

A new class of enzyme acting on damaged ribosomes: ribosomal RNA apurinic site specific lyase found in wheat germ

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A new enzyme, which we named ribosomal RNA apurinic site specific lyase (RALyase), is described. The protein was found in wheat embryos and has a molecular weight of 50 625 Da. The enzyme specifically cleaves the phosphodiester bond at the 3' side of the apurinic site introduced by ribosome-inactivating proteins into the sarcin/ricin domain of 28S rRNA. The 3' and 5' ends of wheat 28S rRNA at the cleavage site are 5'-GUACG- α -hydroxy- α,β -unsaturated aldehyde and pGAGGA-3', demonstrating that the enzyme catalyzes a β -elimination reaction. The substrate specificity of the enzyme is extremely high: it acts only at the apurinic site in the sarcin/ricin domain of intact ribosomes, not on deproteinized rRNA or DNA containing apurinic sites. The amino acid sequences of five endopeptidase LysC-liberated peptides from the purified enzyme were determined and used to obtain a cDNA sequence. The open reading frame encodes a protein of 456 amino acids, and a homology search revealed a related rice protein. Similar enzyme activities were also found in other plants that express ribosome-inactivating proteins. We believe that RALyase is part of a complex self-defense mechanism. **Keywords:** apurinic site/cleavage/lyase/rRNA/sarcin-ricin domain

Introduction

Ribosome-inactivating proteins (RIPs) (Endo and Tsurugi, 1987; Endo *et al.*, 1987; Wool *et al.*, 1992; Barbieri *et al.*, 1993) are a group of plant enzymes that inhibit polypeptide chain elongation by depurinating a specific nucleotide in the RNA of large ribosomal subunits. RIPs are classified into two groups: type 1, single-chain polypeptides; and type 2, two-chain proteins such as ricin, which consist of a catalytic A-chain and a B-chain that has lectin properties. The most commonly found RIPs are of type 1. The biological role of RIPs is as yet unclear, however, a possible role in defense against pathogens such as fungi and viral infections has been proposed (Ready *et al.*, 1986) and demonstrated experimentally (Taylor *et al.*, 1994; Massiah and Hartely, 1995). The molecular basis of ribosome inactivation is the hydrolysis of the N-glycosidic bond between the base and the ribose at position A4324

in 28S rRNA of rat or A2660 in 23S rRNA of *Escherichia coli* (see Wool *et al.*, 1992 for a review). The cleavage site is embedded in a purine-rich, single-stranded segment of 14 nucleotides that is nearly universal.

α -sarcin, a different type of ribosome-inactivating protein produced by the mold *Aspergillus giganteus*, catalyzes cleavage of a single phosphodiester bond within the same rRNA domain, between G4325 and A4326 in 28S rRNA (or G2661 and A2662 in 23S rRNA) (Endo and Wool, 1982; Endo *et al.*, 1983; Wool *et al.*, 1992). The sarcin/ricin domain (S/R domain) is crucial for ribosome function because: (i) it is involved in elongation factor (EF)-1 (or EF-Tu in *E.coli*)-dependent binding of aminoacyl-tRNA and EF-2 (or EF-G)-catalyzed translocation (Montanaro *et al.*, 1975; Hausner *et al.*, 1987); and (ii) EF-Tu and EF-G protect only four nucleotides in the 50S ribosomal subunit of *E.coli* against chemical modification, and these include the α -sarcin G2661 and the ricin A2660 (Moazed *et al.*, 1988).

In the course of our examination of the inactivation of wheat germ ribosomes by wheat's endogenous RIP, tritin (K.Madin, T.Sawasaki, T.Ogasawara and Y.Endo, in preparation), we noticed that wheat embryos contain a unique enzyme that cleaves the phosphodiester backbone at the tritin-induced apurinic site in the S/R domain. Here we report the purification and characterization of this enzyme and the cloning and sequencing of its cDNA. Although there have been a number of reports on apurinic/apurimidinic (AP)-endonucleases and AP-lyases that cleave phosphodiester bonds at AP sites during DNA repair (Seeberg *et al.*, 1995), to our knowledge this is the first report of an AP site-specific enzyme that acts on an RNA molecule. We also discuss the possible biological role of this enzyme in the context of RIPs.

Results

Wheat germ S100 fraction contains RALyase, a unique enzyme that cleaves the phosphodiester bond at an AP site in 28S rRNA of wheat ribosomes

During the course of our studies to examine the stability of protein synthesis in the wheat germ cell-free system (K.Madin, T.Sawasaki, T.Ogasawara and Y.Endo, in preparation), we noticed that a significant percentage of the ribosomes was inactivated by tritin. Tritin is a specific RNA-N-glycosidase, the hallmark of whose action is the creation of a 28S rRNA fragment of ~460 nucleotides (the α -fragment) after performing an aniline-catalyzed β -elimination reaction (Endo and Tsurugi, 1987; Endo *et al.*, 1987) (lanes 4 and 6 in Figure 1A). Quantitation of depurination revealed that 24% of ribosomes were depurinated after 4 h of incubation. Furthermore, at the start of the incubation 7% of the ribosome population had

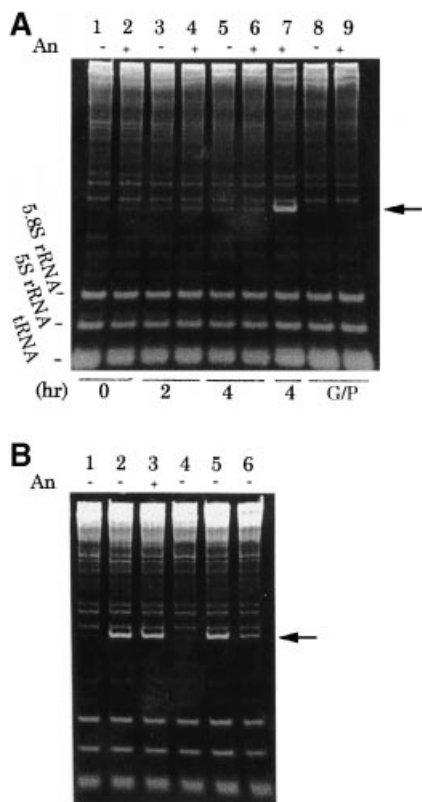


Fig. 1. Wheat germ S100 contains a unique enzyme that cleaves the phosphodiester backbone at an AP site in 28S rRNA of wheat ribosomes. (A) A wheat cell-free protein synthesis system was incubated at 30°C, and at the times indicated samples were withdrawn and their RNA was extracted and separated on a polyacrylamide-gel with (+) or without (-) aniline treatment (An). In lane 7, the incubation was carried out in the presence of 10 ng gypsophilin. In lanes 8 and 9, the RNA was extracted directly from wheat embryos by the guanidine-phenol method (G/P). (B) Ribosomes were first treated with gypsophilin (lanes 2, 3, 5 and 6), then incubated with 20 μ l of S100 for 0 min (lane 4) or 60 min (lanes 1-3 and 5). In lane 6, gypsophilin-treated ribosomes were incubated with 20 μ l of S100 fraction from endosperm for 60 min. Arrows mark the α -fragments.

already been depurinated (Figure 1A, lane 2). When RNA was extracted directly from wheat germ by the guanidine-isothiocyanate/phenol method, little fragment formation was observed (Figure 1A, lane 9), showing that depurination occurred during extract preparation. As shown later, the site of depurination was confirmed by direct sequencing to be in the universally conserved S/R domain of 28S rRNA (5'-AGUACGA*GAGGAAC-3'; the asterisk marks the target residue) (Figure 3B). These results demonstrated that wheat germ ribosomes are susceptible to the endogenous tritin, a result inconsistent with earlier reports that showed that tritin does not act on homologous ribosomes (Taylor and Irvin, 1990; Massiah and Hartley, 1995). An important and interesting observation was that a fragment similar to the α -fragment was present even without aniline treatment (Figure 1A, lanes 3 and 5). It was unclear at this point whether wheat seeds contain a new enzyme that recognizes the depurinated nucleotide and then cleaves the phosphodiester bond at the damaged site, or whether they contain a ribonuclease similar to α -sarcin, which cleaves a specific phosphodiester bond in the S/R domain and creates a RNA fragment almost identical to the α -fragment (Endo and Wool, 1982).

