J. Biochem. **92**, 1655–1661 (1982)

#12

Substrate Specificity and Mode of Action of the Zinc-Metallo Nuclease from *Physarum polycephalum*

Jaap H. WATERBORG[1] and Charles M.A. KUYPER

Department of Chemical Cytology, Faculty of Science, University of Nijmegen, Toernooiveld, Nijmegen, The Netherlands

Received for publication, October 5, 1981

The alkaline zinc-metallo nuclease of *Physarum polycephalum* is an endonuclease with a high specificity for single-stranded nucleic acids. Single-stranded DNA was cleaved at least 6,000 times faster than double-stranded DNA under identical conditions. In the supercoil-induced single-stranded region of Form I PM2 DNA only a single nick was made.

The nuclease showed nucleotide specificity. Poly(A), poly(I), and poly(dT) were preferentially hydrolyzed.

Product analysis showed that it acted by an endonucleolytic mechanism: long polynucleotides were fragmented *via* intermediate length products to oligo- and mono-nucleotides with the phosphate group at the 5′-terminal position.

Extensive similarities exist with the single-strand-specific nuclease S1 from *Aspergillus*. The zinc-metallo endonuclease from *Physarum* could be used as a similar probe for single-stranded nucleic acids at neutral or alkaline pH conditions.

The alkaline nuclease from microplasmodia of the acellular slime mold *Physarum polycephalum* was a zinc-metallo protein with maximal activity at low ionic strength and at slightly alkaline pH conditions (*1*). It was active on single stranded DNA and RNA. Here we wish to report studies on substrate specificity and mode of action of this enzyme.

Initial results indicated that the nuclease showed differential rates of enzyme synthesis during sclerotization and that the enzyme could be involved in the extensive degradation of cellular nucleic acids during this differentiation process (*2*). We also wished to investigate the possibilities to use this nuclease as a tool for the analysis of nucleic acids, similar to other RNases from *Physarum* which have been used in the sequencing of RNA (*3*, *4*).

MATERIALS AND METHODS

All studies were performed with a nuclease preparation obtained after a 25,000 fold purification (Fraction V) from salt-induced microplasmodia of *Physarum polycephalum* (*1*). It contained the nuclease at a concentration of 180 Units per ml and a specific activity of 140,000 Units per mg protein. The Unit of enzyme activity was defined as the

[1] Present address: Department of Biological Chemistry, Medical School, University of California at Davis, Davis, CA 95616, U.S.A.

amount of enzyme which produced from denatured DNA under the assay conditions described below during an incubation of 1 h at 30°C an increase in the absorbance at 260 nm of 1.0 in the acid supernatant.

Nuclease Assays—The nuclease assay contained 0.1 ml 0.5 M Tris-HCl pH 8.0, 0.1 ml nuclease in buffer A (10 mM Tris-HCl, 10 mM KCl, pH 7.0) and 0.5 ml heat-denatured salmon sperm DNA (5 mg/ml in distilled water, Sigma). The mixture was incubated at 30°C and the incubation was stopped by chilling in melting ice. 0.05 ml bovine serum albumin (Sigma, BSA fraction V, 50 mg/ml in distilled water) and 0.5 ml 2.5 N $HClO_4$ were aded. The tubes were shaken vigorously, left stainding in ice for 15 min and clarified by centrifugation for 10 min at $1,500 \times g$ in the cold. The absorbance of the supernatants was measured at 260 nm and corrected for blank values.

In nuclease assays with RNA or polynucleotides the BSA solution was replaced by one containing 2.5 mg/ml heat-denatured salmon sperm DNA (Sigma) and 25 mg/ml BSA (Sigma). Native and heat-denatured salmon sperm DNA (Sigma), yeast RNA (Schwarz) purified according to Shortman (*5*), and polynucleotides (Sigma) were used at the final concentrations given in the legends to the figures.

