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Characterization of a Recently Purified Thermophilic DNase from a Novel Thermophilic Fungus

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Abstract A newly isolated thermophilic fungus was found to produce a partially inducible extracellular DNase. This manuscript focuses on the characterization of this novel thermophilic DNase in terms of optimal enzyme conditions, molecular weight, and certain kinetic properties. The DNase was found to be inactivated by the presence of EDTA demonstrating its dependence on metal cofactors for activity. Maximum activity occurred at pH 6.0 with no activity at pH 2.0 or 10.0. The optimal temperature for the purified DNase was 65 °C. The thermophilic DNase was found to be an exonuclease with an estimated molecular weight of 56 kDa.

Keywords DNase · Enzyme kinetics · Enzyme characterization · Thermophilic fungus

Introduction

Nucleases are enzymes which catalyze the cleavage of the internucleotide bridges without the release of inorganic phosphate [14]. These internucleotide bridges are extremely resistant to chemical hydrolysis and are necessary for the stability and geometric integrity of molecules such as DNA and RNA [6]. Due to the overall ruggedness of nucleic acids, nucleases must possess qualities that allow the rapid hydrolysis of such molecules. Nucleases are divided into two major subgroups based on substrate specificity.

Nucleases may show some specificity towards either double- or single-stranded nucleic acids [13]. With double-stranded nucleic acids, nucleases can only approach from the outside of the helix where external nucleophiles are limited [19]. Single-stranded nucleic acids are much easier for nucleases to attack because bases are not stacked allowing access to the nucleophiles [19]. Nucleases themselves are subdivided into two specific classes, endonucleases and exonucleases. Both cleave phosphodiester bonds, but substrate recognition and product formation determine if they are endo- or exonucleases [5]. Endonucleases attack

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phosphodiester bonds situated in the middle of nucleic acid chains, forming oligonucleotides and some mononucleotide fragments. Exonucleases also break the phosphodiester bonds holding nucleic acids together but only attack from one end (5′ or 3′ end) sequentially cleaving mono and/or dinucleotides.

Endonucleases exhibit site specificity and have the ability to recognize certain sequences of nucleotides [7]. These sequences direct the nuclease to cut either within or outside these sequences [13]. Along with sequence specificity, nucleases also demonstrate sugar specificity. There are three possibilities for the pentose sugar specificity of nucleases: DNA specific (deoxyribonuclease or DNase), RNA specific (ribonuclease or RNase), and nucleases that are sugar nonspecific and can cleave both RNA and DNA. Nonspecific nucleases show no specificity towards the nature of the pentose sugar moiety. Nonspecific nucleases have been shown to have some specificity towards the nature of the linkages, specifically the purine or pyrimidine linkages [14]. Nonspecific nucleases have varying degrees of "blindness to sugar" according to Wechter and Mikulski [18].

Fungal nucleases adhere to the classical definition of nucleases and are either intra- or extracellular in nature. In 1975, Hanking and Anagnostakis [8] examined ten species of thermophilic fungi for the production of extracellular enzymes. They found that eight out of the ten species examined produced extracellular DNase activity and five out of seven produced extracellular RNase activity [8]. We recently isolated a novel thermophilic *Chaetomium* spp. from horse manure compost that produced extracellular nucleases [10, 11]. From this, the extracellular DNase was purified 145-fold using a combination of traditional and novel purification techniques including ultra-membrane filtration, size exclusion chromatography, and affinity membrane purification. This manuscript describes the partial characterization of the purified DNase.

Materials and Methods

Fungal Identification and DNase Purification

Fungal identification and enzyme purification were carried out as explained by Landry, Vu, and Levin [11] using a combination of traditional and novel affinity membrane purification techniques [9].