To determine whether fragment formation without aniline treatment is dependent on depurination, isolated ribosomes were treated first with an exogenous RIP similar to tritin, and then incubated with S100 prepared from wheat embryos. We chose gypsophilin as depurinating enzyme because this RIP has high RNA-*N*-glycosidase activity on the ribosomes from several species (Yoshinari *et al.*, 1997). In the conditions of Figures 1B, depurinated 28S rRNA in ribosomes was 100% cleaved as judged by comparison of the intensity of the fragment band with an aniline-induced control reaction (Figure 1A, lane 7); little fragment formation was observed without the prior gypsophilin treatment (Figure 1B, lane 1). The cleavage activity was recovered mostly from wheat embryos but not from endosperm (Figure 1B, compare lanes 5 and 6). The activity remained with the embryos even after extensive washing with detergents such as NP-40 (data not shown). These experiments suggested that wheat embryos contain a highly active enzyme that acts on depurinated ribosomes and cleaves a phosphodiester bond at or close to the AP site in the S/R domain. Since this activity disappeared completely after brief boiling of the S100 and also was sensitive to proteinase K treatment while being resistant to micrococcal nuclease treatment (data not shown), we assumed that the enzyme is a protein. We next attempted purification and characterization of this unique enzyme, which we named RALyase (see below).

Purification of RALyase

The details of the purification procedure are described in the Materials and methods. Figure 2A shows a typical chromatogram obtained from the MiniQ column used in step 6 of the purification; RALyase activity binds to the column, and was recovered with a KCl gradient in three fractions (fractions 23-25). The pooled fraction was separated as final step on a Superdex column (Figure 2B). RALyase activity coinciding with the absorbance peak was pooled (fractions 8-10), and when assayed with depurinated ribosomes, produced two fragments: the α -fragment (arrow) and the β -fragment (the remaining 5' end fragment of 28S rRNA, arrowhead). The molecular weight of RALyase as estimated by gel-filtration chromatography is 55-60 kDa. Figure 2C shows an SDS gel of the pooled fractions from the final three steps. The purification procedure yielded an apparently homogeneous protein with a molecular weight of ~54 kDa. This value is fairly close to the value obtained by gel filtration, suggesting that RALyase is a monomer. Micrococcal nuclease or DNase I treatment had little effect on the activity of the purified enzyme, excluding the possibility that RALyase contains nucleic acids as a functional element (data not shown). The yield of RALyase was as low as 7.6 μ g of purified protein per 5 kg of wheat germ in spite of the fact that the S100 fraction retained high enzymatic activity (Figure 1B). As noted in the Materials and methods, addition of proteinase inhibitors to the buffer solutions stabilized the enzyme activity. These results suggest that endogenous proteinases are responsible for the low yield because of enzyme degradation during the purification.

Mechanism of action of RALyase

Since the treatment of depurinated wheat germ ribosomes with the enzyme led to the production of an α -fragment

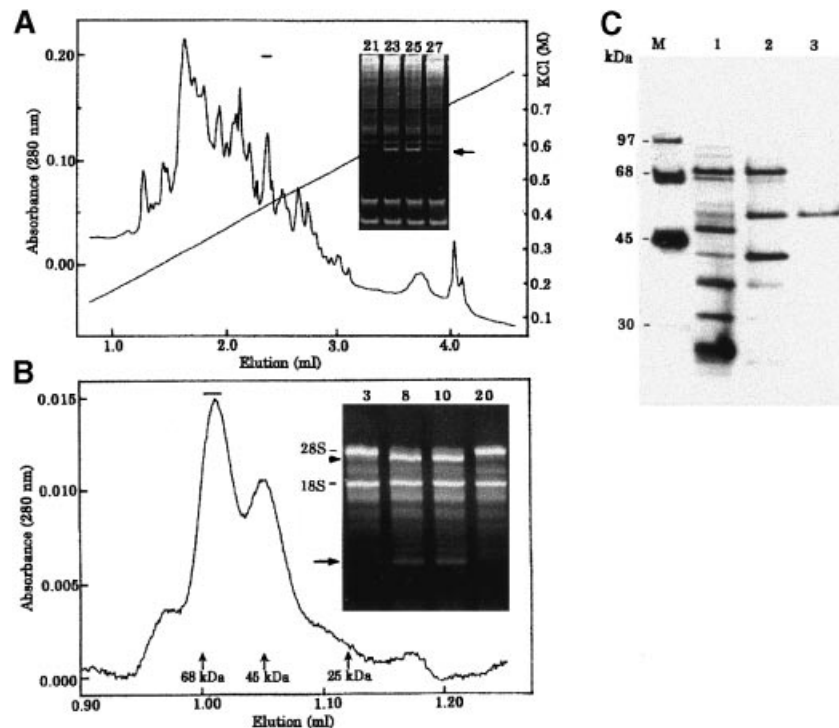


Fig. 2. Purification of RALyase. Chromatograms of the MiniQ (A) and Superdex (B) columns. Depurinated wheat ribosomes were incubated with 5 μ l of each fraction. The insets show the activity of the fractions by separating extracted RNAs on polyacrylamide (A) or agarose gels (B). (C) SDS-PAGE of protein fractions before (lane 1) and after the MiniQ [see bar in (A), lane 2] column and a purified sample after the Superdex column [see bar in (B), lane 3]. The molecular weight markers used were chymotrypsinogen A from bovine pancreas (25 kDa), carbonic anhydrase b (30 kDa), albumin from hen egg (45 kDa), bovine serum albumin (68 kDa) and phosphorylase b (97 kDa).

apparently identical to the aniline-induced fragment (Figure 1A), it was likely that the cleaved phosphodiester bond is close to the depurinated site in the S/R domain. To determine the mechanism of action we first analyzed the structure at the 5'-terminus of the α -fragment. The strategy we applied was to treat gypsophilin-treated wheat ribosomes with RALyase, extract the RNA, and label it with [γ - 32 P]ATP after treatment with or without alkaline phosphatase. As shown in Figure 3A, the result demonstrated that the 5'-terminus of this fragment retained a phosphate as in the case of the aniline-induced fragment: the fragment was labeled only after treatment with phosphatase (which removes the 5'-fragment) similar to the 5'-phosphorylated 5S and 5.8S rRNAs, which is different from the behavior of an α -sarcin induced α -fragment, which is known to have a 5' hydroxyl group (Endo and Wool, 1982). We next determined the site of cleavage by direct nucleotide sequencing using the enzymatic method. As shown in Figure 3B, the sequence at the 5'-terminus of the α -fragment is pGAGGAA-3'OH, the same as for the aniline-induced α -fragment (Endo *et al.*, 1987).

