATGTTCTG-3′
	Gln	5′-TTTGGCCCCAAAAATGTTCTG-3′
	Lys	5′-TTTGGCCCAAGAAATGTTCTG-3′

Circular Dichroism Spectra—CD spectra were measured with a JASCO J-600 spectropolarimeter at room temperature. The cells used for the wavelength regions between 200-250 and 250-300 nm had 0.05- and 1.0-cm light paths, respectively. The enzyme concentration was *ca.* 42 μM.

Release of Four Nucleotides from RNA—A small volume of the enzyme was added to a reaction mixture (2 ml) comprising 5 mg of yeast RNA in 20 mM sodium acetate buffer (pH 5.0). The final concentrations were about 45-344 nM for RNase RNAP Rh and its mutants. The reaction were carried out at 37°C. Samples (100 μl) were withdrawn at appropriate intervals. Ten volumes of ice-cold alcohol was added to the samples, which were then kept in a freezer overnight. The precipitate formed was removed by centrifugation, and the supernatants were dried *in vacuo*. The dried samples were dissolved in the buffer for HPLC, 20 mM sodium phosphate buffer (pH 6.45). The nucleotides were separated by HPLC on a column of TSKgel 80™ (4.4×250 mm). The column was eluted with a linear gradient of acetonitrile (0.8%/15 min) in the same buffer. The flow rate was 1 ml/min. The eluate was monitored by measuring the absorption at 254 nm

RESULTS

Preparation of Mutant Enzymes as to Asp51—D51N, D51E, and D51A were prepared as described previously (*22*). Four other mutant enzymes, D51S, D51T, D51Q, and D51K, were prepared by the procedures described under "MATERIALS AND METHODS" using the oligonucleotides listed in Table I. All the enzymes were purified, each giving a single band on SDS-PAGE, and their secondary structures were found from the CD spectrum in the region between 200-240 nm to be the same as that of the native enzyme within the limits of experimental error (data not shown).

Enzymatic Properties of Asp51 Mutants—The enzymatic activities of the Asp51 mutant enzymes toward RNA at pH 5.0 are shown in Table II. The pH optima of the Asp51 mutants were pH 5.0-5.5. These values are similar to that of the wild-type enzyme (pH 5.0). Therefore, the decrease in enzymatic activity was not due to a change in the optimal pH. The RNA hydrolysis activities of all the mutant enzymes were markedly lower than that of the native enzyme.

The rates of hydrolysis of the four homopolynucleotides, poly A, poly I, poly U, and poly C, by the D51 mutant enzymes and RNase RNAP Rh are also shown in Table II.

TABLE III. **Kinetic constants of Asp51 mutant enzymes derived from RNase Rh.**

	ApG			GpG			CpG			UpG		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Rh	0.52	13,400	25,700	0.45	40,900	90,900	1.08	55,400	51,300	0.63	39,900	65,300
D51N	1.1	204	185	1.08	43,400	40,200	1.38	30,800	22,300	0.76	8,700	11,400
D51E	0.84	45	54	0.80	616	770	1.29	44	34	3.80	370	97
D51Q	0.47	478	1,010	0.70	467	667	0.83	2,520	3,300	0.79	470	590
D51S	0.39	1,300	3,300	0.96	11,800	12,300	1.36	378	278	1.48	566	382
D51T	0.67	344	513	0.72	14,300	19,800	0.64	2,910	4,040	1.45	1,100	758
D51K	1.54	5	3	1.13	23	21	2.9	15	5	1.60	72	45

K_m, V_{max}, and V_{max}/K_m values are expressed as follows; K_m, $M \times 10^4$; V_{max}, min^{-1}; V_{max}/K_m, $M^{-1} \cdot min^{-1} \times 10^{-4}$.

TABLE II. **Relative rates of hydrolysis of RNA and homopolynucleotides by RNase RNAP Rh and its Asp51 mutants.**

Enzyme	Relative rate of hydrolysis[a]				
	RNA	Poly A	Poly I	Poly C	Poly U
RNase RNAP Rh	100	100	100	100	100
D51N	34	1.8	82	37	2.4
D51E	11	0.4	48	38	1.5
D51Q	23	4.6	17	12	2.4
D51T	41	1.2	235	61	2.5
D51S	40	1.1	138	24	2.0
D51K	8.2	0.27	11.6	11	0.6
D51A	4.7	2.8	4	4.3	3.5

[a]Enzymatic activities are based on that of native RNase Rh as 100.