Tritiated PM2 DNA was isolated as described (*6*). The DNA was centrifuged through a preparative neutral sucrose gradient (35 ml, 5–20% (w/v) in 1 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.5) for 18 h in the Spinco rotor SW 27 at 25,000 rpm and 4°C. Isolated superhelical DNA (Form I) and relaxed Form II DNA were used as double-stranded DNA substrates. Single-stranded DNA was prepared from Form II DNA by heat-denaturation for 10 min at 100°C and rapid chilling in melting ice. The nuclease assay, based on acid solubilization of substrate, was performed with approximately 1 μg of tritiated PM2 DNA and treated as described above for the assay on RNA. The incubations for gradient analysis were stopped by chilling in ice and the addition of 0.3 ml 100 mM EDTA. Samples of 0.1 ml were centrifuged through a neutral sucrose gradient (4 ml, 5–20% (w/v) in 1 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.5) for 2.5 h in the Spinco rotor SW 60-Ti at 60,000 rpm and 4°C, or through an alkaline sucrose gradient (4 ml, 5–20% (w/v) in 0.5 M NaCl, 0.4 N NaOH, 1 mM EDTA) (*7*) for 1 h in the Spinco rotor SW 60-Ti at 60,000 rpm and 4°C. Fractions of approximately 0.1 ml were collected and radioactivity was determined.

Product Analysis—The fragmentation of DNA and poly(A) and the composition of the acid soluble products was studied during a series of incubations with the assays as described above. The reactions were stopped in ice and samples of 1.0 ml, containing 20 *A* at 260 nm, were chromatographed in the cold over a column of Sephadex G-200 (2.6×28 cm) with buffer A at 0.85 ml/min. Fractions of 0.85 ml were collected and the absorbance was measured at 260 nm. The acid soluble fraction of the samples, made without BSA or DNA, containing 20 *A* at 260 nm, was chromatographed over a DEAE-cellulose column (1.6×3.5 cm) according to Tomlinson and Tener (*8*).

The localization of phosphate groups in the products was determined by thin layer chromatography on PEI-cellulose, according to Wyers *et al.* (*9*). PEI-cellulose F-sheets (Merck) were prechromatographed with distilled water, spotted with nucleic acid digests and 2′/3′-AMP and 5′-AMP (Sigma) marker compounds, and developed by ascending chromatography.

RESULTS

The nuclease was active on denatured salmon sperm DNA. During a series of digestions of this DNA at various concentrations by 1 Unit of enzyme activity apparently linear correlations were found between the time of incubation and the amount of acid soluble material produced, at least during the initial phase of the substrate hydrolysis (Fig. 1). The apparent K_m, calculated from these experiments according to Hofstee (*10*), was 0.6 mg single-stranded salmon sperm DNA per ml incubation mixture or approximately 2 mM nucleotide concentration (Fig. 1, inset). The activity on double-stranded DNA was low. The initial rate on denatured DNA was 25 to 50 times higher than on an identical concentration of native salmon sperm DNA (Fig. 1). The activity on native DNA was higher during incubations at higher temperatures. Native DNA was hydrolyzed at 5,

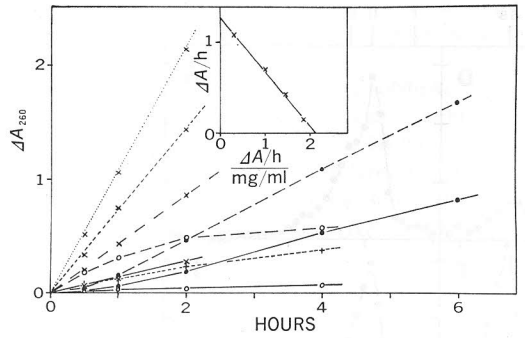

Fig. 1. Digestion of nucleic acids by the nuclease. The nuclease Fraction V (1.05 Units) in buffer A was incubated as described in "MATERIALS AND METHODS" at the indicated final concentrations: ·····, 3.6 mg/ml; ----, 0.7 mg/ml; – – –, 0.3 mg/ml; ——, 0.07 mg/ml. The absorbance of the acid supernatants at 260 nm, obtained with carrier DNA added to the BSA solution, is corrected for blanks. The substrates used were denatured salmon sperm DNA (×), native salmon sperm DNA (+), yeast RNA (○), and poly(A) (●). The inset shows a Hofstee plot, calculated from the initial reaction rates on denatured DNA.

