J. Biochem. 124, 848-856 (1998)

Characterization and Primary Structure of a Base Non-Specific and Acid Ribonuclease from *Dictyostelium discoideum*

Norio Inokuchi,* Shigeru Saitoh,* Hiroko Kobayashi,* Tadashi Itagaki,* Takashi Koyama,* Saburo Uchiyama,† Masanori Iwama,‡ Kazuko Ohgi,‡ and Masachika Irie‡,1

Department of Microbiology, College of Pharmacy, Nihon University, Narashinodai 7-7-1, Funabashi, Chiba 274-0063; †Department of Biology, Dokkyo University School of Medicine, Mibu, Tochigi 321-0200; and ‡Department of Microbiology, Hoshi College of Pharmacy, Ebara 2-4-41, Shinagawa, Tokyo 142-0063

Received for publication, June 18, 1998

A base non-specific and acid RNase was isolated from cellular slime mold (*Dictyostelium discoideum*) cells in a homogeneous state (about 2.4 kDa) by SDS-polyacrylamide gel electrophoresis. The RNase (RNase DdI) has a pH optimum of 5.0. The amino acid sequence of RNase DdI was determined by a combination of protein chemistry, a search of Data base, Dicty cDB and further sequence analysis of cDNA from the same bank. RNase DdI consists of 198 amino acid residues, and about 13.3, 0.9, 1.2, 3.3, and 1.0 residues of mannose, xylose, glucose, GlcNAc, and GalNAc, respectively. RNase DdI has two characteristic conserved segments of the RNase T2 family, and thus belongs to the RNase T2 family. Considering the fact that most of the RNase activity of *D. discoideum* is present in the lysosomal fraction [Wiener and Ashworth (1970) *Biochem. J.* 118, 505-512], it was concluded that the lysosomal RNase in *D. discoideum* is a member of the RNase T2 family. The amino acid sequence of RNase DdI is highly homologous with that of *Physarum polycephalum* RNase (RNase Phyb), and its amino acid sequence seems to be similar to those of plant/animal type RNases, rather than fungal RNases. The location of RNase DdI in the phylogenetic tree of the RNase T2 family was estimated.

Key words: acid RNase, base non-specific RNase, *Dictyostelium discoideum*, lysosomal RNase, ribonuclease.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

That lysosomal RNases in animal cells belong to the RNase T2 family was indirectly shown by the following facts (*1*); (i) lysosomal RNases are acidic enzymes (*2*), (ii) the uniquely sharp pH-profile of the RNase activity of rat liver lysosomal RNase (*3*) is very similar to those of bovine and porcine spleen acid RNases (RNase Bsp1 and RNase Psp1, respectively), which were isolated by Ohgi *et al.* (*4*) and Kusano *et al.* (*1*), respectively, and (iii) both RNases have been identified as members of the RNase T2 family (*1, 5*). On the other hand, Wiener and Ashworth (*6*) demonstrated that 70% of the RNase activity of myxoamoebae of *Dictyostelium discoideum* was localized in the lysosomal fraction. In this paper, to obtain direct evidence of the nature of lysosomal RNases, we report the isolation of an RNase from *D. discoideum* cells, characterization of its enzymological properties and determination of its amino acid sequence.

In the previous paper, we reported the enzymatic properties and primary structure of the RNase of an amoebae, *Physalum polycephalum* (*7*). The RNase clearly belonged to the RNase T2 family (*7*), since it had unique structural characteristics of RNase T2 family enzymes (*8*). In this paper, we will also compare the enzymatic characteristics and structural relationship of RNase from *D. discoideum* with RNase Phyb and RNase Rh (a typical fungal RNase belonging to the RNase T2 family).

MATERIALS AND METHODS

Cultivation—One or two plates of cells of *D. discoideum* strain NC4 on bacto-peptone agar containing glucose, Na_2HPO_4 and KH_2PO_4 (*9*) were harvested and suspended in 1.5 liters of pre-cultured *Escherichia coli* cells at 37°C for 8 h in bacto-peptone medium (pH 7.2). The cells were then shaken for 4 days at 22°C (pre-cultured cells). To 15 liters of bacto-peptone medium was added 300 ml of an *E. coli* culture, followed by aeration for 36 h at 22°C. Pre-culture cells were added to the *E. coli* culture, followed by further aeration for 5-8 days. The cultivated *D. discoideum* cells were centrifuged at 4,500 rpm (1,740×*g*). The pellet was washed with Bonner's salt medium (*10*) 8 times and then centrifuged at 2,000 rpm (400×*g*) for 5 min. The washed cells were suspended in an equal volume of deionized water and kept at −20°C until use.

Chemicals—TSKgel G2000SW_{XL}, DEAE-Toyopearl, and SP-Toyopearl were purchased from Tosoh (Tokyo). Ultrogel AcA54 and heparin-Sepharose CL6B were obtained from Pharmacia Japan (Tokyo). Yeast RNA was a product

[1] To whom all correspondence should be addressed.
Abbreviations: DTT, dithiothreitol; RNase DdI, a base non-specific and acid ribonuclease from *Dictyostelium discoideum*; RCM RNase DdI, reduced and *S*-carboxymethylated RNase DdI.

© 1998 by The Japanese Biochemical Society.

of Marin Bio (Tokyo).

Lysyl endopeptidase was obtained from Wako Pure Chem. (Osaka). Asparaginyl endopeptidase was purchased from Takara Shuzo (Shiga, Japan).

Enzyme Assay—Enzyme activity was measured as described in the previous paper (*11*). To 1 ml of sodium acetate buffer (50 mM, pH 5.0) containing yeast RNA (2.5 mg) was added a small volume of the enzyme solution, and then reaction mixture was incubated at 37°C. The reaction was stopped by the addition of MacFadyen reagent (0.5 ml). The supernatant (150 μl) obtained on centrifugation of the reaction mixture at 3,000 rpm for 5 min was diluted with 1 ml of deionized water and then the absorbance of the diluted solution was measured at 260 nm. One enzyme unit was defined as the absorbance increase at 260 nm per 5 min under the experimental conditions described above.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—SDS-PAGE was conducted in a 12% polyacrylamide gel according to the method of Laemmli (*12*). The gel was stained with a silver staining kit (Daiichi Kagaku, Tokyo). Molecular weight determination was performed by SDS-PAGE with molecular weight marker proteins (Oriental Yeast, Tokyo).

