Phy M: an RNase activity specific for U and A residues useful in RNA sequence analysis

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ABSTRACT

Physarum polycephalum, an acellular slime mold, produces an endoribonuclease activity (Phy M) useful in direct RNA sequence determination. Under conditions previously described for direct enzymatic RNA sequencing (7M urea, 50°C, pH 5.0)[1] Phy M cleaves almost exclusively and uniformly at Up↓N and Ap↓N. In the absence of urea UpN, ApN, and GpN are attacked in a sequence specific way identical to that found with RNase Phy I[2] digests performed under the same reaction conditions.

INTRODUCTION

Direct RNA sequence analysis by enzymatic methods[1,3] relies on uniform base-specific endonucleolytic cleavage by ribonucleases under partial digestion conditions. Although monospecific purine RNases are available (RNase T_1, G specific; RNase U_2, A specific[1]), analogous pyrimidine-specific RNases have not been found.

This paper describes an enzymatic activity extracted from the culture fluid of Physarum polycephalum which cleaves uniformly ApN and UpN bonds in RNA when used in 7M urea at 50°C, pH 5.0. In conjunction with the B. cereus RNase (which cleaves CpN and UpN[4]) the Physarum activity allows the distinction of the pyrimidines.

Other Physarum RNase activities with different specificities have previously been described[2,5,6]. Because more than one of these already has been called Phy I, and because the activity here described is likely to be due to a mixture of proteins, I propose the U + A specific Physarum activity be called Phy M. R. Braun and K. Bherens[5] first described an endonucleolytic activity they designated Physarum ribonuclease I which hydro-

lyzed UpN, ApN, GpN, but not CpN bonds in RNA. Another analysis[2] showed that this enzymic activity contained two separable RNases termed Phy I and Phy II and an acid phosphatase. With both Phy I and Phy II, the nucleotides on either side of the phosphodiester bond attacked affect the rate of cleavage. Pilly et. al.[2] found that with Phy I the order of cleavage susceptibility is UpN > ApN > GpN > CpN and NpA > NpC > NpG > NpU; therefore UpC, UpA, ApA and ApC are the most susceptible and CpU and CpC the most resistant to cleavage.

I show below the Phy M ribonuclease activity is present in relatively unfractionated preparations made essentially as described by Braun and Bherens[5]. When used under partial digestion conditions (7M urea, 50°C, pH 5.0), Phy M cleaves at A and U residues; although in the absence of urea it cleaves with a complex specificity essentially the same as that reported for Phy I by Pilly et. al.[2]. However, the converse is not the case; commercial Phy I is inactive at 50°C in 7M urea, pH 5.0. Therefore, with respect to use in sequence analysis the less fractionated Phy M preparation is more useful.

PHY M GROWTH AND PURIFICATION

Physarum Growth Conditions In order to insure reproducibility in the Physarum cultures, inbred haploid amoebal strains CH262 and CH272[7] (kindly provided by Dr. C.E. Holt, M.I.T.) were crossed to produce plasmodia. The plasmodia were grown at 25°C in shaker culture in PYE media[8] which contains per liter 10g tryptone, 3g yeast extract, 9g dextrose, 3.6g citric acid, 0.5g $MgSO_4/7\ H_2O$. The pH of the solution was adjusted to 4.6 with 30% KOH and autoclaved for 45 min and 10 ml/liter sterile hematin solution (0.05% hematin (Cal Biochem), 1% NaOH) added. The plasmodia were grown to a density of 1.0-1.5 ml packed cell volume. Packed cell volume was assayed by a 1 min centrifugation in a table top clinical centrifuge of 10 ml culture medium in a graduated test tube.

Purification Procedure The purification procedure was adapted from that described by Braun and Bherens[5]. The culture broth containing plasmodia was centrifuged at 1600 x g for 10 min, the

supernatant decanted, 10 ml 1 M HCl per 100 ml supernatant added, and the solution allowed to stand for 1 hr at 4°C. After centrifugation of the solution at 2400 x g for 30 min at 4°C, the brownish precipitate was disgarded and the pH of the supernatant adjusted to 4.5 with 1 M NaOH. 60 g ammonium sulfate (ultra-pure, R-Plus Inc.) per 100 ml solution, was added and the mixture allowed to stand for 12 hr at 4°C. The precipitate was collected by centrifugation at 2500 x g for 1 hr at 4°C, dissolved in a small volume of 10 mM NaAc pH 4.5 and dialyzed (#8 cellulose tubing, Union Carbide) for 12 hr against 10 mM NaAc pH 4.5. Undissolved particulate matter was removed by centrifugation for 60 min at 10,000 x g and the solution concentrated by dialysis against 50% glycerol, 10 mM NaAc pH 4.5 and stored at -20°C. The mixture is stable for at least 3 years under these storage conditions. The Phy M preparation is brownish and rather viscous which is probably due to the presence of pigments and polyglycans. However, the presence of these impurities does not interfere with enzyme activity.