Two types of DNA repair enzymes that cleave a phosphodiester bond at AP-sites are known (Seeberg *et al.*, 1995): AP-endonuclease cleaves the 5'-adjacent phosphodiester bond hydrolytically, yielding 5'-terminal deoxyribose-5'-phosphate and 3'-OH nucleotide residues at the cleavage ends; and AP-lyase catalyzes a β -elimination reaction at the 3'-adjacent phosphodiester bond, yielding a 5'-terminal deoxynucleoside-5'-phosphate residue and a 3'- α,β -unsaturated aldehyde (Mazumder *et al.*, 1989). Since the 5'-terminus of the α -fragment created by RALyase was a phosphorylated G, RALyase is likely

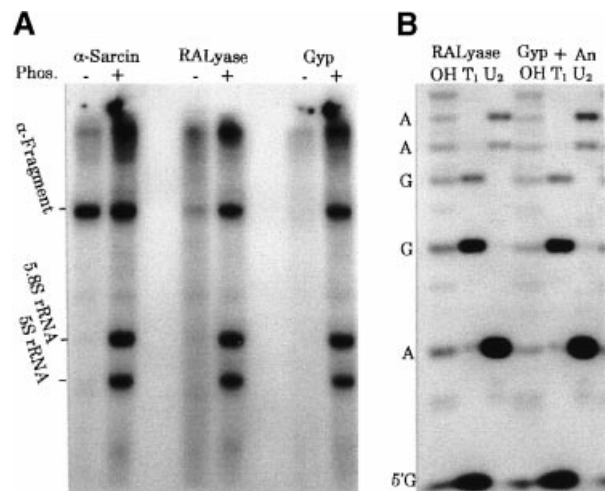


Fig. 3. Structure of the 5'-terminus of the α -fragment. Total rRNA was prepared from α -sarcin-treated, gypsophilin-treated (Gyp), and both gypsophilin- and RALyase-treated ribosomes (RALyase). For the RALyase treatment, ribosomes were incubated with 40 ng of purified enzyme for 60 min. (A) RNA from gypsophilin-treated ribosomes was treated with aniline, and all samples were 5'-end labeled before or after phosphatase (Phos) treatment, then separated on a polyacrylamide gel and a radioautograph was prepared. (B) α -fragments produced by RALyase or gypsophilin/aniline were labeled at the 5' terminus and sequenced enzymatically. The ribonucleases used were T₁ and U₂. OH, alkaline hydrolysis.

to catalyze a β -elimination reaction rather than a hydrolytic event.

To confirm the mechanism of action of RALyase we analyzed the structure of the other cleavage end, the

Table I. Reduction of the RNA with tritiated borohydride and subsequent diol-specific oxidation by sodium periodate

rRNAs from ribosomes	Reaction						
	Reduction with NaB ³ H ₄			Oxidation with NaIO ₄			
	d.p.m./pmol β ^a	(Δ)	Hydrogen incorporated (mol/mol β)	d.p.m./pmol β ^a	(Δ)	Hydrogen remaining (mol/mol β)	Hydrogen released (mol/mol β)
(i) Control (28S)	1326	(0)	–	1393	(0)	–	–
depurinate (28S)	1614	(288)	1.00	1419	(26)	0.10	0.90
α-sarcin (β ^b)	1350	(24)	0.08	–	–	–	–
depurinated/ α-sarcin (β ^c)	1669	(343)	1.19	1353	(–40)	–0.14	1.33
RALyase (β)	1904	(578)	2.01	1712	(319)	1.11	0.90
(ii) Control (28S)	1497	(0)	–	1524	(0)	–	–
depurinate (28S)	1752	(255)	0.89	1552	(28)	0.10	0.79
α-sarcin (β ^b)	1485	(–12)	–0.04	–	–	–	–
depurinated/ α-sarcin (β ^c)	1796	(299)	1.04	1499	(–25)	–0.09	1.13
RALyase (β)	2077	(580)	2.01	1784	(263)	0.91	1.10
(iii) Control (28S)	1650	(0)	–	1548	0	–	–
depurinate (28S)	1899	(249)	0.86	1546	(–2)	–0.01	0.87
α-sarcin (β ^b)	1684	(34)	0.12	–	–	–	–
depurinated/ α-sarcin (β ^c)	1939	(289)	1.00	1506	(–41)	–0.14	1.14
RALyase (β)	2240	(590)	2.05	1795	(247)	0.86	1.19

^aThe concentrations of the RNAs were calculated from the absorbance at 260 nm (Wool, 1979). Since the structure of wheat 28S rRNA is not known, *Oryza sativa* 28S rRNA (3377 nucleotides) (Takaiwa, 1985) was used for the estimation of the molecular weight of the β-fragment (3016 nucleotides). A radioactivity of 2875 d.p.m. corresponds to 10 pmol of hydrogen incorporated.

^b5'-fragment of 28S rRNA produced by α-sarcin.

^c5'-fragment of 28S rRNA produced by α-sarcin from gypsophilin-treated ribosomes.

3'-terminus of the β-fragment of 28S rRNA. For this purpose, we took advantage of the use of tritiated NaB[³H]₄ for the quantitative labeling of aldehyde groups (see Materials and methods for details). In a preliminary control experiment, we used depurinated 28S rRNA, which has a single aldehyde group at the site of depurination. Although this method had a quite high background even after extensive washing of the RNA (possibly due to adsorption of tritiated by-products to RNA), the difference in radioactivity between controls and the depurinated RNA was significant and highly reproducible (Table I). As expected, 1 mol of hydrogen was incorporated into 1 mol of depurinated 28S rRNA, and subsequent treatment of the RNA by the diol-specific oxidant NaIO₄ completely released the incorporated radioactivity, establishing that it is a C1 ribose atom that is reduced, and that this method can be applied for our purpose.

To establish which of the two alternative enzymatic mechanisms, hydrolysis or β-elimination, is responsible for the cleavage of the phosphodiester bond by RALyase (Figure 4), we used as controls two 5' fragments whose 3'-structures are known: one produced by α-sarcin (5'-GUACGAG_{OH}; Endo and Wool, 1982), and one produced by a combined gypsophilin and α-sarcin treatment (5'-GUACG-ribose-G_{OH}; Endo *et al.*, 1991). When the β-fragment from gypsophilin- and RALyase-treated ribosomes was incubated with tritiated borohydride, 2 mol of hydrogen were incorporated into 1 mol of the β-fragment, suggesting that the cleavage is not of a hydrolytic manner; if it were, there would be no aldehyde for reduction by borohydride. The α-sarcin-induced β-fragment had practically no incorporation of radioactivity, while the fragment produced by gypsophilin and α-sarcin treatment incorporated 1 mol of hydrogen and the hydrogen was released from the fragment after the diol-specific periodate oxidation.

Next the aldehyde sites at the 3'-terminus of the β-fragment that were reduced by borohydride were determined. One of the two moles of incorporated hydrogen was released from the RNA after subsequent diol-specific oxidation (Table I), indicating that C1' and C2' have OH groups. The results can only be explained in that the enzyme catalyzes cleavage of the phosphodiester bond by introducing a double bond between C_α and C_β through a β-elimination reaction (Figure 4). Thus we conclude that the isolated enzyme is a lyase that catalyzes cleavage of the phosphodiester bond at the deadenylated site by producing a α-hydroxy-α,β-unsaturated aldehyde at the 3'-terminus of the 5'-fragment, and a phosphate at the 5'-terminus of the 3'-fragment. Based on these results, we named this novel enzyme ribosomal RNA AP site-specific lyase, or RALyase for short.