Activity Staining—Activity staining of RNase on a slab-gel was performed essentially according to the procedure of Blank *et al.* (*13*).

Protein Concentrations—Protein concentrations were determined by the method of Smith *et al.* (*14*) with bovine serum albumin as the standard. The protein concentration of the final enzyme preparation was measured spectrophotometrically assuming the absorbance at 280 nm of a 0.1% protein solution to be 1.82. This value was estimated from the amino acid composition.

Amino Acid Analysis—Amino acid analysis of proteins and peptides was performed by the method of Bidlingmeyer *et al.* (*15*) with a Pico Tag amino acid analysis system (Millipore Japan).

Measurement of Tryptophan—Tryptophan was measured by the method of Pajot (*16*).

Hexosamine Content—The hexosamine content was determined with a Pico Tag amino acid analysis system (Millipore Japan) by the method of Bidlingmeyer *et al.* (*15*) after hydrolysis of glycoproteins with 2 N HCl at 100°C for 12 h as described in the previous paper (*17*).

Carbohydrate Content—The carbohydrate content was measured by the method of Mikami and Ishida (*18*) after hydrolysis with 4 N trifluoroacetic acid at 100°C for 4 h. The neutral sugars were separated with a cation exchanger (Shimpack-ISA07/S2504), and then determined fluorometrically through reaction with arginine.

Reduced S-Carboxymethylation of RNase DdI—RNase DdI (the purified *D. discoideum* RNase in this report) was reduced and *S*-carboxymethylated by the method of Crestfield *et al.* (*19*). The reduced and *S*-carboxymethylated RNase DdI (RCM RNase DdI) was separated from the excess reagents by dialysis against deionized water.

Digestion with Proteases—RCM RNase DdI was digested with lysyl endopeptidase in trimethylamine-acetate buffer (pH 8.9) at 37°C for 3 h, with a protease/substrate ratio of 1 : 400 (w/w). One of the lysylendopeptidase-digested peptides (L2) was further digested with asparaginyl endopeptidase in 20 mM sodium acetate buffer (pH 5.0) containing 1 mM each EDTA and DTT at 37°C for 15 h. The enzyme was used at 0.04 mU/2 nmol peptide.

Amino Acid Sequence Determination—The amino acid sequences of proteins and their lysyl endopeptidase digestion peptides were determined by Edman degradation with an Applied Biosystems 477A protein sequencer with a 120A PTH analyzer, as described by Hewick *et al.* (*20*).

Sequence Determination of an RNase from a cDNA Clone of D. discoideum—A cDNA clone, SSI141, derived from *D. discoideum* Ax4 (Dicty cDB) cloned into pBluescriptIIKS was transformed into *Escherichia coli* cells, one shot competent cell top 10 (Invitrogen, CA), and the transformants were selected in medium with ampicillin. The plasmids were purified from the transformed *E. coli* cells cultivated in LB broth by an alkaline prep method (*21*). The sequencing of cDNA was performed by the dideoxye-method (*22*) with a Thermo Sequenase Fluorescence Labeled Primer Cycle Sequencing Kit (*23*) (Amersham-Pharmacia) with a LI-COR DNA sequencer LIC 4000.

Release of Four Nucleotides upon Digestion of RNA with RNase—RNA (0.5 mg) was dissolved in 1 ml of 50 mM sodium acetate buffer (pH 5.0) and then incubated with 0.8 μg of RNase at 37°C. Aliquots were withdrawn at appropriate intervals and the products were analyzed by HPLC as described previously (*7*).

RESULTS

Purification of a Base Non-Specific and Acid RNase from D. discoideum Cells—A cell suspension of *D. discoideum* (340 ml) in an equal volume of distilled water was frozen and thawed three times, and then centrifuged at 10,000 rpm (17,000 × *g*) for 30 min. The supernatant (crude extract) was further purified by ammonium sulfate fractionation, column chromatographies on DEAE-Toyopearl and SP-Toyopearl, gel filtration on Ultrogel AcA 54, affinity chromatography on heparin-Sepharose CL6B, and gel-filtration on TSKgel G2000SW$_{XL}$, successively. The purified RNase gave a single band on SDS-PAGE followed by silver staining (Fig. 1). Its mobility was the same as that of the RNase activity of the crude extract detected on activity staining. The purification steps are summarized in Table I. Thus, *D. discoideum* RNase was purified 12,000-fold from the crude extract. The electrophoretic mobility of this enzyme was very similar to that of one of the isozymes

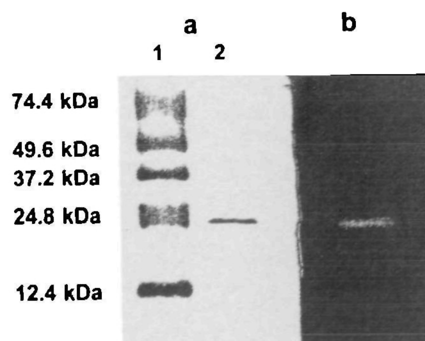

Fig. 1. **SDS-PAGE on a Slab-Gel (12%) of RNase DdI.** (a) Silver staining. Lane 1, molecular weight marker proteins; lane 2, RNase DdI. (b) Activity staining of RNase in a crude extract of *D. discoideum*. The activity staining was performed as described under "MATERIALS AND METHODS."

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

in this organism reported by Uchiyama *et al.* (*24, 25*) (RNase DdI), we designate it as RNase DdI hereafter. (We did not detect any other isozyme of *D. discoideum* under the experimental conditions used in this paper.)

Characterization of RNase DdI—The optimal pH of RNase DdI was 5.0. Among the divalent cations examined and EDTA, only Cu^{2+} and Zn^{2+} inhibited this enzyme, it showing 9 and 67% of the activity of the native enzyme, respectively, at the concentration of 1.0 mM (Table II). RNase DdI was stable up to 55°C (unpublished data). The rates of release of four mononucleotides from RNA with RNase DdI were in the order of GMP > UMP > AMP > CMP (Fig. 2). Thus, the base specificity of RNase DdI seems to be guanylic acid and then uridylic acid, as far as determined with this method.