Enzyme Activity Assay Phy M activity is assayed by serial dilution of the Phy M preparation into 10 µl of a buffer containing 20 mM Na citrate pH 5.0, 1 mM EDTA, 7 M urea, 0.1 µg/µl tRNA, 0.02% xylene cyanol and bromo phenol blue dyes, and 5' end-labeled RNA of known sequence (e.g. yeast 5.8S rRNA[9] purified according to the method of Rubin[10]). The mixture is incubated at 50°C for 10 min then loaded directly onto a 20% polyacrylamide, 7 M urea, 50 mM Tris-borate, 1 mM EDTA pH 8.3 slab gel (20 cm x 20 cm x 1.5 mm). Electrophoresis at 800v (constant power) in 50 mM Tris-borate, 1 mM EDTA pH 8.3 buffer is terminated when the bromo phenol blue dye reaches 12 cm from the origin (approximately 1.5 hrs). The gel is wrapped with saran wrap and autoradiographed at -20°C. A unit of enzyme activity is defined as the amount required to produce a uniform partial of 1 µg RNA in 10 min at 50°C in a buffer containing 20 mM Na citrate pH 5.0, 1 mM EDTA, 7 M urea. One liter of culture broth will normally give a final yield of at least 10^5 units of Phy M; the final volume in glycerol storage buffer is usually 20 mls or 8 x 10^3 u/ml.

PHY M SPECIFICITY

To determine the nucleotide or sequence specificity of the Phy M preparation, terminally labeled RNA of known sequence was partially digested with Phy M, the reaction products separated according to size by electrophoresis through a denaturing polyacrylamide gel, and the gel autoradiographed. Figures 1 and 2 show the different cleavage patterns produced by Phy M on 5' end-labeled 5.8S rRNA under two reaction conditions. In urea-citrate buffer pH 5.0 (lanes B) Phy M cleaves uniformly at each U and A residue with the exception that UpA bonds (see fig 2, U_{60}-A_{61}) are somewhat more resistant than other UpN bonds. In contrast, CpN, GpU, GpA, and GpC bonds are virtually uncleaved. Although GpG bonds are slightly more susceptible than other GpN bonds, cleavage at this position is significantly less than at UpN or ApN bonds. The RNase T_1 and alkali patterns provide markers based on the known sequence of the RNA[9]. In acetate buffer pH 5.0, in the absence of urea, Phy M shows a pronounced sequence specificity (fig 1, lanes A). While cleavage at A,G, and U is apparent, not all phosphodiester bonds are attacked uniformly. ApA bonds are strongly cleaved but ApC, ApU and ApG bonds are weakly cleaved. With the U series, UpU and UpA bonds appear to be more resistant to cleavage than UpC or UpG bonds. GpU and GpC bonds are more resistant than GpA or GpG bonds; cleavage at CpN is negligible although light cleavage at CpA is apparent. Figures 1 and 2 also show that the pattern produced by Phy I[2] (PL Biochemicals) is identical to Phy M (lanes A) when the reaction is done in the absence of urea. The specificity observed for Phy M and Phy I is maintained whether the limited digestions are done in acetate or citrate buffers pH 5.0, at 24°C or 50°C; the enzymes are less active at 50°C or in the presence or urea. The sequence specificity of Phy I under limited digestion conditions is in agreement with reports by others[2] except that they reported that ApC bonds in di-nucleotide substrates were highly susceptible to cleavage in variance with the relatively weak cleavage observed here. Under sequencing conditions (7 M urea, 50°C, pH 5.0)[1] the same commercial preparation of Phy I shows no activity (with 2,5,or 10 units of Phy I per reaction). Therefore if Phy I and Phy M

