Novel specificity of an endoribonuclease of yeast

Audrey Stevens

Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

Received 30 June 1986

Investigations of the cleavage specificity of a cyclizing endoribonuclease of yeast show that it hydrolyzes Y–A bonds of single-stranded regions of RNA and that oligonucleotides are hydrolyzed poorly. The stringent specificity of the enzyme suggests that it may be useful for generating specific fragments of polyribonucleotides. An isolation technique employing ethanol extraction has now been used to isolate the enzyme free of other RNases, and the studies show that it is a small protein, 10 kDa or less in molecular mass.

Endoribonuclease RNase Pyrimidine-specific cleavage Peptide extraction $tRNA^{Phe}$ (Yeast)

1. INTRODUCTION

An endoribonuclease isolated from *Saccharomyces cerevisiae* was found to give a limited cleavage of yeast mRNA and rRNA, yielding products that were terminated with pyrimidine nucleoside 2′,3′-cyclic phosphate [1]. By determination of the 5′-OH end groups of the fragments, it was shown that bonds between pyrimidine and A residues constituted more than 90% of the cleavage sites. Homopolyribonucleotides were hydrolyzed poorly, while poly(A,U) was cleaved rapidly at U–A bonds. The enzyme did not hydrolyze long double-stranded polymers, but was inhibited by ethidium bromide, which suggested that the cleavages might occur at short double-stranded regions. The specificity of the enzyme has now been studied further by analyses of partial digests of yeast [5′-^{32}P]$tRNA^{Phe}$ and determinations of the reactivity of oligoribonucleotides, and has been compared directly with RNase A. A simple isolation procedure using an ethanol extraction step frees the yeast RNase of other RNase activity, and its size has been examined.

2. MATERIALS AND METHODS

The yeast endoribonuclease was isolated as described [1] or by a peptide extraction technique which was a modification of that described by Hillar et al. [2] for the isolation of nucleic acid-binding peptides from calf thymus nuclei and other tissues. All reagents and glassware were autoclaved to prevent contamination with extraneous RNase activity. Operations were carried out at 0–4°C unless otherwise indicated. 15 g of yeast (grown as in [3]) were washed with 50 ml water and collected by centrifugation. The cells were suspended in 45 ml of 100 mM Tris-HCl buffer (pH 9.0) containing 500 mM NaCl, 20 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The suspension was passed twice through a French pressure cell at 20000 lb/inch^2 and centrifuged for 10 min at 3500 × *g*. The supernatant solution was poured off, diluted with 30 ml of the Tris buffer described above and adjusted to pH 9.5 by the addition of 3 N NH_4OH. After stirring for 1 h, the solution was homogenized with a Polytron homogenizer for two periods of 2 min each. The homogenate was stirred slowly and 3 vols 95% ethanol were added. The suspension was then homogenized again as described above, stirred for 1 h, and centrifuged for 20 min at 10000 × *g*. The supernatant solution was evaporated to dryness using a rotary evaporator at 22°C. The residue was suspended in 6 ml water and centrifuged for 15 min at 12000 × *g* to remove insoluble material.

The peptide fraction was diluted by the addition

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

of an equal volume of 20 mM Tris-HCl buffer (pH 8.7) containing 100 mM KCl, incubated 10 min at 22°C, and filtered at 22°C using an Amicon YM-10 filter (M_r cut-off 10000). The flow rate was 1–2 ml per h.

The filtered fraction was chromatographed on a Sephadex G-25 (Pharmacia, medium grade) or a Sephadex G-75 (Pharmacia, 40–120 μm) column (2 × 24 cm). The columns were equilibrated by first washing with 100 ml of 20 mM Tris-HCl buffer (pH 8.7) containing 100 mM KCl, then with 100 ml of the same buffer containing 5 mg acetylated bovine serum albumin (Bethesda Research Laboratories), followed by 150 ml of the same buffer without albumin. The sample was layered on the column and fractions (1.3 ml) were eluted with the same buffer. Marker proteins were obtained from Sigma.