Fig. 2. **Release of nucleotides upon digestion of RNA with RNase DdI.** The hydrolysis and separation conditions are given under "MATERIALS AND METHODS" and in our previous report (7), respectively. ●, GMP; ○, UMP; ▲, AMP; △, CMP. The nucleotides released were expressed as percentages, the released nucleotides at 48 h being taken as 100%.

Amino Acid Sequence of RNase DdI—The N-terminal amino acid sequence of RCM RNase DdI was determined by Edman degradation up to the 42nd amino acid residue (Fig. 3). RCM RNase DdI was digested with lysylendopeptidase and the products were separated into six peaks (L1-L6) on HPLC on a Capcellpak C-18 (Shiseido) column (Fig. 4a). The amino acid sequence of L1 was included in the sequence of N-terminal amino acid sequence of RCM RNase DdI. The N-terminal part of L2 overlapped with the 31st to 42nd residues of the N-terminal sequence, thus the amino acid sequence of RCM RNase DdI was determined up to the 65th residue from the N-terminus. L2 was further digested with asparaginyl endopeptidase, and the products were separated by HPLC on a TSKgel ODS-80_{TM} column into 3 peaks (Fig. 4b). The amino acid sequences of two of the peptides, L2-N1 and L2-N2, are shown in Fig. 3. They were Ser45-Asn61 and Ala62-Lys91, respectively (Fig. 3). With these

TABLE I. **Purification of RNase DdI from *D. discoideum* (349 ml).**

Step	Column size	Elution conditions	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification
1. Crude extract			523	3,920	0.13	100	1
2. Ammonium sulfate fractionation (0.4-0.9 saturation)			323	360	0.90	61.8	7
3. DEAE-Toyopearl	1.5×25 cm	10 mM Tris-HCl (pH 7.0) NaCl, 0-0.5 M (700 ml)	467	386	1.21	89.2	9
4. SP-Toyopearl	1.0×40 cm	10 mM Na acetate (pH 3.5) NaCl, 0-0.4 M (600 ml)	356	22	16.2	68.1	124
5. Ultrogel AcA 54	3.5×180 cm	10 mM Na acetate (pH 4.5)	168	–	–	32.1	
6. Heparin-Sepharose CL6B	1.0×13 cm	10 mM Na acetate (pH 4.5) NaCl, 0-1 M (600 ml)	189	0.40	473	36.2	3,640
7. TSKgel G-2000SW_{XL}	0.78×30 cm	20 mM trimethylamine-acetate (pH 6.0)	79	0.05	1,590	15.2	12,200

Fig. 3. **The amino acid sequence of RNase DdI estimated by superimposing on the sequence of RNase Phyb (7).** RCM RNase DdI, the six peptides obtained on digestion of RCM RNase DdI with lysylendopeptidase, and some of the peptides obtained on asparaginyl endopeptidase digestion of L2 were superimposed on the amino acid sequence of RNase Phyb from *P. polycephalum* (7). "L" and "LN" indicate the lysylendopeptidase digestion product of RCM RNase DdI and the asparaginyl endopeptidase digest of L2, respectively. The L and LN peptides are numbered from the N-terminus to the C-terminus. The numbers at the top of the matrix are the RNase DdI numbering from sequencing of the cDNA. The heavy lines with an arrow head at the right end indicate the amino acid sequences determined by Edman degradation. The dotted lines indicate the amino acid sequences estimated only from the amino acid compositions of the peptides.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

Fig. 4. **a: Separation of the lysylendopeptidase digest of RCM RNase DdI on a Capcellpak C18 column (10×250 mm) equilibrated with 40 mM trimethylamine-acetate buffer (pH 8.0).** The column was eluted with a linear gradient between the same solvent and the solvent containing 60% acetonitrile. The flow rate was 1 ml/min. One ml fractions were collected. **b: Separation of the aspraginyl endopeptidase digest of the L2 fraction in (a) on a TSKgel ODS-80$_{TM}$ column equilibrated with 0.1% trifluoroacetic acid.** The column was eluted with a linear gradient between the same solvent and the solvent containing 80% acetonitrile. The flow rate was 1 ml/min. 0.5 ml fractions were collected.

TABLE II. **Effects of divalent cations and EDTA (1 mM) on RNase DdI activity at pH 5.0.**

Compound	Relative activity (%)
Non	100
EDTA	97
$BaCl_2$	98
$CaCl_2$	96
$FeCl_2$	97
$SrCl_2$	96
$CdCl_2$	95
$MnCl_2$	95
$CoCl_2$	93
$HgCl_2$	91
$NiCl_2$	85
$ZnCl_2$	67
$Cu(CH_3COO)_2$	9

TABLE III. **Amino acid composition of RNase DdI.**

Amino acid	Experimental (residue/mol)	Amino acid residues (Calcd. from the sequence)
Asp	26.2	26
Glu	17.5	17
CM Cys	n.d.	8
Ser	24.3	24
Gly	14.6	14
His	4.4	5
Arg	1.3	1
Thr	16.5	17
Ala	7.6	8
Pro	12.3	12
Tyr	8.4	8
Val	5.4	6
Met	1.0	1
Ile	14.4	15
Leu	9.9	10
Phe	13.2	13
Lys	8.5	8
Trp[b]	4.5	5
Total		198
GlcNAc	3.3	
GalNAc	1.0	
Mannose	13.3	
Xylose	0.9	
Glucose	1.2	