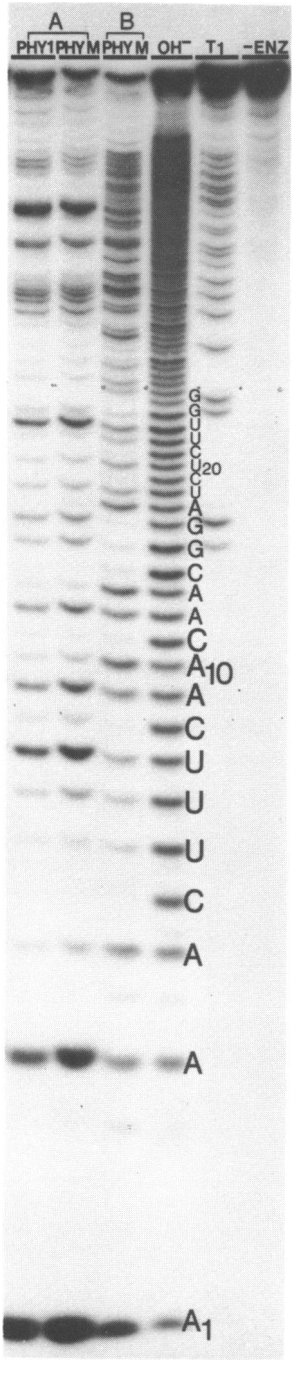
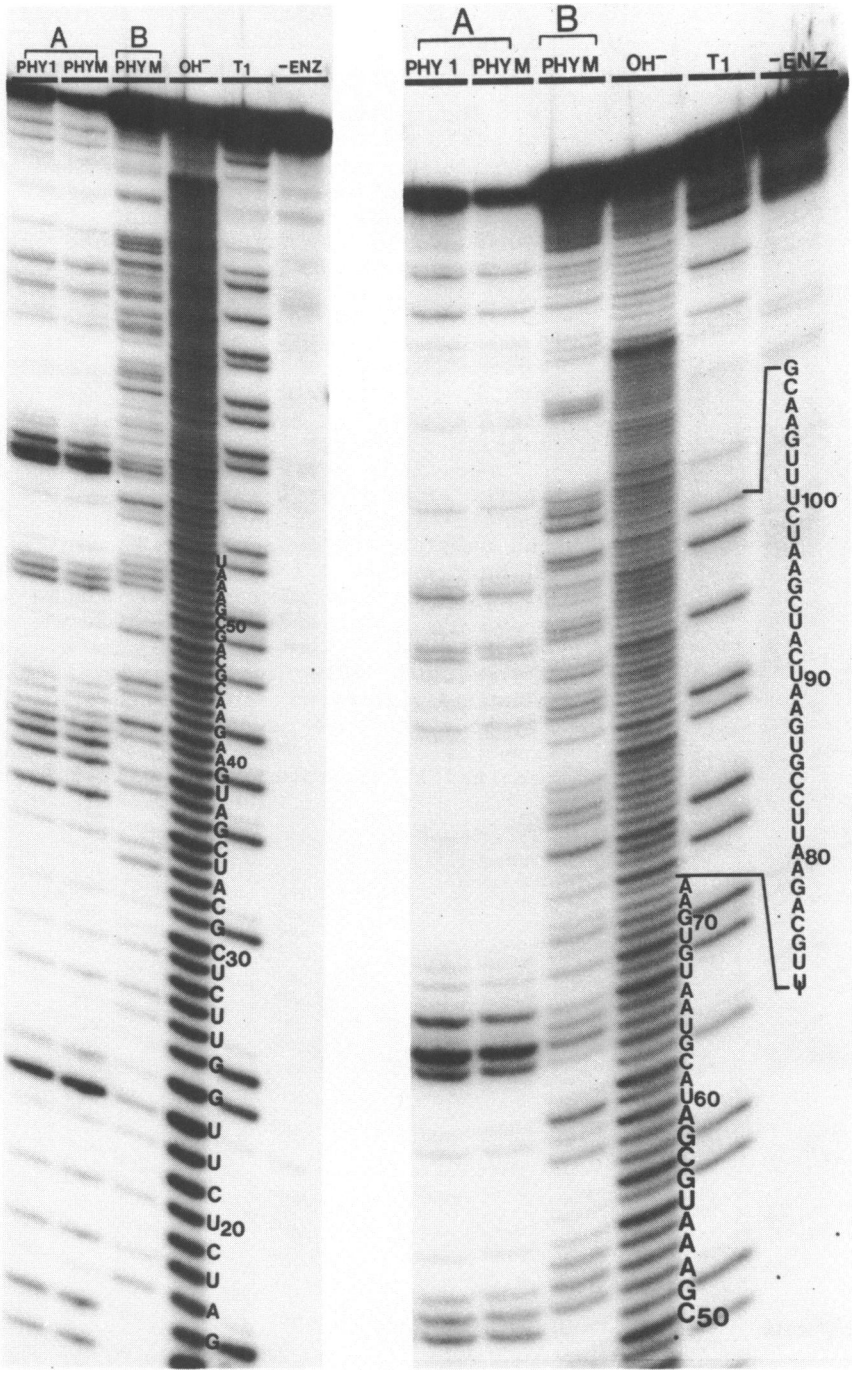