Substrate specificity of RALyase

Ribosomes prepared from rat liver and *E.coli* cells were first completely deadenylated by gypsophilin and then used as substrates for experiments to determine the specificity of RALyase. As shown in Figure 5A and B, the enzyme acted on both substrates at a single site, as shown by the formation of the α-fragments of 28S or 23S rRNA (arrows). Susceptibility, however, was somewhat different: RALyase acted in the order of wheat ribosomes > rat liver ribosomes > *E.coli* ribosomes.

It is known that when naked rRNAs are the substrate some RIPs depurinate a second site in *E.coli*, A1014 of 16S rRNA, which is in the sequence 5'...GA*GA...3' (Endo and Tsurugi, 1988; Habuka *et al.*, 1991). We were able to demonstrate that this site also depurinated in intact 30S subunits if a low magnesium ion concentration, which favors subunit dissociation, is used (T.Sawasaki and Y.Endo, unpublished results). The site of action was confirmed to be A1014 by directly sequencing the 3'

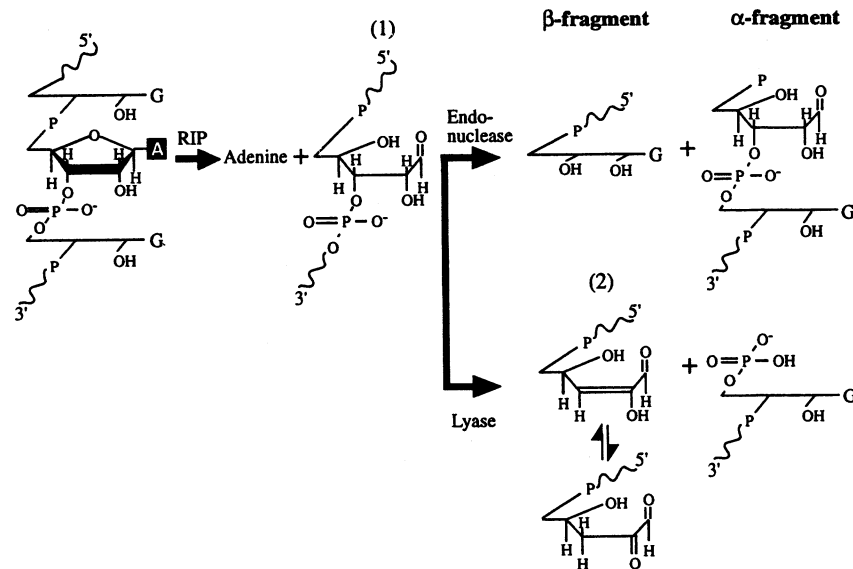
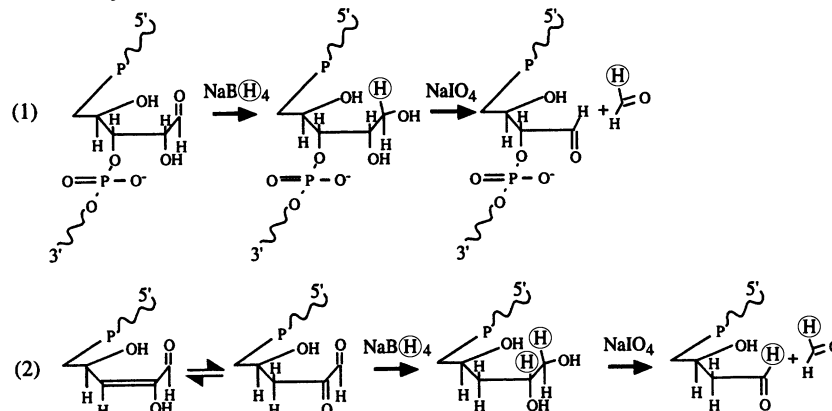
Alternative enzyme action**Chemistry of the analysis**

Fig. 4. Chemistry of the RALyase-catalyzed reaction. Samples were prepared as above and the structure of the 3'-terminal end of the β -fragment produced by RALyase was analyzed using $\text{NaB}[\text{H}]_4$ and NaIO_4 to distinguish between cleavage of the phosphodiester bond by either an endonuclease or a lyase. RIPs hydrolyze the N-glycosidic bond at A4324 (the numbering follows that of rice 28S rRNA) in the S/R domain and RALyase cleaves by β -elimination to yield an α -fragment with a 5'-terminal adenosine-5'-phosphate residue and a β -fragment with a 3'-terminal α -hydroxy- α , β -unsaturated aldehyde. H enclosed in a circle represents a hydrogen from borohydride.

fragment (results not shown). As shown in Figure 5B, gypsophilin also depurinates two sites under the experimental conditions, the 16S rRNA site of the 30S subunit and the 23S rRNA site in the 50S subunit: two specific bands are formed after aniline treatment (an arrowhead marks the 3' fragment of 16S rRNA). As can be seen, RALyase did not cleave at the deadenylated site in 16S rRNA even at a higher concentration, indicating that the enzyme has high specificity for the S/R domain. Thus the enzyme recognizes not only the structure or sequence of a deadenylated site in the tetrad GAGA, but also the specific structure conferred to the S/R domain by the ribosomal proteins (Endo *et al.*, 1991). RALyase did not act on deproteinized rRNA nor DNAs containing AP sites (Figure 5C and D, respectively). Although cells from all organisms possess AP-endonucleases and AP-lyases involved in the DNA repair process, these data demonstrate that RALyase is a new enzyme of completely different character.

Cloning and sequencing of RALyase cDNA

RALyase proved to be insensitive to automated Edman degradation, indicating that the N-terminal amino acid residue is blocked. Therefore, the protein was digested with endopeptidase Lys-C and the products were separated by reversed-phase HPLC and subjected to peptide sequence analysis (Figure 6). A RALyase-specific cDNA was obtained by RT-PCR with RNA isolated from wheat coleoptiles. Figure 7 shows the nucleotide sequence of the RALyase cDNA as well as the deduced amino acid sequence. The complete sequence of the cDNA contains an open reading frame encoding a protein of 456 amino acids. All five peptide sequences are contained in the deduced amino acid sequence. Although the molecular weight of RALyase as calculated from the amino acid residues in the open reading frame of the cDNA (50.6 kDa) is less than that estimated from an SDS gel of the purified protein (~54 kDa), the translation product of the cDNA in a wheat germ cell-free system has the same mobility