[a]Based on one Met residue per mol enzyme. [b]Determined by fluorometry (*15*). n.d., not determined.

```
  1                                                                      ccctcgaggtcgac   14
 15 ccacgcgtccgattatatttttaatcctaaaaaaaacccaacagttttatttagaaactcttttaaaaccttaaagtttg   94
 95 aatttttttttttttttaaaaaaaaatctcttttttttattttttaaattttttatttttttcatatattttttttcaattct  174
175 ttttttttcgaataaacactccaatttataaaaatatcatttatctgatataacctcggccgaagaaaattaccaaaaa  254

255 ATG AGA TTA ATT GCT GCT CTT TTA TCT GTA TTA TTA ATA GCT TCA ACT GCT CAA TCA ACT  314
     M   R   L   I   A   A   L   L   S   V   L   L   I   A   S   T   A   Q   S   T
   -25              -20                                         -10
315 GTT ACA ATT TAT GAA AGT TCA AAA CCA GGT GAT TTT GAC TTT TAT TTA TTT GTT CAA CAA  374
     V   T   I   Y   E   S   S   K   P   G   D   F   D   F   Y   L   F   V   Q   Q
                         1                                   10
375 TGG ATT TAT TCA TAC TGC GAT TCA CAA ACT TGT ATT CAA AAT AAA GAA AGA GAA GCA TTC  434
     W   I   Y   S   Y   C   D   S   Q   T   C   I   Q   N   K   E   R   E   A   F
                     20                                      30
435 ACT ATC CAT GGT TTA TGG CCA GAG AAT AGT GAT GGC AGT TAT CCA TCA TTC TGT TCA GGT  494
     T   I   H   G   L   W   P   E   N   S   D   G   S   Y   P   S   F   C   S   G
                     40                                      50
495 CCA TCA TTC AAT GTT AAT GCT ATC CAA GAT TTA GAA GAT CAA TTA AAC TTT GAT TGG CCA  554
     P   S   F   N   V   N   A   I   Q   D   L   E   D   Q   L   N   F   D   W   P
                     60                                      70
555 TCA CTC ACT GGT CCA AAC ACT GAT TTC TGG ACT CAC GAA TTC AGC AAA CAT GGT ACT TGT  614
     S   L   T   G   P   N   T   D   F   W   T   H   E   F   S   K   H   G   T   C
                     80                                      90
615 TCA ATC ACA CAA CCA ATC ACA GAT ATT CAC GAT TAT TTC GCT ACT GGT ATC AAA CTT TAC  674
     S   I   T   Q   P   I   T   D   I   H   D   Y   F   A   T   G   I   K   L   Y
                    100                                     110
675 ACC GAA TTC AAT ATC ACT GCT GCC CTC GAA TCT GAA AAT ATT TAT CCA TCA GAT AGC AAC  734
     T   E   F   N   I   T   A   A   L   E   S   E   N   I   Y   P   S   D   S   N
                    120                                     130
735 ACT TAC AAA CCA GTC GAT ATC ACT AAT GCT ATC ACC ACT CAT TTT GGT GGT AAA CCA GGT  794
     T   Y   K   P   V   D   I   T   N   A   I   T   T   H   F   G   G   K   P   G
                    140                                     150
795 ATT CAA TGT TCA TCT GGT CAA TTA TCA ACT GTT GCT GTT TGT ATC GAT AAA AAT AGT TTA  854
     I   Q   C   S   S   G   Q   L   S   T   V   A   V   C   I   D   K   N   S   L
                    160                                     170
855 TCA ATC ATG GAT TGT CCA GAT CTC CAA GGT TGG AGT TGT TCT GGT AGT GTT AAA TTC CCA  914
     S   I   M   D   C   P   D   L   Q   G   W   S   C   S   G   S   V   K   F   P
                    180                                     190
915 AGC ACT GCT TAA aaatatttaacaaaactatcatataaaaaaaaaaaaaaaaaaaaaaaaaatattatatata  990
     S   T   A   *
            198
991 ggttttattttaatacatacccaatatatataatatttttaatgtgtgttttaacaaaaatttcaaaaaaaaaaa     1063
```

Fig. 5. **The nucleotide sequence of cDNA of RNase DdI and the amino acid sequence of RNase DdI deduced from the nucleotides sequence.** The amino acid numbering for RNase DdI is shown below the sequence as plus 1 to 198. The signal peptide sequence is numbered from −1 to −25. The probe used for the first search of the cDNA library is shaded. The amino acid sequence in by italics indicates the amino acid sequence of the signal peptide. The asterisk indicates the stop codon.

sequences, the sequence of RNase DdI was determined up to Lys91. The amino acid sequences of the rest of the lysylendopeptidase peptides (L3-L6) were also determined, as shown in Fig. 3. The alignment of these peptides was performed in two ways. First, they were aligned as to the homology to the amino acid sequence of RNase Phyb from *P. polycephalum* (*7*). By this method the entire amino acid sequence of RNase DdI was determined except for an about 9 amino acid residue sequence (probably in the C-terminal of L4) not yet determined by protein chemistry.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