Figure 1. Autoradiograph of partial digests by Phy I and Phy M on 5' end-labeled 5.8S rRNA. Lanes A: Left, 0.5 u Phy I (PL Biochemicals); Right, 0.2 u Phy M. Incubation was for 15 min at 24°C in 10 µl 10 mM Na Ac pH 5.0, 1 mM EDTA, 0.1 µg/µl tRNA, and 5' end-labeled 5.8S rRNA. An equal volume of 10 M urea, 0.02% xylene cyanol and bromo phenol blue dyes was added to the reaction mixture prior to loading on the polyacrylamide gel. Lane B: 1 u Phy M, incubation at 50°C for 10 min in 10 µl 20 mM Na citrate pH 5.0, 1 mM EDTA, 7 M urea, 0.1 µg/µl tRNA, 0.02% xylene cyanol and bromo phenol blue dyes, and 5' end-labeled 5.8S rRNA. OH^- lane: limited alkaline hydrolysis of 5' end-labeled 5.8S rRNA, RNase T_1 partial digest (G specific); -ENZ, incubation of the RNA in the absence of RNase. Reaction conditions were as described[1] except that the reactions were done for 10 min in 10 µl buffer with 0.1 µg/µl RNA. 3 µl aliquots of each reaction mixture were loaded onto a 20% polyacrylamide 7 M urea, 50 mM Tris-borate pH 8.3, 1 mM EDTA, (33 cm x 40 cm x 0.4 mm) gel. Electrophoresis at 1500 v (constant power) was terminated when the xylene cyanol dye marker reached 10.5 cm from the origin. This gel resolves the 5' terminal 25 nucleotides of yeast 5.8S rRNA.


A
B
PHY1
PHYM
PHYM
OH⁻
T1
-ENZ
50
40
30
20
100
90
80
70
60
50

are identical then the Phy M preparation must contain something which stabilizes the RNase activity in the presence of 7 M urea.

APPLICATION OF PHY M TO RNA SEQUENCE ANALYSIS

The RNase activity of Phy M in urea-citrate buffer is useful for two reasons. In 7 M urea the RNA substrate is mainly denatured and exists as a single-stranded molecule thereby accessible to the RNase (most RNases including Phy M cleave only single-stranded RNA). For example fig 2 shows three regions in 5.8S rRNA, nucleotides 44-50, 54-62, and 68-87, which are hydrolyzed by Phy M in the presence of urea (lanes B) yet resistant under less denaturing conditions (lanes A). RNA secondary structure may interfere with cleavage by Phy I and Phy M of the normally susceptible UpG, GpG,GpA,ApA, and UpC bonds located within these regions. Phy M's uniform U+A cleavage specificity in urea-citrate buffer is easy to interpret, allows confirmation of A residues, and, when compared with the B. cereus U+C specificity[4], distinguishes U from C residues. Figure 3 shows the application of Phy M to the sequence analysis of 5.8S rRNA; the Phy M digest is fractionated in parallel with RNase T_1 (G specific), U_2 (A specific), B. cereus (C+U specific), and limited alkali hydrolysis (all residues) digests. Bands common to the U_2 and Phy M lanes represent RNA fragments whose 3' termini are Ap, and bands common to Phy M and B. cereus lanes represent fragments ending with Up.

CONCLUSION

For a ribonuclease to be useful in the direct RNA sequencing method[1,3] not only is base -specificity essential but the endonuclease must also cleave uniformly under partial digestion conditions so that each occurrence of a given base can be

Figure 2. Autoradiograph of partial digests by Phy I and Phy M on 5' end-labeled 5.8S rRNA. These gels resolve nucleotides G16 to U55 (left panel) and nucleotides C50 to G107 (right panel). Reaction and electrophoresis conditions were as described in fig 1 except that the partial digests were fractionated on 10% polyacrylamide gels.