Acid Soluble Assay for Enzyme Activity

DNase activity was determined by measuring acid-soluble nucleic acids. The method used in this study was a modified version of that of Eaves and Jeffries [3]. The enzyme sample (0.75 mL) was added to 0.75 mL of substrate (salmon sperm DNA, USB Lot No. 126531, 10 μmol $MgSO_4$ in 0.1 M imidazole, pH 7.0) and incubated in a water bath set to the desired temperature. The reaction was stopped by adding 0.5 mL of uranyl acetate-perchloric acid reagent (0.25 % uranyl acetate in 10 % perchloric acid). Reaction tubes were cooled in an ice bath for 15 min. The mixture was diluted with 2.0 mL of deionized water and the precipitate removed by centrifugation at 13,400 rpm for 5 min at ambient temperature. The supernatant was diluted as required, and the absorption at 260 nm was measured against a reagent blank prepared by adding the uranyl acetate-perchloric acid reagent to the substrate prior to the addition of the enzyme. One unit of enzyme activity is defined as an increase in absorbance of 0.05 units in a cuvette of 1 cm light path at 260 nm.

Real-Time Spectrophotometric Assay of DNase Activity

The method used for this study was a modified version of the one used by Wang and Levin [17] and involves the real-time detection of DNA hydrolysis. Approximately 2.25 mL of substrate (40 μg/mL salmon sperm DNA, USB Lot No. 126531, 10 μmol $MgSO_4$ in 0.1 M imidazole, pH 7.0) was added to a cuvette (1 cm path length) and placed into a temperature-controlled cuvette holder equilibrated to the desired temperature. Purified enzyme (0.75 mL) was added and mixed with a cuvette mixing device, for a total volume of 3 mL. The increase in absorbance at 260 nm was recorded. The blank consisted of 2.25 mL of substrate solution and 0.75 mL of deionized water.

Enzyme Destruction Assay

Two water baths were used for this assay, one set to 55 °C (assay temperature) and another set to the desired destruction temperature (65 or 68 °C). The enzyme sample was placed in the water bath set to the 65 °C destruction temperature and allowed to equilibrate. Once the enzyme preparation reached the desired temperature, the enzyme (0.75 mL) was removed after various time intervals and added to 0.75 mL of substrate (salmon sperm DNA, USB Lot No. 126531, 10 μmol $MgSO_4$ in 0.1 M imidazole, pH 7.0) equilibrated at 55 °C. The sample was allowed to incubate for 1 h before assessment of DNase activity. For the 68 °C destruction temperature, the enzyme sample and water bath previously equilibrated to 65 °C was adjusted to 68 °C (come up time ~40 s), and the previous procedure was repeated.

Molecular Weight Estimation via Sephadex G-75 Chromatography

The molecular weight of the purified DNase was estimated using a column of Sephadex G-75 (Pharmacia Fine Chemicals Lot No. 8591) with a gel bed of 2.5×30 cm. Sephadex G-75 (Pharmacia Fine Chemicals Lot No. 8591) was hydrated in deionized water for 3 h at 100 °C prior to loading the column. The column was equilibrated with 0.5 M imidazole buffer at pH 7.0 containing 0.02 % sodium azide. Samples were loaded onto the bottom of the gel bed and eluted ascendingly at a rate of 0.8 mL/min (48 mL/h) in a 2–5 °C chromatography refrigerator. Fractions (6.6 mL) were collected using a Gilson FC 203B fraction collector.

The K_{av} values of enzymes with known molecular weights were used to determine the molecular weight of the purified nuclease from a standard curve. Highly purified ribonuclease A (13,700 Da; Sigma Chemical, Lot 80 K1561), α-chymotrypsinogen (25,000 Da; Sigma Chemical, Type II No. C-4879, Lot 111 F-8055), α-amylase (51,000 Da; Sigma Chemical, Type VIII-A No. A-2771, Lot 57 F-0552), and enolase (62,000 Da; Sigma Chemical, Type III, Lot 27B-7890) as well as Blue Dextran 2,000 (Pharmacia Fine Chemicals) were used to generate the standard curve. The K_{av} values were determined using the equation: $K_{av}=(V_e-V_o)/(V_t-V_o)$ where V_e=elution volume of the protein, V_o=elution volume for Blue Dextran 2,000, and V_t=total bed volume.