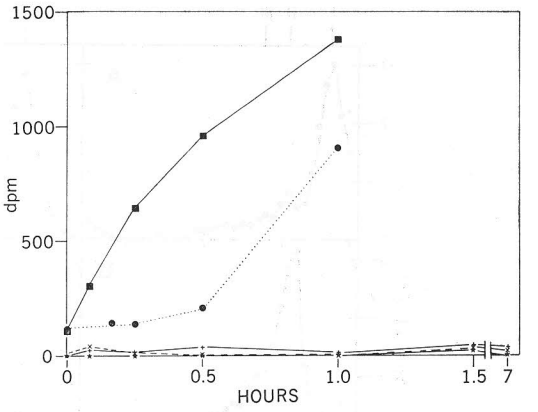

Fig. 2. Digestion of PM2 DNA to acid solubility. Approximately 1 μg of PM2 DNA was used for each digestion and the acid solubilized DNA was determined as described in "MATERIALS AND METHODS." 0.18 Units nuclease were used to digest single-stranded DNA (17,800 dpm) (···●···). 1.8 Units were used to digest single-stranded DNA (13,000 dpm) (—■—), relaxed double-stranded Form II DNA (11,500 dpm) (—+—) and supercoiled double-stranded Form I DNA (13,000 dpm) (—★—). 18 Units nuclease were used to digest Form II DNA (11,800 dpm) (– –×– –).

30, and 58 percent activity, relative to denatured DNA, during incubations for 1 h at 45, 75, and 80°C, respectively.

The specificity of the nuclease was further studied on PM2 DNA. Approximately 1 μg of single-stranded PM2 DNA was digested by 1.8 Units of enzyme activity and an initial rate of acid solubilization of 17.4 percent per h was found. A digestion of the same amount of Form II double-stranded PM2 DNA by 18 Units of enzyme activity gave an upper limit of less than 0.2 percent acid solubility in 7 h (Fig. 2). With the difference in enzyme concentration taken into account, the rate of hydrolysis of single-stranded DNA is more than 6,000 times higher than that of double-stranded DNA.

The analysis of digested PM2 on neutral and alkaline sucrose gradients made clear that the nuclease nicked Form I DNA to yield Form II, but the rate of this hydrolysis was much lower than the rate at which an identical amount of single-stranded DNA was cleaved. During gradient analysis of digests of Form I or Form II PM2 DNA, no Form III [linear, double-stranded DNA] or smaller products were found even after digestions of 1 μg PM2 DNA with an amount of nuclease sufficient for the solubilization of more than 5 mg single-stranded salmon sperm DNA (Fig. 3).

Yeast RNA was hydrolyzed at an initial rate similar to that found with denatured DNA. However, this rate declined to zero after 15 to 25 percent of the RNA had become acid soluble (Fig. 1). The rate of hydrolysis of poly(A) was, after an initial lag phase, similar to that found with denatured DNA (Fig. 1). Poly(I) and poly(dT) were hydrolyzed at similar rates and low or insignificant activity was found on all other homopolynucleotides tested (Table I). On all adenine-containing heteroribopolynucleotides activity was found, although this activity was low on poly(AC). The insignificant activity on the alternating poly(dAdT) at 30°C increased sharply when high temperatures melted the double-stranded structure. An activity of 70 percent was found on poly(dAdT), relative to the activity on denatured DNA. No activity on poly(G) was found at 30, 60, 70, and 80°C.