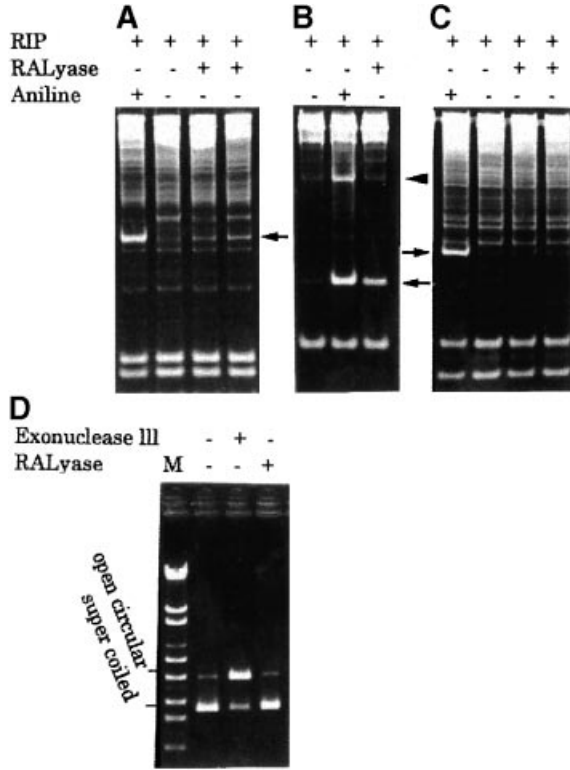


Fig. 5. Substrate specificity of RALyase. Ribosomes from (A) rat liver and (B) *E. coli* were first deperinated with gypsophilin and then incubated with RALyase [0, 10, 40 ng (left to right) in (A), 90 ng in (B)] for 60 min. Deproteinized RNA from (C) wheat ribosomes and (D) plasmid DNA containing AP sites were also used as substrates, with Exonuclease III (an AP-endonuclease) as positive control for DNA cleavage. Incubations were carried out with 0, 40 or 100 ng (left to right) in (C) or with 100 ng of RALyase in (D). The RNA samples were analyzed as in Figure 1 and the DNA was separated on a 0.8% agarose gel. Arrows mark the fragments produced, and the arrowhead points to the 3' fragment of 16S rRNA.

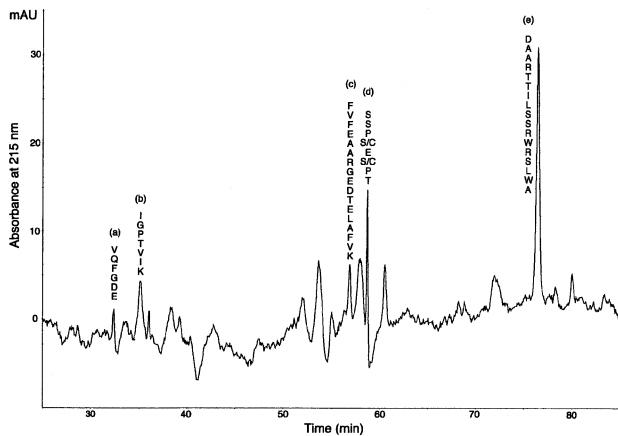


Fig. 6. Reverse-phase HPLC separation of an endopeptidase Lys-C digest of RALyase. The sequences of five peptides were determined and are given at the respective peaks.

as authentic RALyase in an SDS–polyacrylamide gel (Figure 8). Furthermore, the sequence of the RALyase gene (result not shown, DDBJ/EMBL/GenBank accession No. AB032124) has the same open reading frame except that it is interrupted by two introns of 85 and 82 bp at nucleotide positions 917 and 1067 (arrowheads in Figure 7). From these results we conclude that the cDNA

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CCCTGTTCCGCTTCCTCTCCGCGGTGTCAGCAAAACCACGGCGATGGACGAAACCCCTCG  60
                                         M D E T L A
CCACCGTCTCTCTCTACCTCCCGCTCCCTCGGTCTCCACCACCGCATCCCGTCTCCTCT  120
T V L S Y L P P P S V S T T A S P S S S
CGTCCACCGCTCCGATGACGATGACCGCATCACTCCCGGACGTGCTCTCCGCA  180
S T A S D D D D R I N C L P D V L L R N
ACATCATCTCTCGCCTCCCAACGAAGACGGCGCGCACCAACCTCTCTCTCCGCT  240
I I S R L P T K6D A A R T T I L S S R W
GGCCAGCCTCTGGGCTCCACCCACTCCGCGTCCGACGCTGGACTCGTTCTTACC  300
R S L W A S T P L R L D D A G L V P T A
CCGTCCACCGCTCCGATGAACTCCACCCCGGCTCGGTCACTCCGCCCGCTCTCTCC  360
V T A A L N S H P G S V T S A R L S S E
AGCACCTCCGTTACAGGAACCGACGCTCTCGACTCCTGGTTCGCTCTCTCAGCA  420
H L A Y Q E P D V L D S W F A S L S A K
AGAAGCTCAGGAACTCTCCGTCGTAACGGATCTCGCTGCTGAATGTGAGTGGCGT  480
N V E E L S V V N G S W P A E C E W R P
CTCCCCCAGCTCTGGATGCGCGTCCCTCCGCGCTCTGGCTCCGGCTCTGCCAGT  540
P P S L L G C A S L R R V W L G L C Q F
TCCCGACATTTCCGCGCTCGCTCCGCGCTCCCGCTGCAAGAGCTCGGATGTCC  600
P D I S G L A P A F P S L Q E L G I V H
ATTGCTCCATGACAGACCGGAGCTCCACGCTGTGCTCCCGCTGCCCGAGCTTGAG  660
C S M Q D R E L H A V L P R C P E L E R
GGCTCGCTTTGGTCTGACAGGACTACCCAAAGTACATCCACATCTGGTCAGGAG  720
L A F V L T Q D Y P R Y I H I W S G S L
TCCAGTCCCTGGTGTGGAGGTCATGGTCAGAGAGGTGACCTTGACACTGCCCA  780
Q S L V V W R S M V R E V H L D D A P N
ACTTGGAGCGGTGCTGTGGAGCGGATTGGCGGTGCCTTACTCACGTCGAAGATT  840
L E R L L L E P I G G A S T H V K I N
ATGCACCGAGGCTGAAAATTTTCGGTACTTTGACGTTGGACTGCATCAGCTCAAG  900
A P R L K I F G Y F D V G L H Q L K1I2G
GCCCACTGTGATCAAGGATGGGATAGGATGAAGTGAACCGAATGCGATGGTCC  960
P T V I K D G I G M K V K P N A M V R T
CCCTTAGCATTGGCATTGAAGTGCAGTTTGGAGCAGAGAAGCAAGTCAAGCTGG  1020
L R T L A L K2V Q F G D E K Q V K L V P
CCCTCTGTAGATGCTTCCCATGCTAGAAACCTGTATATCAAGTCACTCTCTCT  1080
L L L R C F P C L E T L Y I K3S S P S E
AATCAACCAACATGTTGATGTTGATTTCTGGGATCAAGTAGCCACACTGAAATG  1140
S P T N V D V D F W D Q V G H T E C V T
CCTCACATATTAAAGATTGTTGTTTGAAGCTGCCAGGAGAGGATACCAGCTGG  1200
S H I K K4F V F E A A R G E D T E L A F
TTGTCAAGTTGTGATGGAAAGAGCCCAATATTGGAGGAGATGCGTGTTCGTGG  1260
V K F V M E R A Q I L E E M R V F V D D
ATGGTGTCTAGAGATGTTGTGCTAAGGCGTTATCTTCAGAAGAATGTGTCTG  1320
G C S R D V V L R R L S S E E C V S A D
ATGTACCGTGGTGGAGAGGCAAGACATGSCCTGGAGCTCGAAGAAGACTA  1380
A S V V V E R Q D M S H A W S F E R A S
GCAACATGTCACAGTGTGACCCCTTGTGTTGCTAAGTGGAGGATAACAAGAAG  1440
N M S Q C D P F G C
AAGATCACGCTCTTCAAGCCTTACCAGATGGAATGAAAAAAGAGATGGTCTG  1500
CGAGTCAAGCCATAGGCCCTGAAGCAAGTTGTAGTCTTTTGTCTCTATACCT  1560
CAGTCAAGCTTCGACCTTTGGTTTGTCTGTAGACGCTGCTTGTGGACCTGA  1620
TCGGCTGTGTAATGAAATGATTTATGTGGATTGTGCTTTATGGAGCTGCACT  1680
TCAACTTGTAAAGATCGTGTGCTGCAAAA
    