Determining the Mode of Attack

The method by which the DNase attacks the substrate was examined using agarose gel electrophoresis. Purified enzyme (0.75 mL) was added to 0.75 mL of substrate (salmon sperm DNA, USB Lot No. 126531, 10 μmol $MgSO_4$ in 0.1 M imidazole, pH 7.0) and incubated in a water bath set at 55 °C. The incubation time intervals were 0, 30, 60, and 90 min. The reaction

was stopped by adding 0.5 mL of a 2.0 mM EDTA solution to the reaction tubes followed by placing them in an iced bath.

The degradation products were resolved using 0.3 % agarose gels (DNA grade agarose, cat no. BP164-100, Fisher Scientific) in TAE buffer (40 mM Tris base, 40 mM acetic acid, 1 mM EDTA) at 100 V for 45 min. Digested substrate (20 μL) was loaded to the agarose gel wells. The bands were stained using EZ-Vision® Three Dye (Amresco, Code: N313-KT, Lot No. 0871C433) and were visualized at 302 nm with a transilluminator (Spectronics Corp., NY, USA) and photographed with a PowerShot G10 digital Canon camera equipped with a green filter lens. Relative fluorescence of digested DNA was determined using the NIH photo-analyzing program Image J. Average values of digested DNA fluorescence intensity were generated from triplicate gels.

Results and Discussion

Effect of Temperature on the Activity of the Purified DNase

The optimal temperature for enzyme activity was found to be 65 °C with little activity at 68 °C (Figs. 1 and 2) and no measurable activity at 70 °C (data not shown). Enzyme destruction assays illustrate the rate of enzyme destruction/denaturation at specific temperatures. Based on the correlation between temperature and enzyme activity, 65 and 68 °C were chosen for the enzyme destruction assay. The first temperature, 65 °C, was chosen because it was the highest temperature without any apparent decrease in activity of the course of the assay. The higher of the two temperatures, 68 °C, was chosen because the enzyme only exhibited activity for the first 20–30 min. After the first 30 min, no activity was detected, suggesting that the majority, if not all, of the enzyme had been destroyed.

No destruction of the purified DNase was detected at 65 °C during 30 min, which was expected since the optimum temperature for the DNase was determined to be

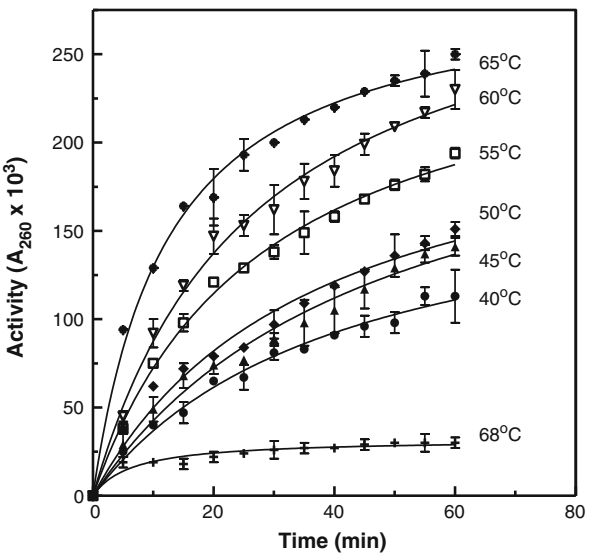

Fig. 1 The effect of temperature on the activity of the purified DNase. The assay used for this experiment was the real-time spectrophotometric assay which was performed in triplicate