The RNase activity was assayed by acid-solubilization measurements using reaction mixtures (50 μl) containing 1 nmol [^{3}H]poly(A,U) (3.6 10^4 cpm), prepared as in [1], 50 mM Tris-HCl buffer (pH 7.0), 20 mM EDTA and enzyme. Blank values were obtained by omitting enzyme. The reaction mixtures were incubated for 30 min at 37°C and the reactions were stopped by the addition of 50 μl of 7% perchloric acid and 50 μg bovine serum albumin. After 10 min in ice the mixtures were centrifuged in an Eppendorf centrifuge for 5 min, and radioactivity was determined with 50 μl of the supernatant solution.

Yeast tRNA^{Phe} (Boehringer-Mannheim) was labeled at the 5′-end with ^{32}P and purified on 10% polyacrylamide-urea gels according to Donis-Keller et al. [4].

3. RESULTS AND DISCUSSION

3.1. *Ethanol extraction and size of the yeast RNase*

A peptide extraction technique utilizing ethanol extraction was tried to isolate the yeast endoribonuclease described previously [1] because its contamination of RNA preparations of yeast in our laboratory suggested that it might be a small protein which binds tightly to RNA. Y-A specific endoribonuclease activity is detectable upon Sephadex column chromatography of a peptide fraction of yeast. As shown in fig.1A, RNase activity is eluted predominantly in the excluded fraction from Sephadex G-25. A small second peak, accounting for about 20% of the total activity, is eluted later. It is possible that the second peak is due to some binding of the RNase to the column. A comparison of the amount of RNase activity found in the Sephadex G-25 fractions upon chromatography of unfiltered and filtered (through Amicon YM-10 membrane) peptide fractions showed that 30–40% of the RNase activity is filterable. When RNase A is filtered under the same conditions, 12% of the activity is found in the filtrate.

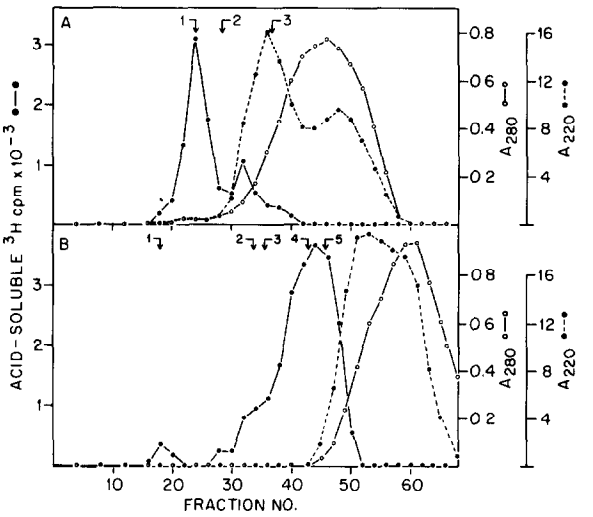

Fig.1. Sephadex column chromatography of peptide fractions of yeast. (A) 4 ml of a peptide fraction filtered through an Amicon YM-10 filter were chromatographed on a Sephadex G-25 column and aliquots (40 μl) of the fractions were assayed as described in section 2. (B) 4 ml of a filtered peptide fraction were chromatographed on a Sephadex G-75 column and aliquots (40 μl) of the fractions assayed. The elution positions of marker compounds are shown by arrows: (A) 1, blue dextran; 2, insulin A chain; 3, polymyxin B. (B) 1, blue dextran; 2, cytochrome *c*; 3, RNase A; 4, ACTH; 5, insulin B chain.

The results of Sephadex G-75 column chromatography of a filtered peptide fraction are shown in fig.1B. The RNase activity is eluted from the column close to the positions of elution of ACTH (M_r 4800) and insulin B chain (M_r 3900). Although the chromatography shows that the RNase activity is eluted with peptides in the range M_r 4000–5000, small basic proteins may behave anomalously on molecular sieve chromatography

[5], and thus it is not possible to estimate conclusively the size of the RNase. The chromatography and ultrafiltration results strongly suggest that the enzyme is 10 kDa or less in molecular mass. Omission of phenylmethylsulfonyl fluoride from the extraction buffer or inclusion of the protease inhibitors, leupeptin (0.1 μg/ml) and antipain (1 μg/ml), does not alter the amount of RNase activity found. About 0.005% of the 280-nm-absorbing material of the original extract is found in the Sephadex column fractions that contain RNase activity. The amount of RNase activity recovered is similar to that found in the purified fraction described in [1].