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Fig. 7. Nucleotide and deduced amino acid sequence of RALyase cDNA. Amino acids representing the sequenced peptides are underlined and labeled according to the peptides shown in Figure 6. The arrowheads denote the sites of introns in the genomic sequence. The cDNA and gene sequences are deposited in EMBL/DDBJ/GenBank under accession Nos AB032123 and AB032124.

of RALyase has a length of 1711 bp with an open reading frame ranging from position 45 to 1412, and that RALyase consists of 456 amino acids and has a calculated molecular mass of 50 625 Da. The cDNA has a 5' non-coding region of 44 bp, a 3' non-coding region of 293 bp and a short poly(A) tail. A homology search in the NBRF protein database revealed that there are no homologous structures among the proteins reported so far. A short, nearly homologous stretch of amino acids was, however, found in valyl tRNA synthetase of *Chlamydia trachomatis* (Stephens *et al.*, 1998). The sequences are YQEPD-VLDSWFAS (amino acids 110–122) in RALyase and YQDPDVLDTWFSS (position 447–459) in valyl tRNA synthetase. Also, a search among rice cDNAs deposited at the MAFF DNA bank (Nihon) found a partial cDNA sequence (accession No. C74581) encoding a highly homologous protein (results not shown).

It may also be worth mentioning that RALyase is most likely neither a secretory protein nor a membrane protein since no signal peptide sequence could be identified at its N-terminus.

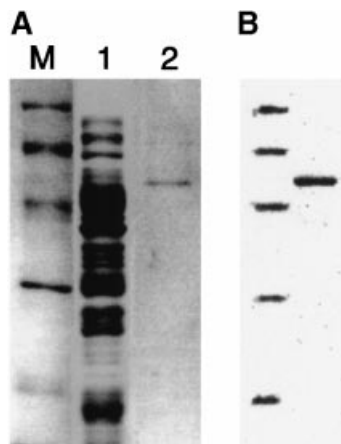


Fig. 8. *In vitro* expression of RALyase from cDNA. Capped mRNA synthesized *in vitro* was translated in a wheat germ cell-free system. (A) Portions of the translation reaction mixture (lane 1) and purified RALyase (lane 2) were separated on an SDS-polyacrylamide gel and protein bands were stained with Coomassie Brilliant Blue. (B) An autoradiogram of the gel shown in (A).

Distribution of RALyase among plants

The biological role of tritin and other RIPs is not well understood although it is believed that they have a defensive function, protecting plants from attack by pathogens (Barbieri *et al.*, 1993). To further our understanding of the biological role of both RIPs and RALyase, we conducted a survey focusing on RIP-producing plants to determine whether they also contain enzymes with RALyase activity. We prepared DE-52 column-bound fractions from the leaves of *Gypsophiia elegans* (in the conditions used, gypsophilin passes through the column (Yoshinari *et al.*, 1997), the root of *Mirabilis jalapa* (Habuka *et al.*, 1991), and the sarcocarp of *Cucurbita pepo* (Yoshinari *et al.*, 1996), and used them as crude enzyme sources. The samples tested produced fragments of apparently the same size as the aniline-induced fragment (Figure 9). We could not detect any significant RALyase activity in seedlings of *Arabidopsis thaliana*, which has no RIP activity (data not shown). The results suggest wide distribution of RALyase among RIP-producing plants, which further suggests cooperativity of RIP and RALyase in a biologically important process. In this context it needs to be mentioned that rice, which contains a wheat RALyase homolog, also contains a tritin-like RIP [MAFF DNA bank (Nihon) accession No. C73224].

Discussion

We report a new class of enzymes, ribosomal RNA AP site-specific lyases, enzymes that acts on damaged ribosomes. Although the biological significance of RALyase is at present obscure, we can offer some speculations. Tritin, which is found in the endosperm of wheat seeds (Massiah and Hartley, 1995), and most other type 1 RIPs are known to be localized in intercellular space (Ready *et al.*, 1986; Barbieri *et al.*, 1993; Yoshinari *et al.*, 1996, 1997); RALyase, on the other hand, was recovered from wheat embryos. Since depurination of ribosomes results

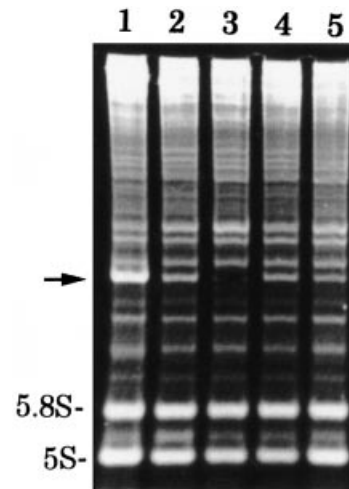


Fig. 9. RALyase activity in other RIP producing plants. Wheat germ ribosomes were treated with gypsophilin and then incubated without (lane 3) or with 20 μ l of an S100 fraction from either *G.elegans* (lane 2), *M.jalapa* (lane 4), or *C. pepo* (lane 5) for 60 min. RNA samples were directly separated and analyzed as above. Lane 1, an RNA sample from depurinated ribosomes treated with aniline as size marker. The arrow marks the α -fragments.

in an irreversible impairment of EF-catalyzed functions, the question of why plants have an additional enzyme emerges. Is it simply a case of unnecessary over-kill, or is the assurance provided by doubly killing ribosomes necessary? Depurinated ribosomes still retain the ability to synthesize polyphenylalanine under saturating amounts of both EF-1 and EF-2 (Olsnes *et al.*, 1975; Fernandez-Puentes *et al.*, 1976), whereas cleavage of the phosphodiester bond at the 3' adjacent guanosine by α -sarcin results in total loss of EF-dependent activities (Hausner *et al.*, 1987). Nilsson and Nygard have reported that the EF-2-catalyzed hydrolysis of GTP stimulated by depurinated rabbit reticulocyte ribosomes increased by >400% compared with normal ribosomes (Nilsson and Nygard, 1986). We also observed in an *E.coli* system that depurination of ribosomes by gypsophilin leads to increased EF-G catalyzed GTPase activity (by 250%), but that treatment with α -sarcin results in the loss of this activity (T.Sawasaki and Y.Endo, unpublished results).

As we have pointed out, the loop of the S/R domain has a complex structure, which is affected by ribosomal proteins since the ricin A-chain is able to depurinate α -sarcin treated ribosomes (Endo *et al.*, 1991; for the crystal structure of an S/R domain oligonucleotide see Correll *et al.*, 1998). These results strongly suggest that there is a difference in the functional impairment between RIP-treated ribosomes and α -sarcin-treated ribosomes, even though the two sites of action are in close proximity. Thus, if the phosphodiester bond cleavage of depurinated ribosomes by RALyase leads to a complete inactivation of polypeptide synthesis, it is possible that plants acquired this system of completely inactivating their depurinated ribosomes in order to stop protein synthesis reliably, since it is conceivable that viral proteins, for example, have found ways of exploiting the residual ribosomal functions.