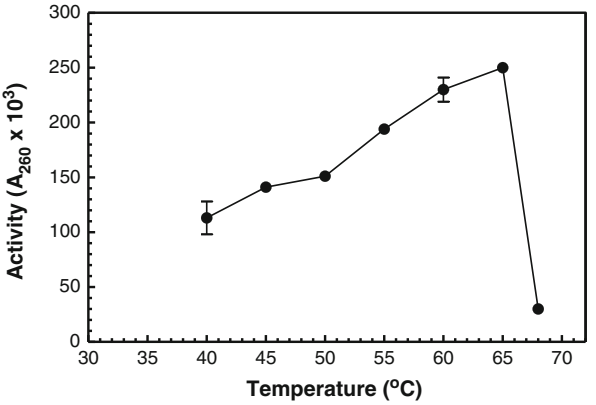

Fig. 2 The effect of temperature on the activity of the purified DNase plotted as activity vs. temperature. Data points represent the amount of activity after a 60-min digestion time using the real-time spectrophotometric assay. All assays were performed in triplicate

65 °C (Fig. 3). However, at 68 °C, a biphasic destruction plot was observed, which may reflect the presence of enzymatic subunits. The biphasic nature of the plot suggests that one subunit or a particular part of the enzyme may have been more susceptible than another to destruction temperatures.

Effect of pH on the Activity of the Purified DNase

The pH profile for the purified DNase was determined using the acid soluble assay with a pH-adjusted substrate. The digestion time for the acid soluble assay was 60 min throughout the pH range. A buffer system comprised of 0.1 M imidazole, malic acid, and glycine was used to provide a substrate that could buffer throughout the desired pH range. With this buffering system, a continual plot of the response to various pH values indicates that the optimal pH for the activity was pH 6.0 with no activity at pH 2.0 or 10.0 (Fig. 4). A close examination of the pH profile for the activity indicates that the majority of the enzyme activity occurs at pH values below a neutral pH, indicating that the enzyme is an acidic DNase.

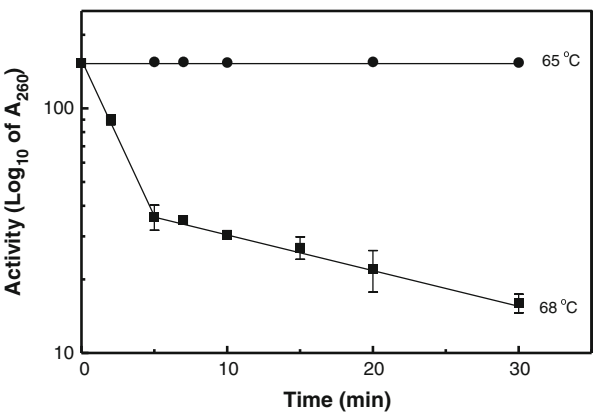

Fig. 3 Destruction plot of the purified DNase at 68 °C. The assay used for this experiment was a modified version of the acid soluble assay which is explained in detail in the "Materials and Methods" section

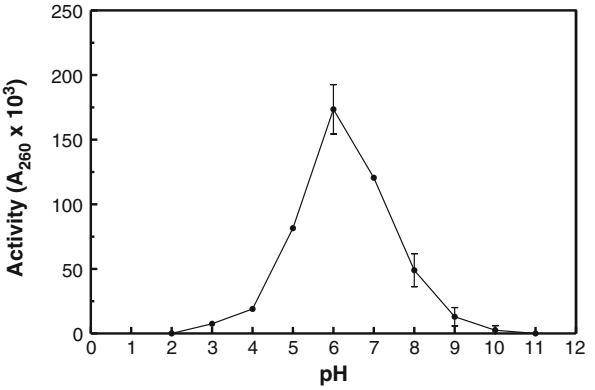

Fig. 4 Plot of the purified DNase pH profile in regard to activity. The activity was measured using the acid soluble assay at 55 °C with varying pH levels

Effect of EDTA on Purified DNase Activity

EDTA at various concentrations (0, 0.5, 1.0, 1.5, and 2.0 mM) was added to the substrate to determine the inhibitory effect of removing the cofactor. Results indicated that 1 and 2 mM EDTA/mL reduced the activity by 51 and 98 % respectively, showing that the DNase was dependent on metal cofactors (Fig. 5). These findings are typical among nucleases since activity is often dependent on the presence of metal cofactors.