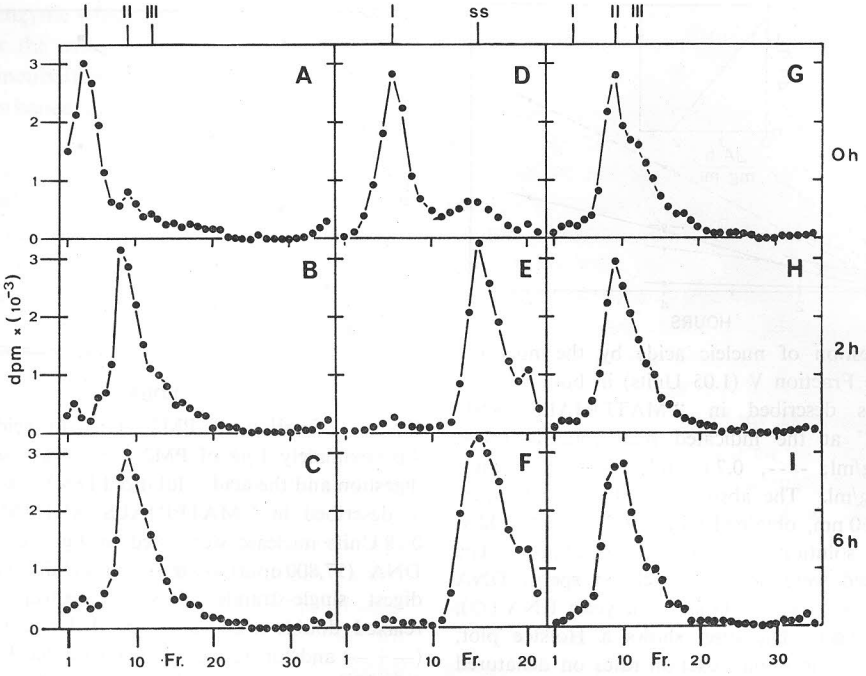

Fig. 3. Digestion of PM2 DNA and gradient analysis. 18 Units nuclease were used to digest approximately 1 μg of Form I (A–F) and Form II (G–I) PM2 DNA for 2 h (B, E, H) and 6 h (C, F, I). The DNA, also undigested (A, D, G), was analyzed on neutral (A–C, G–I) and alkaline (D–F) sucrose gradients as described in "MATERIALS AND METHODS." The location of Form I, Form II, and Form III DNA in the neutral gradient profiles and of Form I and single-stranded DNA in the alkaline sucrose gradient profiles is indicated. Sedimentation was from right to left.

The enzyme activity on circular PM2 DNA and the lag phase during the digestion of poly(A) (Fig. 1) and of single-stranded PM2 DNA (Fig. 2) indicated that the nuclease acts by an endonucleolytic mechanism. The analysis of the product composition of DNA and poly(A) digests at various stages during the reaction (Fig. 4) confirmed this conclusion. Gelfiltration showed the fragmentation of the substrate and chromatography the decrease in length of the acid soluble oligonucleotides (Fig. 5). The endonucleolytic action is most clearly seen in a combination of these results, which shows the gradual shift from long polynucleotides to short oligo- and mononucleotides (Fig. 6). In no case were nucleosides or free bases found among the reaction products. The presence of a non-digestible core in the denatured DNA was noted, which was absent in poly(A) (Figs. 5 and 6). The acid soluble products from digested DNA were longer than those of poly(A), digested to a similar extent (Fig. 5).

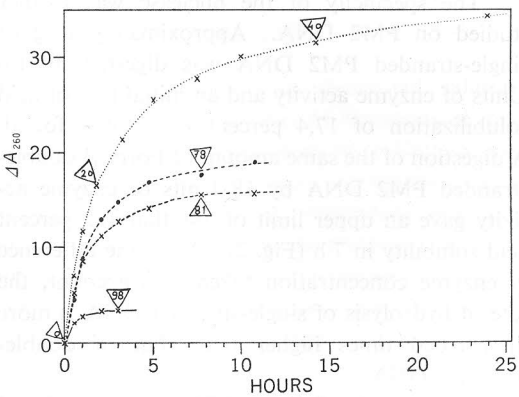

Fig. 4. Extensive digestions of DNA and poly(A) by the nuclease. 10.5 Units nuclease was incubated as described in "MATERIALS AND METHODS" with denatured salmon sperm DNA at final concentrations of 3.6 mg/ml (···×···), 0.7 mg/ml (--×--), and 0.3 mg/ml (—×—) and poly(A) at 0.7 mg/ml (--●--). The aparently higher concentration of poly(A) relative to DNA as measured by the absorbance at 260 nm was caused by the higher molar absorptivity of the adenine nucleotides (*11*). The figures within the triangles give the percent of acid solubility.

TABLE I. Nucleotide specificity. DNA and polynucleotides were used at a final concentration of approx. 0.7 mg/ml. All heteropolynucleotides were structured at random, except for the alternating poly(dAdT). The level of complete acid solubility was determined by hydrolysis for 1 h at 70°C in 0.5 N $HClO_4$. The nuclease (Fraction V, 9 Units) hydrolyzed the denatured DNA during an incubation for 1 h at 30°C to 69 percent acid solubility. This activity was taken as 100%.