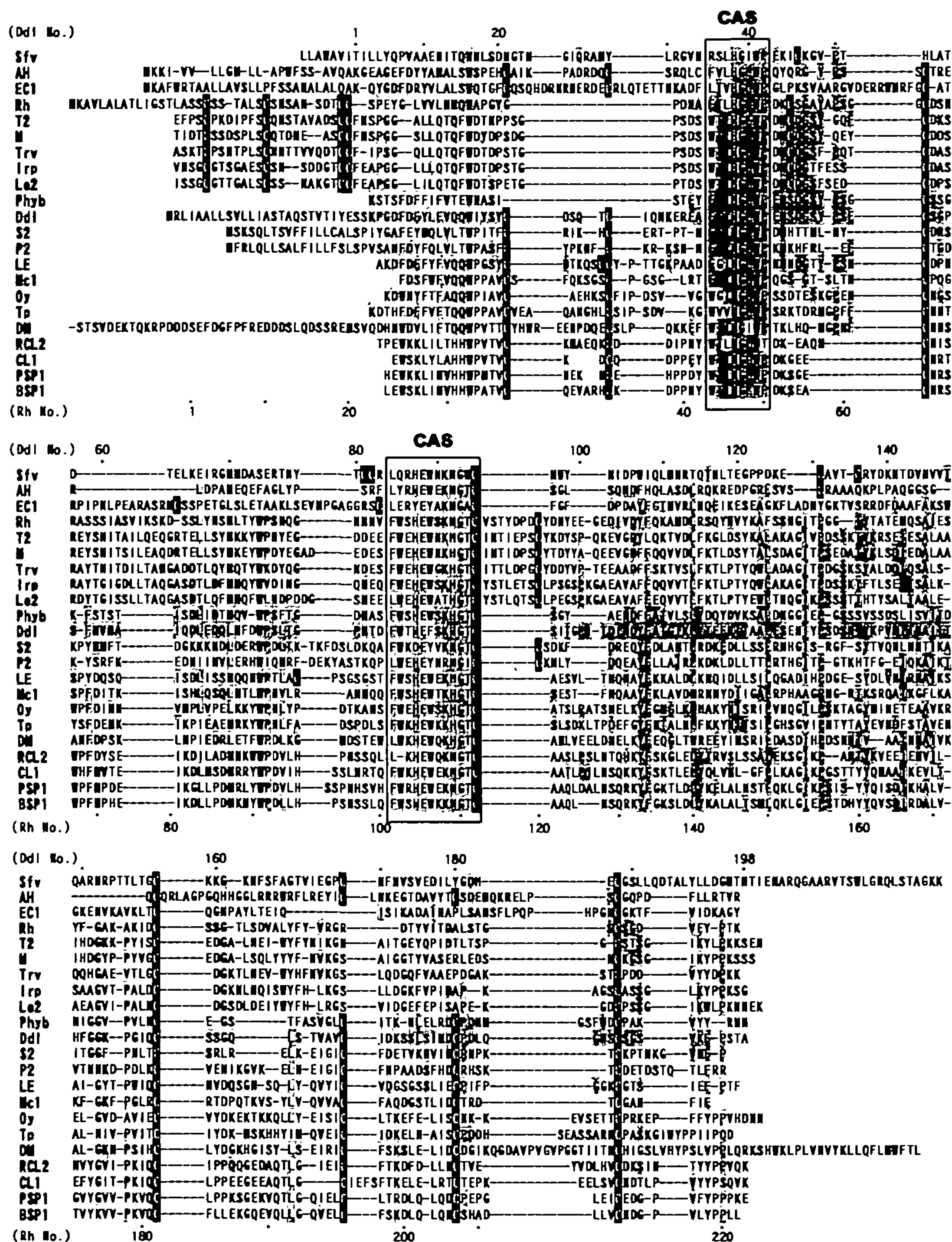

Fig. 6. **Comparison of the amino acid sequence of RNase DdI with those of other RNases belonging to the RNase T2 family.** SFV, swine fever virus RNase (*26*); AH, RNase from *Aeromonus hydrophila* (*27*); EC1, RNase I from *Escherichia coli* (*28*); Rh, RNase Rh from *R. niveus* (*29*); T2, RNase T2 from *A. oryzae* (*30*); M, RNase M from *A. saitoi* (*17*); Trv, RNase Trv from *Trichoderma viride* (*31*); Irp, RNase Irp from a mushroom, *Irpex lacteus* (*32*); Le2, RNase Le2 from a mushroom, *Lentinus edodes* (*33*); Phyb, RNase Phyb from *P. polycephalum* (*7*); DdI, RNase from a cellular slime mold; S2, a self-incompatibility RNase from *Nicotiana alata* (*34*); P2, a self-incompatibility RNase from *Petunia inflata* (*35*); LE, a tomato RNase from *Lycopersicon esculentum* (*36*); MC1, RNase from the seeds of bitter gourd (*Momordica charantia*) (*37*); Oy, oyster RNase (*38*); TP, RNase from squid (*Todarodes pacificus*) (*39*); DM, RNase from *Drosophila melanogaster* (*40*); RCL2, bullfrog (*Rana catesbeiana*) RNase RCL2 (*41*); CL1, chicken liver RNase CL1 (*42*); Psp1, porcine spleen RNase Psp1(*1*); Bsp1, bovine spleen RNase Bsp1 (*5*). The numbers at the top and bottom of the matrix are the RNase DdI and RNase Rh numbering, respectively. The amino acid residues which are the same as those in RNase DdI are shaded.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

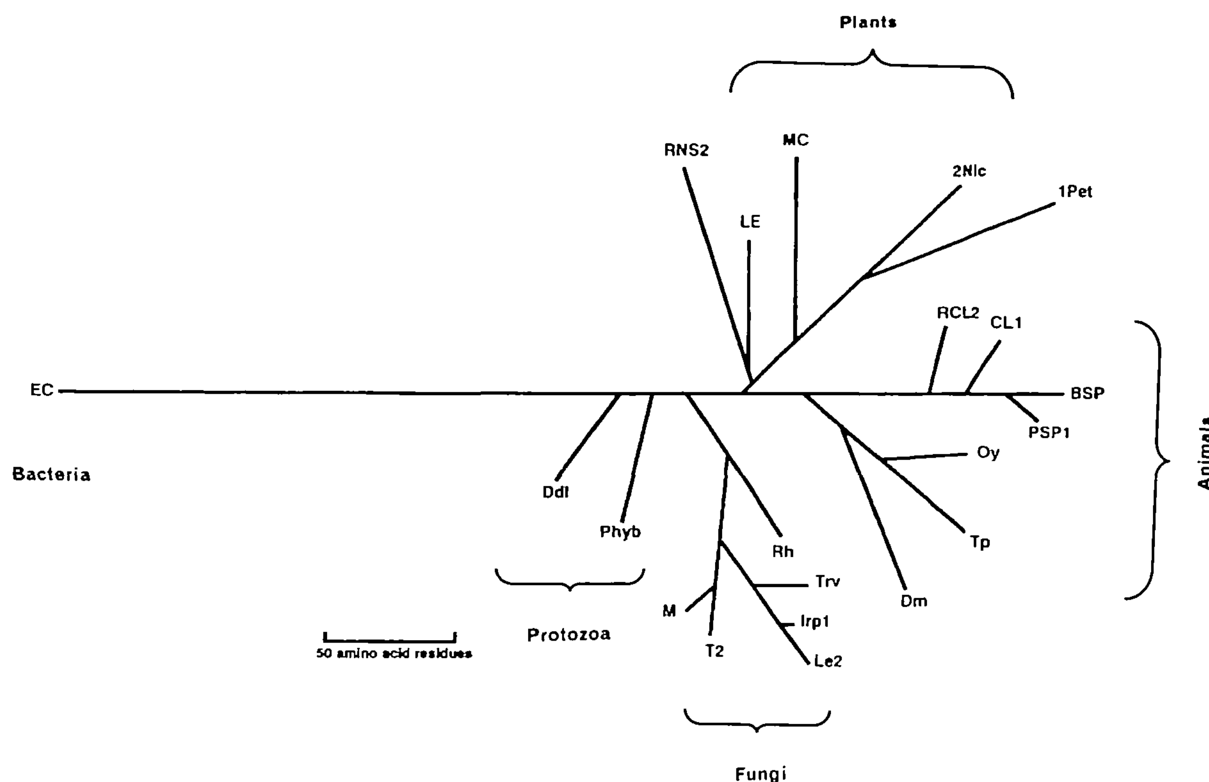

Fig. 7. **Phylogenetic tree of RNase T2 family enzymes.** The tree was constructed using the data shown in Fig. 6 by means of the method of Adachi and Hasegawa (*43*).