Since poly G has been shown to aggregate under the used reaction conditions, poly I was used instead of poly G. The rates of hydrolysis of the four homopolynucleotides by D51A and D51K were very low. The rates of hydrolyses of poly A and poly U decreased more markedly, but that for poly I increased in the cases of D51T and D51S. The rates of hydrolysis of poly I by D51N, D51Q, and D51E were higher (or comparable to) than those of poly C. Thus, the base specificities of these mutant enzymes changed, they becomming more preferential for I , then C, as compared to that of RNase Rh.

Kinetic Constants of the Cleavage of Dinucleoside Phosphates, XpG, by D51 Mutant Enzymes—The kinetic constants of the D51 mutant enzymes toward XpG where X=A, G, U, and C, were measured and the results are shown in Table III. Judging from their V_{max}/K_m values, the three mutant enzymes, D51N, D51S, and D51T, were most reactive toward GpG, and then CpG and UpG, while ApG was the poorest substrate for these enzymes. D51K was less reactive even to GpG, probably due to a large or positively charged side chain unfavorable for the interaction with the base moiety. The reactivity of D51Q was between those of D51E and D51N, and its base specificity toward CpG was the highest of the four substrates.

From the data shown here, it is evident that D51 mutants, except D51Q and D51K, show a preferential guanine base preference. Their specific activities are, however, less than that of RNase Rh having D51. The replacement of Asp51 by the amino acids described herein appeared to effectively change the base specificity.

Release of Four 2′,3′-Cyclic Nucleotides and 3′-Nucleotides from RNA on Digestion with Mutant Enzymes as to Asp51—The release of four 2′,3′-cyclic nucleotides and 3′-nucleotides from RNA on digestion with the D51 mutant enzymes other than D51A was measured, and the results are shown in Table IV. The order of the release of nucleotides by the wild type enzyme was A>G>U,C (*38*).

TABLE IV. **Release of four nucleotides from RNA on treatment with RNase Rh mutants as to Asp51.**

Enzyme	Released nucleotides
RNase Rh	A>G>>U>C
D51N	G>C>U>A
D51E	G>C>U>A
D51Q	C>G,U,A
D51S	G>C>U,A
D51T	G>C>U>A

D51S, D51T, D51N, and D51E released these nucleotides in the order of G>C>U>A, while that in the case of D51Q was C>G,U,A. The order of nucleotide release from RNA by D51S, D51T, and D51N appeared to be related to the guanylic acid preference for the three naturally occurring enzymes described above.

DISCUSSION

X-Ray crystallography of the RNase Rh-2′-AMP complex showed that Asp51 interacts with the amino group of the adenine base. The RNase Rh mutant enzymes as to this position are more or less reactive toward RNA. However, the specific activities of all the mutant enzymes were less than that of the native RNase Rh having Asp at this position. This is probably due to the decrease in adenylic acid preference of all the mutant enzymes.

Among the fungal RNases belonging to this family, Asp51 is conserved, but in RNase Oy (*17*), RNase Le (*9*), RNase Le_2 (*16*), and RNase Dm (*19*), Asp51 is replaced by Ser, Asn, Glu, and Thr, respectively. It is known that these RNases, except for RNase Dm (the specificity of which has not yet been determined), are more or less guanine base preferential RNases. These results coincided well with the data given above for the mutant enzymes, D51S, D51N, and D51E.

We observed that the CD spectrum of tomato RNase in the shorter wavelength region (200-250 nm) is somewhat different from that of RNase Rh (A. Löffler and M. Irie, unpublished results), indicating a lower β-sheet or α-helix content than that of RNase Rh. The conformational difference between tomato RNase and RNase Rh is possibly due to the N-terminal part of tomato RNase, which exhibit very low homology with the sequence of RNase Rh. In spite of such a conformational change, we could mimic the base specificities of oyster RNase, RNase Phyb, and RNase Le by replacing Asp51 with Ser, Glu, and Asn, respectively. This indicated that the spatial relationship of the active site components around the B_1 and P_1 sites is very similar in plant and fungal RNases.