Substrate Specificity of the DNase

The specificity of the DNase towards single-stranded (ssDNA) or native DNA (double-stranded DNA (dsDNA)) was assessed using the acid soluble assay with a 60-min digestion time at 55 °C. To make ssDNA, the substrate (fish sperm DNA, USB Lot No. 126531, 10 μmol $MgSO_4$ in 0.1 M imidazole, pH 7) was boiled in a microwave for 30 s, rapidly cooled in an ice bath to 55 °C, and then placed in a water bath set to 55 °C. The DNase demonstrated a significant difference in substrate preference, with a 121 % higher specificity for ssDNA than native DNA (dsDNA) (Fig. 6). The difference in enzyme activity between substrates can be explained. With double-stranded nucleic acids, nucleases can only approach from the outside where external nucleophiles

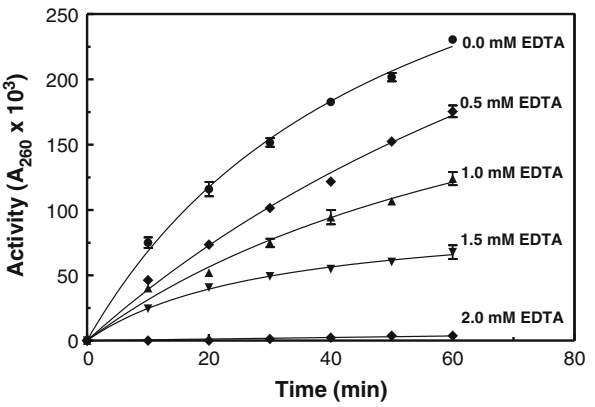

Fig. 5 Effect of EDTA on DNase activity. Results were obtained using the real-time spectrophotometric assay at 55 °C with the designated concentration of EDTA added to the substrate

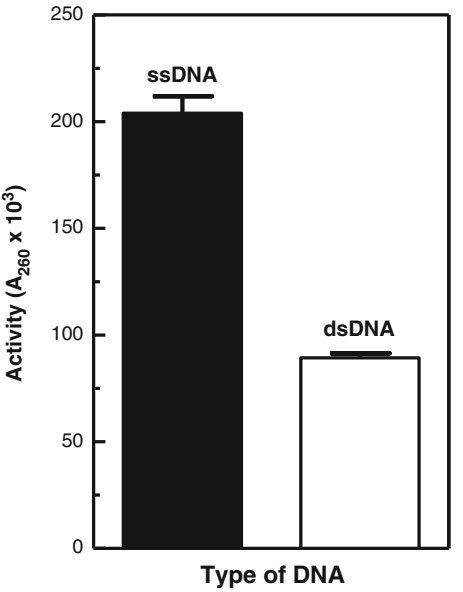

Fig. 6 Difference in DNase specificity towards ssDNA and dsDNA. The activity was determined using the acid soluble assay at 55 °C with a 60-min digestion time

are limited [19]. Single-stranded nucleic acids are much easier for nucleases, especially exonucleases, to attack because bases are not stacked allowing access to the nucleophiles [19].

Molecular Weight Estimation of the Purified DNase

The molecular weight of the purified DNase was estimated using a column of Sephadex G-75. The calculated K_{av} values were plotted against the log molecular weight of the standard proteins. Plotting the K_{av} value for the purified DNase resulted in a log molecular weight of 4.75 which is roughly equal to a molecular weight of 56,000 Da (Fig. 7).