Secondly, the cDNA sequence library of Data base, Dicty cDB (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html) was searched using HGTCSITGPITDIHDYFATGS (L3) as a probe, and we found the same sequence in SSI 141 covering 528 bases (from the C-terminus up to Leu70). This sequence included L3, L4, L5, and L6, and 22 residues of L2-N2 and 137 bases of the 3′-flanking region (Fig. 5), *i.e.* not the whole sequence. However, only one differece was found, *i.e.* the N-terminus of L5, Asn, was Ser in the cDNA sequence in this data base. To confirm this discrepancy, cDNA obtained from Dicty cDB was re-sequenced from the 3′-side up to 712 bases, and it was found that the cDNA sequence also contains an Asn residue at this position. Thus it was clearly indicated that SSI141 includes the C-terminal 107 residues of RNase DdI. To confirm the structure further, the N-terminal part of RNase DdI, *i.e.* the nucleotide sequence of the minus strand of cDNA SSI141, was analyzed, a 689 base sequence consisting of a 254 base 5′-flanking region, a 75 base signal peptide and 360 N-terminal bases (up to Ile120) being determined (Fig. 5). Thus, the cDNA sequence overlapped with that determined from the C-terminus and well coincided with the amino acid sequence elucidated by means of protein chemistry, as described above (Figs. 3 and 5). Thus we elucidated the whole sequence of RNase DdI composed of 198 amino acid residues from the cDNA sequence (SSI141) and the results of protein sequencing. The amino acid composition of RNase DdI is shown in Table III. The amino acid composition coincides fairly well with that calculated from the sequence deduced in this study (Figs. 3 and 5).

In the amino acid sequence of L4, Asn119 was not determined on protein sequencing and the sequence, Asn-Ile-Thr, indicated that Asn119 is a *N*-glycoside attachment site. Indeed, this protein contains about 13.3, 0.9, 1.2, 3.3, and 1.0 residues of mannose, xylose, glucose, GlcNAc, and GalNAc, respectively (Table III). Therefore, Asn119 might be the only carbohydrate junction of this RNase. Since RNase DdI is homologous to RNase Phyb and it contains two unique CAS sequences (conserved active site sequence), it is a member of the RNase T2 family (see Fig. 5). Therefore, it was concluded that the lysosomal RNase of this organism is a member of the RNase T2 family.

Comparison of the Amino Acid Sequence of RNase DdI with Those of Other RNase T2 Family Enzymes—Since RNase DdI is a member of the RNase T2 family, the primary structure of RNase DdI was compared to those of typical members of the RNase T2 family (Fig. 6). RNase DdI shared 54-62, 50-77, and 49-65 amino acid residues with the RNase T2 enzymes of animal, plant and fungi origin, respectively. It is most homologous to *P. polycephalum* RNase Phyb (83 amino acid residues), and tomato RNase LE (77 amino acid residues). The most homologous RNase in fungal world so far examined is RNase Rh of *Rhizopus niveus* (65 amino acid residues). The phylogenetic tree constructed by the most likelihood method developed by Adachi and Hasegawa (*43*) is shown in Fig. 7. The results indicated that RNase DdI is most closely related to RNase Phyb. The tree indicated that RNase DdI diverged only slightly before RNase Phyb and fungi did.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

DISCUSSION

The results of this study showed that RNase DdI is a RNase T2 family enzyme, as shown in Fig. 6. The results also support the suggestion in the previous paper (*1*) that the bovine and porcine spleen acid RNases are lysosomal enzymes.

The RNase T2 family enzymes are known to play a variety of roles in living organisms. In the plant world, they function as self-incompatible S-RNases (*34, 35, 44*), defense RNases in many seeds as plant toxins (*37, 45*), and vacuolar enzymes (*45*). Among microbes, RNase I in *E. coli* is localized in the periplasmic space (*28, 46*). Considering these results, it could be stated that the common function of RNase T2 family members seems to be digestion of the exogenous RNA in the orgnellar or periplasmic space of cells.

Two protozoan RNases, RNase Phyb and RNase DdI, are highly homologous enzymes, although RNase DdI has four disulfide bridges in contrast to RNase Phyb, which has three (*7*). The locations of these three/four disulfide bridges of those two protozoan RNases are the same as those in plants and animals RNases (Fig. 6), but differ from those in fungal RNases except for the two disulfide bridges common to whole RNase T2 enzymes. In this respect, RNase DdI is more closely related to plant and animal than fungal RNases. In addition to the locations of disulfide bridges, plants/animal type RNases have several common characteristics as compared to fungal RNases: (i) there is an about 10 amino acid residue loop between the 38 and 39th residues of RNase Rh (RNase Rh numbering) (*29*), and (ii) there are deletion between the 74th-80th residues and shortening of the peptide corresponding to the 113rd to 121st residues, as compared to those of fungal RNases. In this respect, RNase DdI has similar characteristics to plant-animal RNases, but RNase Phyb lacks the first characteristic, thus, it has a slightly more fungal RNase-like structure than RNase DdI.

All of the amino acid residues responsible for the catalysis by RNase Rh, the most active RNase in this family, *i.e.* His46, His104, His109, E105, and K108 (RNase Rh numbering), are conserved in RNase DdI (Fig. 6) (*8, 47*). This is probably the reason why RNase DdI showed relatively higher specific activity towards RNA.

The base specificity of RNase DdI determined as the rates of release of four mononucleotides on digestion of the RNA was G>U>A≫C, but that of RNase Phyb was G≧A>U,C (*7*). The base specificity of RNase Phyb is very similar to those of many fungal RNases (A≧G>C,U), but that of RNase DdI is more similar to those of plants/animal type RNases (*8*).