3.2. *Substrate specificity*

Characterization of the yeast RNase isolated by ethanol extraction showed that its pH optimum, divalent and monovalent cation requirements, and ethidium bromide and protease sensitivities are the same as described for the endoribonuclease purified by conventional steps [1]. Its activity and mode of hydrolysis of poly(A,U) are also similar.

To characterize further the specificity of cleavage by the yeast enzyme, in particular, its cleavage of single-stranded or double-stranded regions, hydrolysis of [5′-^{32}P]tRNA^{Phe} (structure shown in fig.2) was studied using both the small yeast RNase and RNase A. Polyacrylamide gel electrophoresis of the ^{32}P-labeled products of limit digests with both enzymes was carried out and the results are shown in fig.3. Fig.3A shows that the main cleavage product formed at 37°C with the yeast RNase isolated by ethanol extraction or by the conventional procedure [1] is the oligonucleotide resulting from cleavage at the single-stranded U–A bond in position 8–9. RNase A cleaves in a similar manner, showing, however, more cleavage of the double-stranded C–A bond at position 28–29. The results at 37°C under conditions of more extensive hydrolysis with both the yeast RNase and RNase A are shown in fig.3B. Cleavage of the C–G bond at position 2–3 is found with RNase A. At 55°C (fig.3C), both enzymes show more cleavage of the partially double-stranded C–A bond at position 13–14 and the double-stranded C–A bond at position 28–29. RNase A shows considerably more cleavage of the C–G bond at position 2–3 than the yeast RNase. Comparison of the yeast RNase (lanes 3,5) with an

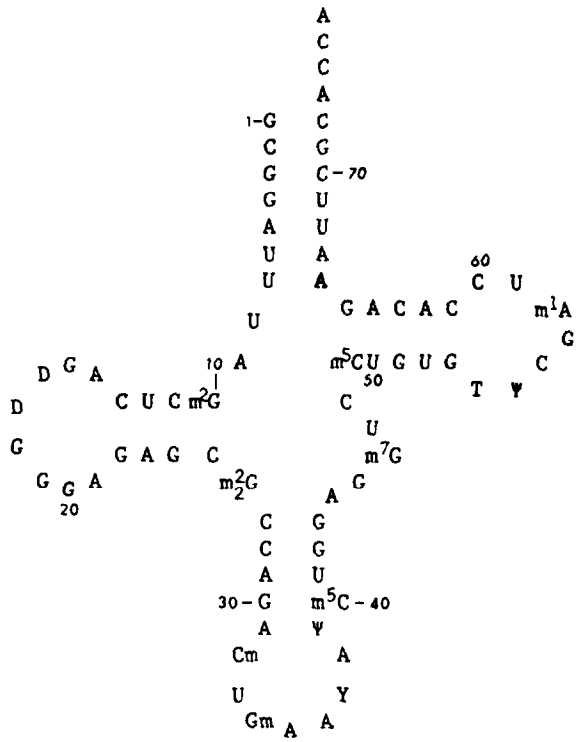

Fig.2. Structure of yeast tRNA^{Phe}.

equal amount of RNase A (lane 9) shows that cleavage of the C–G bond at position 2–3 is at least 4–5-times faster with RNase A. Analyses of end groups of the oligonucleotide products formed from rRNA with the yeast RNase also showed that Y–G bonds are cleaved very poorly [1], while under very similar conditions, RNase A cleaves Y–G bonds at greater than 10% of the rate of Y–A bonds [6,7].

Studies of the time course of digestion of rRNA by the yeast RNase suggested that short fragments were hydrolyzed more slowly than longer molecules [1]. The oligonucleotides, [5′-^{32}P]-pUpA, [5′-^{32}P]pCpA and [5′-^{32}P]pCpApU, have now been tested as substrates for both the yeast RNase and RNase A. As shown in table 1, the oligonucleotides are hydrolyzed by the yeast RNase (prepared by either method) at less than 10% of the rates obtained with RNase A.