Mode of Attack of the DNase

The mode of attack (endo- or exo-activity) of a nuclease can be determined using the acid soluble assay with varying digestion times and resolving the digested products via

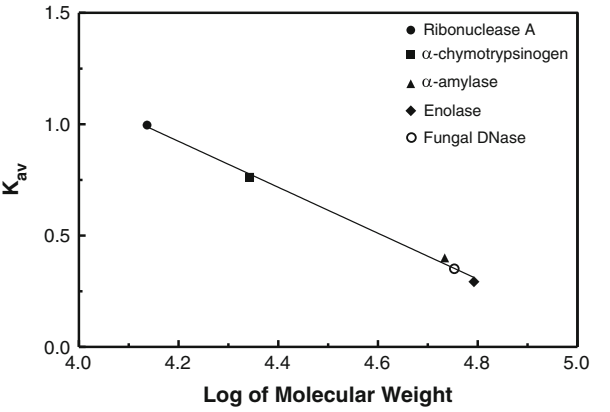

Fig. 7 Molecular weight estimation of the purified DNase. Molecular weights of the protein standards were the following: ribonuclease A, 13,700; α-chymotrypsinogen, 25,000; α-amylase, 51,000; and enolase, 82,000. The log molecular weight of the DNase was determined to be 4.753. This is roughly equal to a molecular weight of 56 kDa

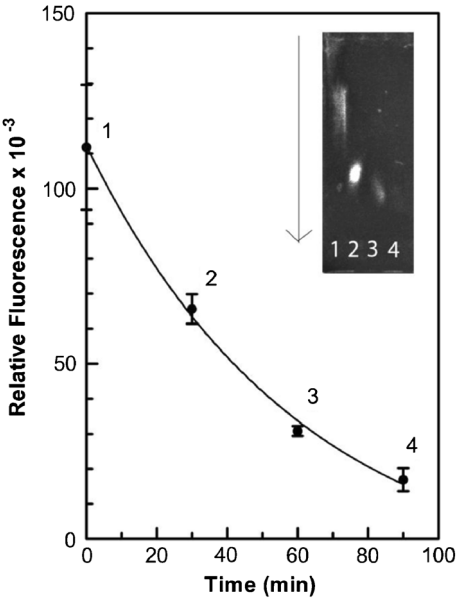

Fig. 8 Determining the method in which the DNase attacked the substrate by plotting the decrease in relative fluorescence over time. Each *point* can be referenced to the corresponding *numbered lane* presented in a typical agarose gel image of digested substrate with lanes: *1* undigested substrate, *2* 30-min digestion, *3* 60-min digestion, *4* 90-min digestion. Relative fluorescence from three agarose gels was used to generate the plot

agar gel electrophoresis. The migration of salmon sperm DNA through an agarose gel is limited due to its large molecular weight. The majority of the undigested substrate would stay in the well, and any smaller fragments would be visualized as a smear (Fig. 8).

By resolving substrate that has been digested for various time intervals, the method of hydrolysis can be determined. As a nuclease hydrolyzes DNA, it can result in the formation of either smaller DNA fragments (endonuclease) or mono and/or dinucleotides (exonucleases), the latter of which cannot be visualized. An endonuclease would give rise to the visualization of multiple DNA bands of varying sizes with an inverse relationship between the number of bands and size of the DNA fragments. Exonucleases, on the other hand, cleave off one or two nucleotides at a time, slowly decreasing the molecular weight of the substrate over time. This would result in the presence of one band or spot that migrates down the gel as digestion