AP endonucleases and AP lyases functioning in the repair of DNA damaged by endogenous radicals as well as exogenous chemicals and UV light have been characterized (Seeberg *et al.*, 1995). By analogy to these DNA repair

enzymes, one can alternatively speculate that RALyase has a role in an RNA repair mechanism; this would provide a safety net for plants expressing RIPs, enabling the repair of accidentally depurinated ribosomes. We are currently testing these hypotheses.

Materials and methods

General

The following procedures were either described or cited previously (Endo and Wool, 1982; Endo *et al.*, 1983, 1987; Endo and Tsurugi, 1987): preparation of ribosomes, extraction of RNA with phenol and sodium dodecyl sulfate, separation of rRNA by sucrose density gradient centrifugation, plasmid preparation, phosphatase treatment, 5'-end labeling with [γ - 32 P]ATP, analysis of nucleic acids by polyacrylamide gel electrophoresis, and RNA sequencing.

Materials

Wheat germ was obtained fresh from a local mill and stored at 4°C. Wheat (*Triticum aestivum* L.) plants were grown under regular conditions and coleoptiles were collected from 10-day-old wheat seedlings. Materials for the ion-exchange column chromatographies and for the affinity chromatography were from Tosoh Co. Ltd and Nakarai Tesque, Inc., respectively. Proteinase inhibitors (E64, APMSF), endopeptidase Lys-C, and molecular weight standard proteins were purchased from Boehringer Mannheim Biochemica, the tritiated borohydride (521.6 mCi/mmol) was from NENTM Life Science Products, Inc., the MicroSpinTM G-25 Columns and μ RPC C2/C18 SC 2.1/10 columns were from Amersham Pharmacia Biotech., and the protein assay kit from Bio-Rad Co. Ltd. PVDF membrane, pGEM-T easy, a 5' RACE kit, and the BigDye sequencing kit were from Millipore Co., Promega Co., Takara Shuzo, and Perkin-Elmer, respectively.

Cell-free protein synthesis

The method used here is practically the same as that reported previously (Erickson and Blobel, 1983; Endo *et al.*, 1992). Briefly, a 50 μ l reaction mixture contained 15 μ l of extract (not treated with micrococcal nuclease) and the following ingredients (the final concentrations are given): 24 mM HEPES-KOH pH 7.8, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 0.45 mg/ml creatine kinase, 2 mM dithiothreitol (DTT), 0.4 mM spermidine, 25 μ M of each of the 20 amino acids, 2.5 mM magnesium acetate, 100 mM potassium acetate, and 40 μ g/ml of capped dihydrofolate reductase mRNA as template. The incubation was carried out at 26°C. For the translation of RALyase its ORF was transferred from pGEM-RALyase into the *in vitro* expression vector pSP65, and capped mRNA was synthesized by *in vitro* transcription of the linearized plasmid carrying the ORF under SP6 RNA polymerase promoter control (Endo *et al.*, 1992). The transcript is ~2100 nucleotides long and consists of the sequence m⁷GpppGAAUACACGGAAUUCGAGCUCGCCCG-GGAAAUCUCAAUG (the underlined sequence is the initiation codon) at its 5' end, a 1368 nucleotide coding sequence, and a 3' non-coding region of 565 nucleotides with a poly(A) tail of ~100 adenosines. The cell-free protein synthesis mixture containing [14 C]leucine (2 μ Ci/ml) was incubated as above. The autoradiogram of the gel was obtained using a BAS-2000 phosphorimager (Fuji Photo Film Co. Ltd).

Purification of RALyase

All steps of the purification were carried out at 4°C.

Step (1). Five kilograms of wheat germ were homogenized with 2 vol. of ice-cold 25 mM Tris-HCl pH 7.6, 100 mM KCl, 5 mM MgCl₂, 15 mM mercaptoethanol for 10 min in a Waring blender. The homogenate was centrifuged at 30 000 g for 30 min, and the extract was centrifuged again at 150 000 g for 2 h to remove ribosomes. The supernatant (S100) was diluted with an equal volume of homogenizing buffer lacking KCl and was centrifuged at 10 000 g for 30 min to remove turbid material; the supernatant (1.5 l) was used for the purification of RALyase.

Step (2). The sample was loaded onto a DE-52 column (35 \times 5.0 cm) equilibrated with a solution of 25 mM Tris-HCl pH 7.6, 50 mM KCl, 5 mM MgCl₂ and 15 mM mercaptoethanol. The column was extensively washed with this solution, and bound proteins were eluted with a linear gradient from 100 to 600 mM KCl in the same buffer. The effluent was collected in 20-ml fractions; fractions showing RALyase activity eluted around 300 mM KCl and were pooled (400 ml total).

Step (3). Solid (NH₄)₂SO₄ was slowly added under stirring to the collected pool. The supernatant at 50% saturation was recovered by

centrifugation and additional solid (NH₄)₂SO₄ was added to 70% saturation. The precipitate formed contained RALyase, and was dissolved in 40 ml of 25 mM Tris-HCl pH 7.6, 500 mM KCl, 0.5 mM EDTA and dialyzed against this buffer.

Step (4). After dialysis, the supernatant recovered after centrifugation (50 ml) was dialyzed for 16 h against two 1000-ml portions of 10 mM potassium acetate buffer pH 5.7, 50 mM KCl, 0.5 mM EDTA. The soluble fraction recovered after centrifugation contained 294 mg of protein. This acid-extracted fraction was loaded onto a 40 ml phosphocellulose column that had been washed and equilibrated with the above acidic buffer. The column was washed with 150 ml of this same buffer, and bound proteins were eluted with a linear gradient from 50 to 600 mM KCl again using the same buffer. RALyase fractions, eluting around 380 mM KCl, were pooled (28 mg protein) and the pH was brought to 7.6 by adding 1/20 vol. of 1 M Tris-HCl pH 7.8. At this point, thiol- as well as serine-type proteinase inhibitors (E64 and APMSF) were added to concentrations of 1 and 2 μ M, respectively. Without addition of these reagents RALyase activity was lost rapidly. All solutions used in the subsequent steps contained both inhibitors.

Step (5). The potassium chloride in the sample was diluted to 200 mM by addition of buffer minus KCl and the sample was loaded onto a 250 μ l Affi-Gel 501 column that had been washed and equilibrated with a solution containing 25 mM Tris-HCl pH 7.6, 200 mM KCl and 0.5 mM EDTA. The column was washed twice with 1.5 ml of this buffer, resulting in most of the protein appearing in the effluent but RALyase activity remaining bound to the column. The activity was then eluted with 2 mM DTT in the above buffer, indicating that RALyase contains free cysteine residues on the surface. The sample (2.4 mg of protein in 2 ml) was stored at -20°C in a solution of 12.5 mM Tris-HCl pH 7.6, 100 mM KCl, 2 mM DTT, 0.25 mM EDTA and 50% glycerol with only a slight loss of activity.

Step (6). A second anion column chromatography was done with a Mini-Q column (MiniQTM PC3.2/3) mounted on a SMART System (Pharmacia, Biotech). The stored sample was directly loaded onto the column pre-equilibrated with a similar solution as above but lacking glycerol, and bound protein was eluted with a linear gradient from 0.1 to 1 M KCl in this solution at a flow rate of 100 μ l/min (Figure 2A). Fractions containing RALyase, eluting around 300 mM KCl (see Figure 2A), were combined (three fractions of 40 μ l containing 81 μ g of protein), and glycerol was added to 50% for the final purification step.