The results show that the yeast RNase hydrolyses RNA at Y–A bonds of single-stranded regions with a greater stringency than RNase A.

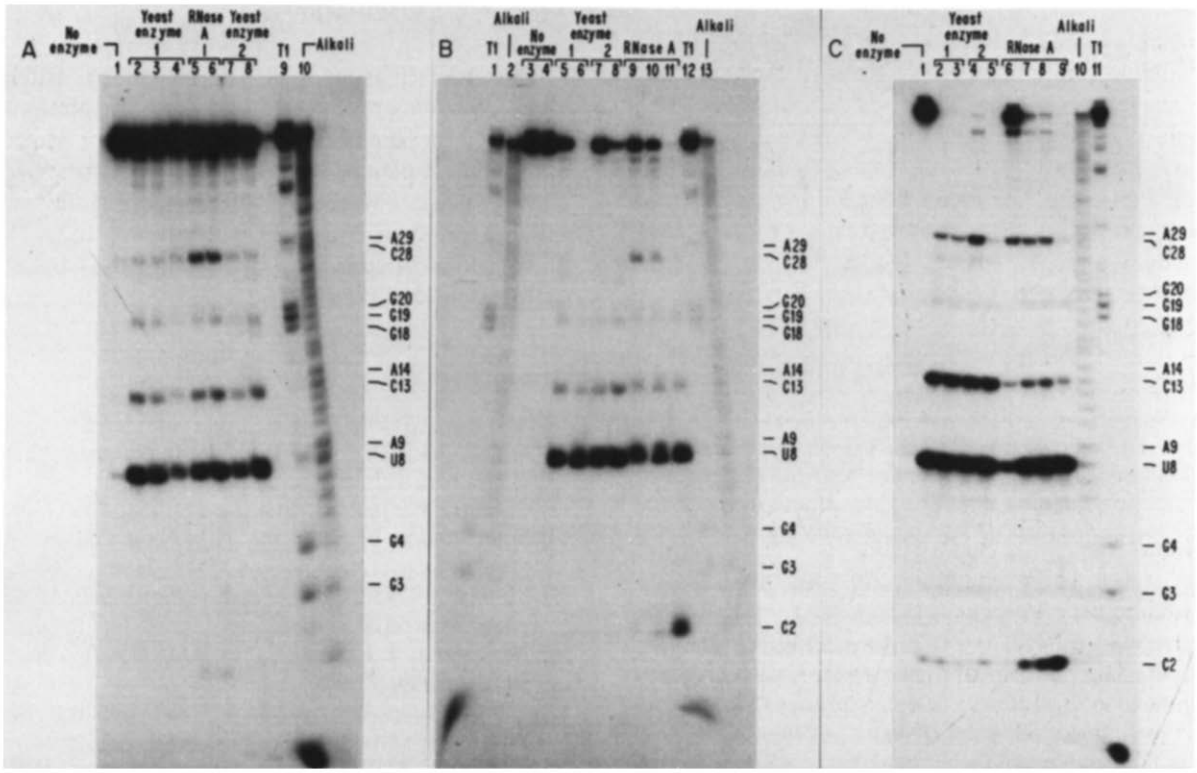

Fig.3. Autoradiographs of limit digests of [5′-^{32}P]tRNA^{Phe} separated by size on a polyacrylamide gel. The RNase T_1 and alkali partial digests were prepared as described by Donis-Keller et al. [4]. The digests were prepared with reaction mixtures (10 μl) containing 2.5 × 10^4 cpm [5′-^{32}P]tRNA^{Phe} and 66 ng of the same unlabeled tRNA. Amounts of enzymes and incubation times at 37°C (A,B) or 55°C (C) were as follows: A. (1) no enzyme, 1 h; (2) 4 μl of a Sephadex G-25 yeast RNase fraction, 1 h; (3) same as (2); (4) same as (2), but 2 μl; (5) and (6) 4.5 and 9.0 pg RNase A, 30 min; (7) 1 μl of a hydroxyapatite fraction prepared as described in [1], 1 h; (8) as (7), but 2 μl. B. (3) no enzyme, 1 h; (4) no enzyme, 2 h; (5) 6 μl of a Sephadex G-25 yeast RNase fraction, 1 h; (6) as (5), 2 h; (7) as (5), but an adjacent fraction, 1 h; (8) as (7), 2 h; (9,10,11) 10, 20 and 40 pg RNase A, 30 min. C. (1) no enzyme, 1 h; (2) 3 μl of an RNase fraction from Sephadex G-25, 1 h; (3) as (2), 6 μl; (4) 3 μl of a second RNase fraction from Sephadex G-25, 1 h; (5) as (4), 6 μl; (6,7,8,9) 5, 10, 20 and 40 pg RNase A, 30 min. Electrophoresis of the samples was carried out using 20% polyacrylamide gels containing 8 M urea as in [4]. The gels were exposed to X-ray film for 48 h at −70°C. The band in position 19 may result from contamination of the tRNA sample with a second tRNA species.

This novel specificity and the fact that oligonucleotides are hydrolyzed very poorly may make the enzyme useful for generating specific fragments of polyribonucleotides. The simple extraction procedure described here may be useful for detecting small RNases from other sources. An RNase purified from Novikoff hepatoma nucleoli was reported to have an M_r of about 7000 and to cleave Y−R bonds [8].

Table 1

Hydrolysis of oligonucleotides by the yeast RNase and RNase A

Substrate	Enzyme	% hydrolysis
[5′-^{32}P]pUpA	yeast RNase	0.32
	RNase A	5.34