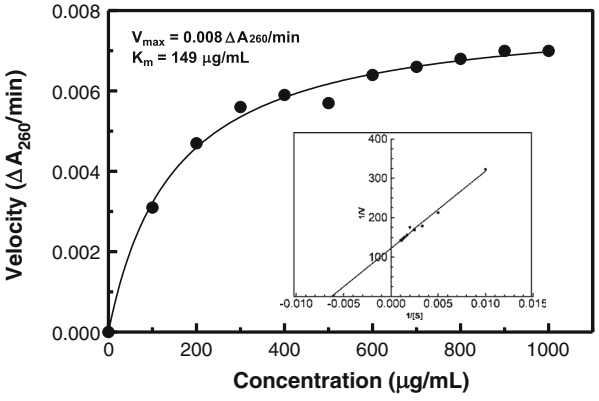

Fig. 9 Michaelis-Menten and Lineweaver-Burk plots of DNase activity. The K_m value was estimated to be 149 μg/mL with a V_{max} of 0.008. The real-time spectrophotometric assay at 55 °C with varying DNA substrate concentrations was used to determine the velocities. The data was then converted to make the Lineweaver-Burk plot

time increases. The observed band intensity, in both cases, would decrease in respect to digestion time. As more of the DNA substrate is hydrolyzed into mono and/or dinucleotides, band UV fluorescence decreases because these is less stainable material present in the gel.

The DNase exhibited exonuclease activity based on the single point progression of the digested substrate through the gel. A decrease in relative band fluorescence over the course of digestion occurred (Fig. 8). This can be explained by the loss of stainable DNA fragments due to nuclease hydrolysis. Following 90 min of digestion, the intensity of relative fluorescence decreased 85 %.

DNase K_m and V_{max}

The Km and Vmax for the purified DNase was estimated by plotting the velocities against the substrate concentration (Fig. 9). The velocities for each concentration were determined using the real-time spectrophotometric assay at 55 °C. The DNase was estimated to have a K_m value of 149 μg/mL and a V_{max} value of 0.008. It is to be understood that for an exonuclease, the precise K_m will vary with the size of the DNA substrate. Therefore, the presented K_m value is an approximate value based on the presented experimental conditions.

Conclusions

In sum, a novel thermophilic DNase previously purified was characterized in terms of optimal conditions, molecular weight, and certain kinetic properties. It can be noted that the optimal and maximum temperatures of any thermophilic enzyme will in theory be higher than most of its mesophilic counterparts. For example, a DNase purified from *Aspergillus sojae* demonstrated no activity at or above 60 °C [16]. The same can be said for a DNase isolated from *Aspergillus oryzae* [1]. This trend is common for most bacterial and fungal nucleases; nevertheless, some thermotolerant organisms produce enzymes comparable to those of thermophilic ones.

One extensively studied fungal extracellular nuclease is nuclease S1. Nuclease S1 is a heat-stable, 32-kDa, endo-exonucleolytic glycoprotein originally isolated from a digestive enzyme preparation from *A. oryzae* [15]. The enzyme attacks both single-stranded DNA and RNA, yielding 5′-monophosphates and small amounts of dinucleotides [4, 15]. Double-stranded DNA is not hydrolyzed [15]. The nuclease is inhibited 50 % by 2 mM phosphate and shows Zn^{2+}-dependent activity with optimal activity at 1 mM concentrations [15, 4]. Nuclease S1 has thermal stability with strong nucleolytic activity at 65 °C [1]. S1 is inhibited by EDTA, with no activity reported at a 0.1 mM concentration [4]. Yet, S1 can be 70 % reactivated with Zn^{++} and Co^{++} ions but not at all with Mg^{++} and Ca^{++} [15].

The estimated molecular weight of the purified DNase (≈56 kDa) is relatively high compared to those of previously purified nucleases. For instance, an extracellular DNase purified from *A. sojae* was found to have a molecular weight of 15,600 Da [11]. Commonly studied DNases from nonfungal origins such as pancreatic (≈30 kDa) and hog (≈38 kDa) DNase are significantly smaller [2, 12].

The secretion of nucleases like S1 sheds light on the main biological role of these enzymes. These enzymes scavenge nucleosides and phosphate necessary for growth. Ultimately, S1 is like the nuclease I of many plant species, all of which have similar dependences, pH optima, molecular weights, and biological function [4].

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