Polynucleotides	Activity (%)
Denatured DNA	100
Native DNA	3
Poly(dT)	120
Poly(dA)	21
Poly(A)	100
Poly(I)	115
Poly(U)	10
Poly(C)	6
Poly(G)	4
Poly(AI)	121
Poly(AG)	100
Poly(AU)	90
Poly(AC)	27
Poly(dAdT)	4

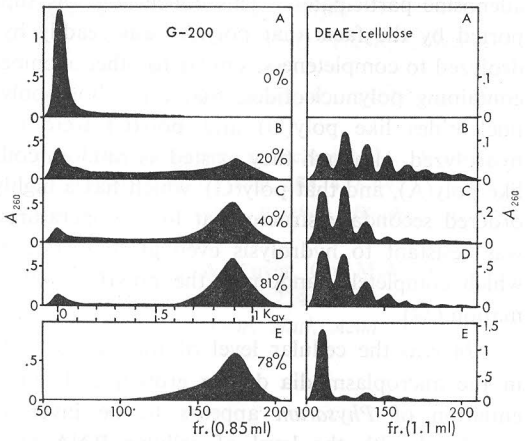

Fig. 5. Product analysis of DNA and poly(A) digests. Samples of 0% (A), 20% (B), 40% (C), and 81% (D) acid solubility of DNA and 78% acid solubility of poly(A) (E) (Fig. 2) were analyzed on Sephadex G-200 and DEAE-cellulose as described in "MATERIALS AND METHODS."

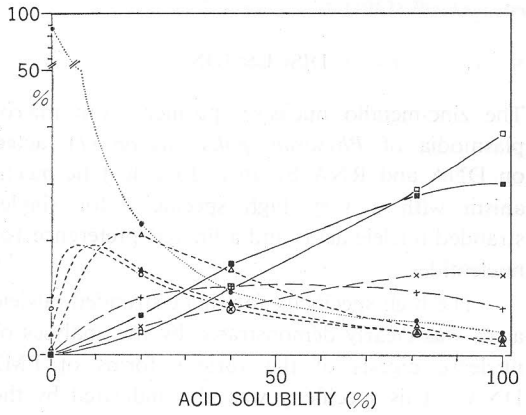

Fig. 6. Kinetic analysis of the nuclease digestion of DNA. The results displayed were calculated from the experiments of Figs. 4 and 5: Polydeoxynucleotides [poly(dN)] longer than 150 nucleotides (*12*) (G-200 K_{av}=0), ···●···; poly(dN) of 120 nucleotides (K_{av}=0.25), --○--; poly(dN) of 50 nucleotides (K_{av}=0.45), --▲--; oligo(dN) of 15 nucleotides (K_{av}=0.70), --△--; oligo(dN) of 6 (range 4–8) nucleotides, --+--; trinucleotides, --×--; dinucleotides, —■—; mononucleotides, —□—. The relative percentages of the various groups of nucleotides are plotted against the percentage of acid solubility of the samples.

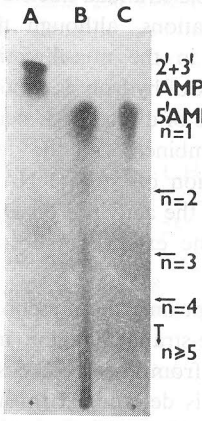

Fig. 7. Thin layer chromatography of a poly(A) digest. A 0.01 ml sample of the poly(A) digest at 78 percent acid solubility (Fig. 4) was spotted in lane B, 0.01 g 2′/3′ AMP was spotted in lane A and 0.05 g 5′-AMP in lane C. The positions of the 2′, 3′, and 5′-AMP, di-, tri-, tetra-, and longer oligo-nucleotides are indicated.

The poly(A) digest was chromatographed on PEI-cellulose and the mononucleotide product was identical to 5′-AMP and different from 2′- or 3′-AMP (Fig. 7). On the basis of this result the conclusion was drawn that all products carried phosphate groups at their 5′-terminal positions.

DISCUSSION

The zinc-metallo nuclease, purified from microplasmodia of *Physarum polycephalum* (*1*), acted on DNA and RNA by an endonucleolytic mechanism with a very high specificity for single-stranded nucleic acids and a limited preference for nucleotides.