However, the base specificity of RNase MC_1, which has

Gln at this position, is very complex, like that of D51Q RNase RNAP Rh. The results of the hydrolysis of 16 dinucleoside phosphates by RNase MC_1 indicated that it hydrolyzed XpU (where X = A, G, U, or C) preferentially, and that its base specificity seemed to be due to the B_2 site (base binding site corresponding to the 3′-side nucleotide of the scissile nucleotidic bond) (*39*). In the case of RNase MC_1, the apparent order of release of mononucleotides from CpXs is U > G > A > C.

D51Q showed a very complex specificity, on the hydrolysis of XpGs it preferentially hydrolyzed CpG. The relative rates of XpG hydrolysis are not the same as those of RNase MC_1. This is probably due to the difference in the constituents of the B_1 site; Tyr57 in RNase Rh is replaced by Leu in RNase MC_1 (Fig. 1) (*11*).

As can be seen from the data obtained, the K_m values of the mutant enzymes were in the range of 0.6 to 3 times of the corresponding values of RNase Rh except for in one case (UpG/D51Q). On the other hand, the V_{max} values varied markedly among the mutant enzymes. X-Ray crystallography of RNase Rh-2′-AMP showed that the B_1 site of RNase Rh consisted of Trp49, Tyr57, and Asp51, while in the D51 mutant enzymes, the former two residues remained unchanged in all cases. The marked effect of substitution on the V_{max} values, rather than the K_m values, could possibly be explained as follows. The amino acid at the 51st position is located close to those of the other active site components of RNase Rh, such as His46 and so on, in the primary structure. Therefore, the interaction of the newly substituted side chain with a substrate induces conformational changes in the catalytic components, which differ in extent depending on the mutant and substrate. Thus, the V_{max} values are affected by the substitution at the 51st position. It is evident from our data that Ser, Thr, Glu, and Asn at the 51st position are preferable for guanine base recognition. This is probably due to proper guanine base binding. The substitution of Glu and Lys for Asp is less favorable for a suitable interaction due to their long side chains or positive charge. Explanation of this phenomenon awaits further X-ray crystallographic experiments.

Among these mutant enzymes, D51Q showed a unique specificity, though the total activity seems to be very low. We do not know, in contrast to in the case of the native enzyme, why Glu at the 51st position becomes more guanylic acid preferential. Is it an effect of the side chain size or the pK_a of carboxylic acid? Clarification of this issue would be quite difficult at present, because the dissociation constants of the amino group of adenine, the substrate, is very close to that of the carboxyl group. It is noteworthy that for adenylic acid preference the presence of Asp at the 51st position is crucial. In other words, the data shown here indicate that the 51st position plays an essential role in the base recognition of RNase T_2 family RNases.

The authors wish to thank Prof. K.T. Nakamura of Showa University, and Prof. Y. Mitsui, Dr. T. Nonaka and Mr. M. Hamashima of Nagaoka University of Technology for the information on the three-dimensional structures of RNase RNAP Rh and its-2′-AMP complex.