In RNase DdI, two of the B1 site (major base recognition site) constituents of RNase Rh, Y57, and W49, are conserved, but D51 is replaced by Glu (*47, 48*). This is one of the possible reasons for why in contrast to many fungal RNases, RNase DdI exhibits a greater preference for guanine base, because the substitution of D51 by Glu in RNase Rh makes the mutant enzyme prefer guanylic acid more (*8, 49*). The B2 site (the second base recognition site located on the 3′-side of the B1 site) of RNase Rh comprises Q32, P92, S93, N94, Q95, and F101 (*48*). Among these amino acid residues, N94 and Q95 are replaced by Leu and Thr, respectively, in RNase DdI. The replacement of hydrophilic and hydrogen bonding forming N94 with hydrophobic Leu may markedly affect the B2 base recognition.

Base non-specific and acid RNases (RNase T2 family members) are known to be distributed among various organisms, from viruses to bacteria, plants, fungi, and animals (*8*). Therefore we could trace the phylogenetic relationship of animal, plant, fungal, and microbial RNases, and study the changes in the structures and physiological functions of this type of enzyme during evolution.

As shown in Fig. 7, the phylogenetic relationship of base non-specific and acid RNases of animals, plants, and fungi is very unusual as compared to those of other proteins (*50*). The phylogenetic trees of many proteins indicate that the plant proteins forms an out-group of the *D. discoideum* and fungi-animal groups. Among base non-specific and acid RNases, fungi RNases form an out-group of the plant and animal groups, although we are not able to explain why RNase T2 enzymes exhibit such an unusual tree. As shown in Fig. 7, RNases Phyb and DdI diverged before the divergence of fungi and plants/animals.

The codon usage of this organism exhibits a quite unique dialectal codon choice; that is a guanine base at the third codon is very rare except in the cases of Met and Trp (Table IV). The data demonstrating this phenomenon have already been presented in a report by Wada *et al.* (*51*).

TABLE IV. **Codon usage of mRNA for RNase DdI.**

Amino acid	Codon	Frequency	Amino acid	Codon	Frequency
Arg	CGA	0	Val	GUA	1
	CGC	0		GUC	1
	CGG	0		GUG	0
	CGU	0		GUU	6
	AGA	2	Lys	AAA	8
	AGG	0		AAG	0
Leu	CUA	0	Asn	AAC	3
	CUC	3		AAU	8
	CUG	0	Gln	CAA	10
	CUU	2		CAG	0
	UUA	10	His	CAC	2
	UUG	0		CAU	3
Ser	UCA	14	Glu	GAA	8
	UCC	0		GAG	1
	UCG	0	Asp	GAC	1
	UCU	4		GAU	14
	AGC	3	Tyr	UAC	3
	AGU	6		UAU	6
Thr	ACA	3	Cys	UGC	1
	ACC	2		UGU	7
	ACG	0	Phe	UUC	8
	ACU	15		UUU	5
Pro	CCA	12	Ile	AUA	1
	CCC	0		AUC	10
	CCG	0		AUU	7
	CCU	0	Met	AUG	2
Ala	GCA	1	Trp	UGG	5
	GCC	1	Ter	UAA	1
	GCG	0		UAG	0
	GCU	10		UGA	0
Gly	GGA	0			
	GGC	1			
	GGG	0			
	GGU	13			