Step (7). Gel-filtration was performed using a Superdex^R 75PC3.2/30 column in the same system as above. The mobile phase was 12.5 mM Tris-HCl pH 7.6, 150 mM KCl, 2 mM DTT, 0.25 mM EDTA and 50% glycerol, and the flow rate was 40 μ l/min. The sample was diluted to adjust the concentrations of each ingredient and 50 μ l of the sample containing 7.6 μ g of protein was loaded onto the column. The eluted proteins were detected by their absorbance at 280 nm (Figure 2B). Fractions containing peak activity were combined, and aliquot portions were stored at -20°C until use.

RALyase assay

Wheat germ ribosomes were suspended in buffer (25 mM Tris-HCl pH 7.6, 50 mM KCl, 5 mM MgCl₂) to give a concentration of 200 A₂₆₀ units/ml. To prepare substrates for RALyase, ribosomes (1 A₂₆₀ unit) were treated with gypsophilin (10 ng), a RIP isolated from *G.elegans* (Yoshinari *et al.*, 1997), for 15 min at 30°C in 30 μ l of buffer (25 mM Tris-HCl pH 7.6, 50 mM KCl, 1 mM MgCl₂). In these conditions, 100% of ribosomes are depurinated as shown by aniline-induced cleavage of the phosphodiester backbone (Endo *et al.*, 1987). To these substrate ribosomes, either RALyase fractions or S100 were added, and final reaction mixtures of 50 μ l (containing 25 mM Tris-HCl pH 7.6, 50 mM KCl, 1 mM MgCl₂, 1 mM DTT) were incubated for 60 min at 30°C. To determine enzyme activity, RNA was extracted from the reaction mixtures and separated on either 4% polyacrylamide or 2% agarose gels, and bands were visualized with ethidium bromide. The number of moles of phosphodiester bond cleaved by RALyase was obtained by the relative intensity of α -fragment produced compared with 5.8S rRNA (Endo and Wool, 1982). To determine the substrate specificity of the enzyme, rat liver or *E.coli* ribosomes were first depurinated as above except that the depurination reaction was done with 1 ng of gypsophilin for rat liver ribosomes and 20 ng for *E.coli* ribosomes. To test for RALyase activity on DNA having AP sites, superhelical pUC19 plasmid was depurinated using the acid method (Shaper and Grossman, 1980).

Determination of the rRNA sequence and structure at the site of cleavage by RALyase

After incubation of wheat ribosomes with purified RALyase, rRNA was extracted and the two fragments of 28S rRNA (the α - and β -fragment)

were purified by sucrose density gradient centrifugation. The sequence at the 5'-end of the α -fragment was determined by direct nucleotide sequencing after labeling of the 5'-terminus with [γ - 32 P]ATP (Endo and Wool, 1982). The structure at the 3'-terminal end of the β -fragment was analyzed by quantitative reduction using tritiated borohydride; the procedure followed a method published previously (Randerath *et al.*, 1972) but had some modifications: a 10 μ l reaction mixture containing 50 mM HEPES-NaOH pH 7.5, 10 pmol of the β -fragment, and 240 nmol tritiated sodium borohydride (521.6 mCi/mmol) was incubated for 1 h at 30°C. The reaction was stopped by adding 10 μ l of 0.1 M acetic acid, and RNA was recovered by ethanol precipitation and purified by passing through a MicroSpinTM Column. The RNA was washed three times by ethanol precipitation to eliminate contaminating tritiated material. The number of moles of reduced groups per mole of β -fragment was calculated from the moles of RNA and the specific radioactivity of borohydride. To confirm further the chemistry of the reaction catalyzed by RALYase, the tritiated β -fragment was oxidized with periodate (Randerath *et al.*, 1972) and the radioactivity remaining with the RNA was measured in a liquid scintillation counter.

Proteolysis of RALYase and sequence analysis of the peptides

Purified RALYase (2 μ g) was separated on an SDS-polyacrylamide gel and the protein was transferred onto PVDF membrane. After visualization of the 54 kDa RALYase with amido black, the protein band was cut out and further cut into smaller pieces, then incubated with endopeptidase Lys-C (0.075 units in 55 μ l of 100 mM Tris-HCl pH 8.0 containing 1% RTX-100 and 10% acetonitrile) (Coligan *et al.*, 1995). The proteolytic products were separated by reversed-phase HPLC using an RPC column equilibrated with 2% solvent A (80% acetonitrile containing 0.05% trifluoroacetic acid) in solvent B (0.065% trifluoroacetic acid) using a Pharmacia SMART system. After loading of the sample, the column was eluted at 100 μ l/min in the following conditions: 2% solvent A in solvent B for 1 min; 3–43% solvent A in solvent B for 80 min. Eluted peptides were detected by following the absorbance at 215 nm. Finally five peptides (a–e) were isolated and their amino acid sequences were determined (Yokogawa Analytical Center, Tokyo, Japan).

Cloning and sequencing of RALYase cDNA

A wheat immature embryo cDNA library constructed with λ gt10 was a generous gift from Dr Kawakami, Meiji University (Kawakami *et al.*, 1996). We used the nested PCR method for the cloning of cDNA fragments encoding RALYase. The degenerated primer 75S1 for the 5'-side of the gene (5'-AARTTYGTTTGGARGCIGC, derived from peptide c) and gt10A1 (5'-TGCATATCGCTCCATCAA) for the 3'-side of the gene were used for the first PCR. For the second PCR, new primers were designed based on the first PCR; the new primers are located downstream of 75S1 (75S2: 5'-GARGAYACIGARYTIGCITT, also derived from peptide c) and upstream of gt10A1 (gt10A2: 5'-CTTATGAGTATTCTCCAG). For the PCR, samples were denatured at 94°C for 5 min, then the reactions were carried out for 25 cycles using the following protocol: denaturation at 94°C for 30 s, annealing at 45°C for 1 min, and elongation at 72°C for 1 min. A fragment of ~550 bp was amplified by the nested PCR, and then sequenced after subcloning into pGEM-T easy. The determination of the 5'-terminus of the mRNA and cloning of the full-length cDNA were as follows. Total RNA of 10-day-old wheat coleoptiles was prepared by the method of Chomczynski and Sacchi (1987). Reverse transcription was carried out with a sequence 5'-GCAGCTCCATAAAG (based on the second PCR) as a primer and the RNA as a template. The 5' terminal end of RALYase mRNA was determined using a 5' RACE kit with 5'-TTTGGT-TTTGCTTGTGTTAG and 5'-GACATGTCTTGCCTCTCCAC obtained from the second PCR product as primers. For the isolation of full-length cDNA of RALYase, we used a primer for the 5'-side, 5'-CCC-TGTCCGCTTCTCCTC, obtained from the above 5' RACE and an oligomer for the 3' end, 5'-AAGCACAAATCCACATAATCAT, derived from the second PCR. RT-PCR was carried out in the same way as above except that annealing was done at 55°C for 1 min, and elongation at 72°C for 2 min. A fragment of ~1700 bp was amplified by RT-PCR, and then subcloned into pGEM-T easy. The nucleotide sequence was confirmed by sequencing of plasmids prepared from three separate RT-PCR experiments, and the plasmid thus obtained was named pGEM-RALYase. Sequencing was carried out on a DNA sequencer (model ABI 310, Perkin-Elmer) using a dye-terminator cycle-sequencing kit. Both strands were sequenced using internal primers.

Protein assay

Protein concentrations were determined using a Bio-Rad assay kit with bovine serum albumin as the standard.

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