[5′-^{32}P]pCpA	yeast RNase	0.32
	RNase A	5.76
[5′-^{32}P]pCpApU	yeast RNase	0.60
	yeast RNase b	0.51
	RNase A	6.40

The labeled oligonucleotides were prepared by phosphorylating UpA, CpA, and CpApU (Pharmacia) in reaction mixtures similar to those described for the labeling of products of RNase digestion [1]. The labeled oligonucleotides were separated by high-voltage paper electrophoresis in pyridine-acetate buffer (pH 3.5). 1 nmol of each oligonucleotide was incubated in reaction mixtures similar to those described in section 2 for 30 min at 37°C except [5′-^{32}P]pUpA was incubated for 90 min. Yeast RNase from a Sephadex G-25 column was used except for yeast RNase b which was prepared as in [1]. Amounts of the yeast RNase and RNase A used showed 40% hydrolysis of poly(A,U) in 30 min. The reaction mixtures were applied to Whatman 3 MM paper and electrophoresed as described above. Spots corresponding to the undegraded oligonucleotide and [5′-^{32}P]pU>p and [5′-^{32}P]pC>p were excised and counted to determine the percentage of hydrolysis. Reaction mixtures lacking enzyme were used as controls – values of approx. 0.10% were subtracted

ACKNOWLEDGEMENTS

This investigation was supported by Public Health Service grant A12098-02 from the National Institutes of Health and by the Office of Health and Environmental Research, US Department of Energy, under contract DE-AC05-840R21400 with the Martin Marietta Energy Systems, Inc. The author acknowledges the fine technical assistance of Marilyn K. Maupin.

REFERENCES

[1] Stevens, A. (1985) J. Bacteriol. 164, 57–62.
[2] Hillar, M., Przyjemski, J. and Wyborny, L.E. (1980) Mol. Biol. Rep. 6, 169–177.
[3] Stevens, A. (1980) J. Biol. Chem. 255, 3080–3085.
[4] Donis-Keller, H., Maxam, A.M. and Gilbert, W. (1977) Nucleic Acids Res. 4, 2527–2538.
[5] Siegel, L.M. and Monty, K.J. (1966) Biochim. Biophys. Acta 112, 346–362.
[6] McLennan, B.D. and Lane, B.G. (1967) Can. J. Biochem. 46, 93–107.
[7] Witzel, H. and Barnard, E.A. (1962) Biochem. Biophys. Res. Commun. 7, 295–299.
[8] Prestayko, A.W., Lewis, B.C. and Busch, H. (1973) Biochim. Biophys. Acta 319, 323–335.