The high specificity for single-stranded nucleic acids was clearly demonstrated by the analyses of nuclease digests of the various forms of PM2 DNA. This specificity was also indicated by the low activity of the nuclease on native DNA — probably at nicks and gaps in the preparation only — which increased sharply at temperatures were melting of the DNA to single-stranded form occurred. The observations of a non-digestible core in the heat-denatured DNA, of the low and declining reaction rates on the native DNA and of the decreasing reaction rates on the yeast RNA preparation, which for a major part consisted of tRNAs, could well be caused by the relative amounts of double-stranded nucleic acid sequences in these preparations, although the absence of preferred bases in the non-digestible core as a cause for the lack of hydrolysis cannot be excluded. The constant rate of digestion of denatured salmon sperm DNA combined with the declining rate of the nuclease action on yeast RNA, resulted in a variable ratio of the activities on DNA and RNA, dependent on the extent of substrate hydrolysis (*1*).

However, not only the presence of hydrolysis-resistant, double-stranded region in the substrate gives deviations from linear kinetics. The activity of the nuclease is determined through the rate at which acid soluble products arise. This means that the enzyme activity is not manifest as long as the products formed are still acid insoluble. This accounts for the lag periods seen in the digestion of poly(A), a preparation of long polynucleotides only (Fig. 1) and of genome-length single-stranded PM2 DNA at low nuclease levels (Fig. 2). A simple endonuclease which hydrolyses phosphodiester bonds irrespective of polynucleotide length, would have given a similar lag period during the digestion of heat-denatured salmon sperm DNA, which is a mixture of long and short polynucleotides. It would also have caused an accumulation of intermediate-length digestion products before short, acid soluble oligonucleotides could be formed. The *Physarum* nuclease did not show a lag when denatured salmon sperm DNA was digested (Fig. 1) and did not accumulate large amounts of intermediate-length products (Fig. 5). Nuclease S1, an endonuclease from *Aspergillus oryzae*, showed a similar behavior: a linear pattern for the solubilization of denatured DNA (*12*, *13*) and almost no accumulation of intermediate-length products (*12*). Apparently nuclease S1 and the *Physarum* enzyme differ from the general group of endonucleases in the characteristic that they degrade short substrate chains at a rate similar to or higher than the rate at which these short chains are formed from the long substrate polynucleotides.

The faster hydrolysis of short substrates relative to long ones may also account to a large extent for the flattening out of the curves for the digestion of denatured DNA, particularly at high substrate concentrations (Fig. 4).

Comparisons of the digests from single-stranded DNA and poly(A) show that in the former oligonucleotides persist to the end, while poly(A) is broken down, almost completely, to mononucleotides. This points to a preference of the nuclease for internucleotide bonds in which adenosine participates. This conclusion was supported by the facts that poly(A) was readily hydrolyzed to completeness, similar to other adenine-containing polynucleotides, that other homopolynucleotides like poly(U) and poly(C) were not hydrolyzed, although they existed as random coils like poly(A), and that poly(G), which has a highly ordered secondary structure at low temperatures, was resistant to hydrolysis even at temperatures which completely randomize the poly(G) conformation (*14*).

In vivo the cellular level of nuclease activity in the microplasmodia during growth and differentiation of *Physarum* appears to be inversely correlated with the level of cellular RNA (*15*). The nuclease could possibly function in the process of the turnover of poly(A) tails in mRNA by the endonucleolytic mechanism suggested by Adams *et al.* (*16*, *17*).

The *Physarum* nuclease and nuclease S1 are

similar in their endonucleolytic mode of action, as shown above. They are also similar in other characteristics. Both are zinc-metallo endonucleases, which degrade single-stranded polynucleotides to 5′-phosphorylated products. They are also similar in molecular weight, temperature stability, inhibition by phosphate, activity in the presence of 5 M urea and action on homopolynucleotides (*1*, *13*, *18–20*). Nuclease S1 has much been used as a probe for single-stranded nucleic acids (*20–25*). The results of the *Physarum* enzyme in one of the most specific systems, the nicking of supercoiled DNA without any further hydrolysis, were identical to the ones obtained with nuclease S1. The reported action of nuclease S1 to cleave the DNA strand opposite an existing nick (*20*, *21*) was not found, possibly because the enzyme concentration employed by us was too low. The major difference between both enzymes, *i.e.* the alkaline pH optimum of the *Physarum* enzyme (*1*) and the acid one for nuclease S1, could be an advantage. With the *Physarum* nuclease single-stranded DNA could be studied under conditions were the acid pH, especially at raised temperatures with its very fast depurination of DNA (*26–28*), should be avoided.

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