REFERENCES

1. Schneider, R., Unger, G., Stark, R., Schneider-Scherzer, E., and Thiel, H.-T. (1993) Identification of a structural glycoprotein of an RNA virus as a ribonuclease. *Science* **261**, 1169-1171
2. Meador, J., III and Kennel, D. (1990) Cloning and sequencing of the gene encoding *Escherichia coli* ribonuclease I, exact physical mapping using a genome library. *Gene* **95**, 1-7
3. Favre, D., Nagai, P.K., and Timmis, K. (1993) Relatedness of a periplasmic broad specificity RNase from *Aeromonas hydrophila* to RNase I of *Escherichia coli* and to a family of eucaryotic RNases. *J. Bacteriol.* **175**, 3710-3722
4. Horiuchi, H., Yanai, K., Takagi, M., Yano, K., Wakabayashi, E., Sanda, A., Mine, S., Ohgi, K., and Irie, M. (1991) Primary structure of a base non-specific ribonuclease from *Rhizopus niveus*. *J. Biochem.* **103**, 408-418
5. Kawata, Y., Sakiyama, F., and Tamaoki, T. (1989) Amino-acid sequence of ribonuclease T_2 from *Aspergillus oryzae*. *Eur. J. Biochem.* **176**, 683-697
6. Watanabe, H., Naitoh, A., Suyama, Y., Inokuchi, N., Shimada, H., Koyama, T., Ohgi, K., and Irie, M. (1990) Primary structure of a base non-specific and adenylic acid preferential ribonuclease from *Aspergillus saitoi*. *J. Biochem.* **108**, 303-310
7. Kobayashi (Shimada), H., Inokuchi, N., Koyama, T., Watanabe, H., Iwama, M., Ohgi, K., and Irie, M. (1992) Primary structure of a base non-specific and adenylic acid preferential ribonuclease from the fruit bodies of *Lentinus edodes*. *Biosci. Biotech. Biochem.* **56**, 2003-2010
8. Inada, Y., Watanabe, H., Ohgi, K., and Irie, M. (1990) Isolation, characterization, and primary structure of a base non-specific and adenylic acid preferential ribonuclease with higher specific activity. *J. Biochem.* **110**, 896-904
9. Jost, W., Bak, H., Glund, K., Terpstra, P., and Beintema, J.J. (1991) Amino acid sequence of an extracellular, phosphate starvation-induced ribonuclease from cultured tomato (*Lycopersicon esculentum*). *Eur. J. Biochem.* **198**, 1-6
10. Löffler, A., Glund, K., and Irie, M. (1993) Amino acid sequence of an intracellular, phosphate-starvation-induced ribonuclease from cultured tomato (*Lycopersicon esculentum*) cells. *Eur. J. Biochem.* **214**, 627-633
11. Ide, H., Kimura, M., Arai, M., and Funatsu, G. (1991) The complete amino acid sequence of ribonuclease from the seeds of bitter gourd (*Momoridica charantia*). *FEBS Lett.* **284**, 161-164
12. Ai, Y., Singh, A., Coleman, C.E., Ioerger, T.P., Kheyer-Pour, A., and Kao, T.-h. (1990) Self-incompatibility in *Petunia inflata*: Isolation and characterization of cDNAs encoding three S-allele associated proteins. *Sex Plant Reprod.* **3**, 130-138
13. McClure, B.A., Haring, V., Ebert, P.R., Anderson, M.A., Simpson, R.J., Sakiyama, F., and Clarke, A.E. (1989) Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. *Nature* **342**, 955-957
14. Tayler, C.B. and Green, P.J. (1991) Genes with homology to fungal and S-gene RNases are expressed in *Arabidopsis thaliana*. *Plant Physiol.* **96**, 980-989
15. Kaufmann, A., Salamini, F., and Thompson, R.D. (1991) Sequence variability and gene structure at the self-incompatibility locus of *Solanum tuberoscum*. *Mol. Gen. Genet.* **226**, 457-466
16. Inokuchi, N., Koyama, T., Sawada, F., and Irie, M. (1993) Purification, some properties, and primary structure of base non-specific ribonuclease from *Physarum polycephalum*. *J. Biochem.* **113**, 425-430
17. Watanabe, H., Narumi, H., Inaba, T., Ohgi, K., and Irie, M. (1993) Purification, some properties, and primary structure of a base non-specific ribonuclease from oyster (*Crussdstra grigus*). *J. Biochem.* **114**, 800-807
18. Yagi, H., Kobayashi, H., Inokuchi, N., Koyama, T., and Irie, M. (1995) Purification and properties of a base non-specific ribonuclease from bullfrog (*Rana catesbeiana*). *Biol. Pharm. Bull.* **18**, 219-222
19. Hime, G., Prior, L., and Saint, R. (1995) The *Drosophila melanogaster* genome contains a member of the Rh/T2/S-glycoprotein family of ribonuclease-encoding genes. *Gene* **158**, 203-207
20. Ohgi, K., Horiuchi, H., Watanabe, H., Takagi, M., Yano, K., and Irie, M. (1991) Expression of RNase Rh from *Rhizopus niveus* in yeast and characterization of the secreted proteins. *J. Biochem.* **109**, 775-783