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

REFERENCES

1. Kusano, A., Iwama, M., Sanda, A., Suwa, K., Nakaizumi, E., Nakatani, Y., Ohkawa, H., Ohgi, K., and Irie, M. (1997) Primary structure of porcine spleen ribonuclease: sequence homology. *Acta Biochim. Polonica* **44**, 689-700
2. de Duve, C., Pressman, B.C., Glanetto, R., Wattlaux, R., and Appelmans, P. (1955) Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60**, 604-617
3. Futai, M., Miyata, S., and Mizuno, D. (1969) Acid ribonucleases of lysosomal and soluble fractions from rat liver. *J. Biol. Chem.* **244**, 4951-4960
4. Ohgi, K., Sanda, A., Takizawa, Y., and Irie, M. (1988) Purification of acid ribonucleases from bovine spleen. *J. Biochem.* **103**, 267-273
5. Narumi, H., Ogawa, Y., Iwama, M., Kusano, A., Sanda, A., Ohgi, K., and Irie, M. (1997) Bovine spleen acid ribonuclease is a member of the RNase T2 family. *J. Biochem. Mol. Biol. Biophys.* **1**, 21-26
6. Wiener, E. and Ashworth, J.M. (1970) The isolation and characterization of lysosomal particles from myxamoebae of the cellular slime mold *Dictyostelium discoideum. Biochem. J.* **118**, 505-512
7. 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**, 425-432
8. Irie, M. (1997) RNase T1/RNase T2 family RNases in "Ribonucleases, Structures and Functions" (D'Alessio, G. and Riordan, J.F., eds.) pp. 101-130, Academic Press, New York
9. Bonner, J.T. (1967) Growth in *The Cellular Slime Molds*, pp. 76-91, Princeton University Press, Princeton, New Jersey
10. Bonner, J.T. (1947) Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum. J. Exp. Zool.* **106**, 1-26
11. Irie, M. (1967) Isolation and properties of a ribonuclease from *Aspergillus saitoi. J. Biochem.* **62**, 509-518
12. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
13. Blank, A., Sugiyama, R.H., and Dekker, C.A. (1982) Activity staining of nucleolytic enzymes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis: use of aqueous isopropanol to remove detergent from gels. *Anal. Biochem.* **120**, 267-275
14. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzono, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85
15. Bidlingmeyer, B.A., Cohen, S.A., and Tarvin, T.L. (1984) Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* **336**, 93-104
16. Pajot, P. (1976) Fluorescence of proteins in 6 M guanidine hydrochloride: A method for the quantitative determination of tryptophan. *Eur. J. Biochem.* **63**, 263-269
17. 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
18. Mikami, H. and Ishida, Y. (1983) Post-column fluorometric detection of reducing sugars in high performance liquid chromatography using arginine. *Bunseki Kagaku* **32**, E207-210
19. Crestfield, A.M., Moore, S., and Stein, W.H. (1963) Preparation and enzyme hydrolysis of reduced and S-carboxymethylated proteins. *J. Biol. Chem.* **238**, 622-627
20. Hewick, R.M., Hunkapiller, M.W., Hood, L.E., and Dreyer, W.J. (1981) A gas-liquid solid phase peptide and protein sequenator. *J. Biol. Chem.* **256**, 7990-7997
21. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Small scale preparation of plasmid DNA in *Molecular Cloning. A Laboratory Manual*, 2nd ed., Book 1, 1.25-1.28. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Sanger, F. and Coulson, A.R. (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* **94**, 441-448
23. Murray, V. (1989) Improved double-stranded DNA sequencing using the linear polymerase chain reaction. *Nucleic Acids Res.* **17**, 8889
24. Uchiyama, S., Isobe, K., and Nagai, S. (1991) Isozymes of ribonuclease and the changes in their relative levels during development in the cellular slime mould *Dictyostelium discoideum. Biochem. Cell Biol.* **69**, 84-87
25. Uchiyama, S., Isobe, K., and Nagai, S. (1992) Determination of the molecular weights of ribonuclease isoenzymes in a cell-free crude extract of *Dictyostelium discoideum*, by activity-staining of gels after SDS-PAGE. *Comp. Biochem. Physiol.* **102B**, 343-347
26. Schneider, R., Unger, G., Stark, R., Schneider-Scherzer, E., and Thiel, H.-J. (1993) Identification of a structural glycoprotein of an RNA virus as a ribonuclease. *Science* **261**, 1169-1171
27. Favre, D., Ngai, P.K., and Timmis, K.N. (1993) Relatedness of a periplasmic, broad-specificity RNase from *Aeromonas hydrophila* to RNase I of *Escherichia coli* and to a family of eukaryotic RNase. *J. Bacteriol.* **175**, 3710-3722
28. Meador, III, J. and Kennell, D. (1990) Cloning and sequencing the gene encoding *Escherichia coli* ribonuclease I: exact physical mapping using the genome library. *Gene* **95**, 1-7
29. Horiuchi, H., Yanai, K., Takagi, M., Yano, K., Wakabayashi, E., Sanda, A., Mine, S., Ohgi, K., and Irie, M. (1988) Primary structure of a base non-specific ribonuclease from *Rhizopus niveus. J. Biochem.* **103**, 408-418
30. Kawata, Y., Sakiyama, F., and Tamaoki, H. (1988) Amino acid sequence of ribonuclease T2 from *Aspergillus oryzae. Eur. J. Biochem.* **176**, 683-697
31. Inada, Y., Watanabe, H., Ohgi, K., and Irie, M. (1991) Isolation, characterization, and primary structure of a base non-specific and adenylic acid preferential ribonuclease with higher specific activity from *Trichoderma viride. J. Biochem.* **110**, 896-904
32. Watanabe, H., Hamid, F., Iwama, M., Onda, T., Ohgi, K., and Irie, M. (1995) Base non-specific acid ribonuclease from *Irpex lacteus*, primary structure and phylogenetic relationships in RNase T2 family enzymes. *Biosci. Biotechnol. Biochem.* **59**, 2097-2103
33. Kobayashi, 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. Biotechnol. Biochem.* **56**, 2003-2019
34. 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
35. Ai, Y., Singh, A., Coleman, C.E., Ioerger, T.R., 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
36. Jost, W., Bak, H., Glund, K., Terpstra, P., and Beintema, J.J. (1991) Amino acid sequence of an extra-cellular, phosphate starvation induced ribonuclease from cultured tomato (*Lycopersicon esculentum*) cells. *Eur. J. Biochem.* **198**, 1-6
37. Ide, H., Kimura, M., Arai, M., and Funatsu, G. (1991) The complete amino acid sequence of ribonuclease from the seeds of bitter gourd (*Momordica charantia*). *FEBS Lett.* **284**, 161-164
38. 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 (*Crassostrea gigas*). *J. Biochem.* **114**, 800-807
39. Kusano, A., Iwama, M., Ohgi, K., and Irie, M. (1998) Primary structure of a squid acid and base non-specific ribonuclease. *Biosci. Biotechnol. Biochem.* **62**, 87-94
40. 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
41. Inokuchi, N., Kobayashi, H., Miyamoto, M., Koyama, T., Iwama, M., Ohgi, K., and Irie, M. (1997) Primary structure of base non-

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

specific and acid ribonuclease from bullfrog (*Rana catesbeiana*). *Biol. Pharm. Bull.* **20**, 471-478
42. Uchida, T., Hayano, K., Iwama, M., Watanabe, H., Sanda, A., Ohgi, K., and Irie, M. (1996) Base specificity and primary structure of poly U-preferential ribonuclease from chicken liver. *Biosci. Biotechnol. Biochem.* **60**, 1982-1988
43. Adachi, J. and Hasegawa, M. (1992) MOLPHY: Program for molecular phylogenetics I. PROTML: Maximum likelihood inference of protein phylongeny. Institute of Statistical Mathematics, Tokyo. *Comput. Sci. Monographs* **27**, 1-77
44. Parry, S.K., Liu, Y.,-H, Clarke, A. E., and Newbigin, E. (1997) S-RNase and other plant extracellular ribonucleases in *Ribonucleases, Structures and Functions* (D'Alessio, G. and Riordan, J.F., eds.) pp. 191-211, Academic Press, New York
45. Bariola, P.A. and Green, P.J. (1997) Plant ribonucleases in *Ribonucleases, Structures and Functions* (D'Alessio, G. and Riordan, J.F., eds.) pp. 163-190, Academic Press, New York
46. New, H.C. and Heppel, L.A. (1964) The release of ribonuclease into the medium when *Escherichia coli* cells are converted to spheroplast. *Biochem. Biophys. Res. Commun.* **14**, 109-112
47. 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
48. Hamashima, M. (1994) Ms Thesis, Nagaoka University of Technology, Nagaoka, Japan
49. Ohgi, K., Takeuchi, M., Iwama, M., and Irie, M. (1997) Enzymatic properties of double mutant enzymes at Asp51 and Trp49 and Asp51 and Tyr57 of RNase Rh from *Rhizopus niveus*. *Biosci. Biotechnol. Biochem.* **61**, 1913-1918
50. Kuma, K., Nikoh, N., Iwabe, N., and Miyata, T. (1995) Phylogenetic position of *Dictyostelium* inferred from multiple protein data sets. *J. Mol. Evol.* **41**, 238-246
51. Wada, K., Wada, Y., Ishibashi, F., Gojobori, T., and Ikemura, T. (1992) Codon usage tabulated from the Gen Bank genetic sequence data. *Nucleic Acids Res.* **20**, 2111-2118

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012