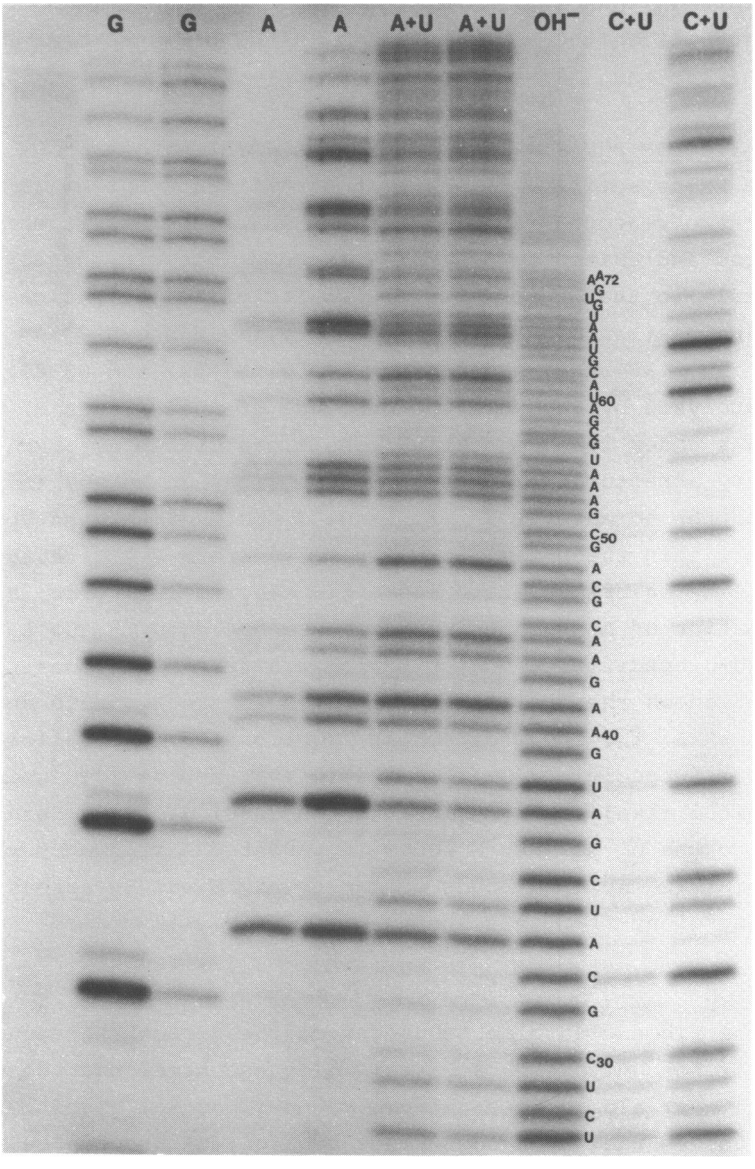


Figure 3. Autoradiograph of partial digests of 5' end-labeled 5.8S rRNA. Lanes G (RNase T_1); A (RNase U_2); A+U (RNase Phy M); C+U (B. cereus RNase); OH⁻, limited alkaline hydrolysis of the end-labeled RNA. Reaction and electrophoresis conditions were as described[1] except that the B. cereus partial digests were carried out in the absence of urea. This gel shows the 5' nucleotides U27 to A72 of yeast 5.8S rRNA.

detected. Two factors affect the uniformity of cleavage: the accessibility of the enzyme to the RNA substrate and the influence of nucleotides on either side of the phosphodiester bond to be cleaved (sequence specificity). Therefore RNases which are active under conditions which discourage the formation of RNA secondary and tertiary structure and provide a simple, uniform base-specific (rather than sequence-specific) cleavage pattern are desirable. RNases T_1 (G specific), U_2 (A specific), and Phy M (U+A specific) fulfill these requirements because they are active under conditions which disrupt RNA secondary and tertiary structure (7 M urea at 50°C, pH 5.0) and provide a simple, easily interpretable cleavage pattern. In combination with a limited alkaline hydrolysis which provides cleavage at every base thereby marking the position of each base in the sequence and the C+U cleavage specificity of the B. cereus RNase[4] the sequence of RNA molecules can be easily and unambiguously determined.

The enzymic activity Phy M is isolated from the culture broth of Physarum polycephalum by a simple procedure which allows recovery of large amounts of activity in highly concentrated from. Extensive purification of the activity is not necessary.

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