21. Ohgi, K., Horiuchi, H., Iwama, M., Watanabe, H., Takagi, M., and Irie, M. (1992) Evidence that three histidine residues of a base non-specific and adenylic acid preferential ribonuclease from *Rhizopus niveus* are involved in the catalytic function. *J. Biochem.* **112**, 132-138
22. Ohgi, K., Horiuchi, H., Watanabe, H., Iwama, M., Takagi, M., and Irie, M. (1993) Role of Asp51 and Glu105 in the enzymatic activity of a ribonuclease from *Rhizopus niveus*. *J. Biochem.* **113**, 219-224
23. Irie, M., Ohgi, K., Watanabe, H., Iwama, M., Nakamura, K.T., Kurihara, H., Nonaka, T., Mitsui, Y., Horiuchi, H., and Takagi, M. (1994) pH-profile of kinetic constants of RNase Rh from *Rhizopus niveus* and its mutant enzymes towards UpU, and possible mechanisms of RNase Rh. *J. Biochem.* **114**, 1083-1087
24. Ohgi, K., Iwama, M., Tada, K., Takizawa, R., and Irie, M. (1995) Role of Lys108 in the activity of RNase Rh from *Rhizopus niveus*. *J. Biochem.* **117**, 29-33
25. Irie, M. (1969) Photooxidation of ribonuclease from *Aspergillus saitoi*. *J. Biochem.* **68**, 569-572
26. Irie, M., Watanabe, H., Ohgi, K., and Harada, M. (1986) Site of alkylation of the major ribonuclease from *Aspergillus saitoi* with iodoacetate. *J. Biochem.* **99**, 627-632
27. Kawata, Y., Sakiyama, F., Hayashi, K., and Kyogoku, Y. (1990) Essential histidine residues of ribonuclease T_2 from *Aspergillus saitoi*. *Eur. J. Biochem.* **187**, 255-262
28. Nakamura, T.K., Ishikawa, N., Hamashima, M., Kurihara, H., Nonaka, T., Mitsui, Y., Ohgi, K., and Irie, M. (1993) Protein nucleic acid recognition in ribonuclease Rh. The 3rd International Meeting on Ribonuclease, Chemistry, Biology, Biotechnology (Capri, Italy, May 9-11), p. 56
29. Ohgi, K., Takeuchi, M., Iwama, M., and Irie, M. (1996) Enzymatic properties of mutant enzymes of RNase Rh from *Rhizopus niveus* at Trp49 and Tyr57. *J. Biochem.* **119**, 9-15
30. Kurihara, H., Mitsui, Y., Ohgi, K., Irie, M., Mizuno, H., and Nakamura, K.T. (1992) Crystal and molecular structure of RNase Rh, a new class of microbial ribonuclease from *Rhizopus niveus*. *FEBS Lett.* **306**, 189-192
31. Kunkel, T.A., Roberts, J.D., and Zakau, R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection in *Methods in Enzymology* (Wu, R. and Grossman, L., eds.) Vol. 154, pp. 415-423, Academic Press, New York
32. Sanger, F. and Coulson, A.R. (1975) A rapid method for determining sequence in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* **94**, 441-448
33. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* **227**, 680-685
34. Kunitz, M. (1946) A spectrophotometric method for the measurement of ribonuclease activity. *J. Biol. Chem.* **164**, 563-568
35. Imazawa, M., Irie, M., and Ukita, T. (1968) Substrate specificity of ribonuclease from *Aspergillus saitoi*. *J. Biochem.* **64**, 556-559
36. Witzel, H. and Barnard, E.A. (1962) Mechanism and binding sites in the ribonuclease reaction. II. Kinetic studies on the first step of the reaction. *Biochem. Biophys. Res. Commun.* **7**, 295-299
37. Lineweaver, M. and Burk, B. (1934) The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**, 658-662
38. Tomoyeda, M., Eto, Y., and Yoshino, T. (1969) Studies on ribonuclease produced by *Rhizopus* sp. I. Crystallization and some properties of the ribonuclease. *Arch. Biochem. Biophys.* **131**, 191-202
39. Irie, M., Watanabe, H., Ohgi, K., Minami, Y., Yamada, H., and Funatsu, G. (1993) Base specificity of two plant seed ribonucleases from *Momordica charantia* and *Luffa cylindrica*. *Biosci. Biotech. Biochem.* **57**, 497-498