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Nuclease Rsn from *Rhizopus stolonifer*: specificity and mode of action

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Abstract

Nuclease Rsn from *Rhizopus stolonifer* catalyzes the hydrolysis of ss- and dsDNA in a ratio of approximately 2:1. Time course of 3′ and 5′ terminal analysis of the hydrolytic products of ss- and dsDNA showed that nuclease Rsn does not show any strict base preference and cleaves DNA in a non-specific manner. Moreover, separation of the hydrolytic products of ss- and dsDNA in the presence of Mg^{2+}, Mn^{2+} or Co^{2+} showed the predominance of tetra-, tri-, and dinucleotides followed by mononucleotides, suggesting an endo mode of action.

Keywords: Nuclease Rsn; Base specificity; Endonuclease; *Rhizopus stolonifer*

Sugar non-specific endonucleases are widespread in distribution. Their ability to recognize different DNA structures has been exploited for the determination of nucleic acid structure [1]. The extracellular nuclease from *Rhizopus stolonifer* (nuclease Rsn) is a sugar non-specific multifunctional enzyme and catalyzes the hydrolysis of ssDNA, dsDNA, and RNA in a ratio of approximately 1:2:0.05 in presence of Mg^{2+}. The enzyme shows an obligate requirement of Mg^{2+}, Mn^{2+} or Co^{2+} for its activity. The major end products of DNA hydrolysis are oligonucleotides ending in 3′hydroxyl and 5′phosphoryl termini [2]. The present communication describes the detailed analysis of the hydrolytic products of DNA to determine the specificity and mode of action of nuclease Rsn to evaluate its potential applications.

Materials and methods

Snake venom phosphodiesterase, spleen phosphodiesterase, and 3′ and 5′mononucleotides (Sigma, USA), calf intestine alkaline phosphatase (Bangalore Genei, India), and HPLC grade acetonitrile (E. Merck, India) were used. High M_r DNA from buffalo liver was isolated according to Mehra and Ranjekar [3]. All other chemicals used were of analytical grade.

Enzyme purification. Cultivation of *R. stolonifer* and purification of nuclease Rsn were carried out as described earlier [2].

Enzyme assay. The DNase activity of nuclease Rsn was determined, at pH 7.0 and 37 °C, according to Rangarajan and Shankar [2]. One unit of DNase activity is defined as the amount of enzyme required to liberate 1 μmol of acid soluble nucleotides/min under the assay conditions.

Terminal base analysis. The total reaction mixture of 4 ml, containing 4 mg of either ss- or dsDNA in 30 mM Tris–HCl buffer, pH 7.0 (containing 2 mM Mg^{2+}), was incubated with 40 U nuclease Rsn at 37 °C. Aliquots of 500 μl were withdrawn at different time intervals and the reaction was terminated by heat treatment (75 °C, 15 min). DNA samples incubated in the absence of nuclease Rsn served as control. The control and nuclease Rsn treated samples were then incubated overnight with 0.2 U of calf intestine alkaline phosphatase, in 1 ml (total volume) of calf intestinal alkaline phosphatase buffer (10 mM Tris–HCl, pH 7.9, containing 50 mM NaCl, 1 mM DTT, and 10 mM Mg^{2+}), at 37 °C. After the incubation period, phosphatase was removed by extraction with an equal volume of chloroform: isoamylalcohol (24:1 v/v). The aqueous phase was collected and subjected to 3′ and 5′ terminal nucleoside analysis. 5′ deoxyribonucleotides (5′dCMP, 5′dTMP, 5′dGMP, and 5′dAMP) treated with calf intestinal alkaline phosphatase served as nucleoside standards.

3′ *termini.* An aliquot (500 μl) of the alkaline phosphatase treated sample was incubated overnight with 0.06 U spleen phosphodiesterase at 37 °C. The 3′ nucleosides obtained, on treatment with spleen phosphodiesterase, were then separated by HPLC (Waters model fitted with 515 HPLC pump) on a Symmetry C18 column (250 × 4.6 mm, 5 μm, Waters, USA). The mobile phase comprising of a discontinuous gradient of acetonitrile in 100 mM triethylammonium acetate, pH 6.2 (0% v/v for 3 min, 0–5% v/v for 5 min and continued at 5% v/v for 5 min, 5–10 % v/v for 5 min, and continued at 10% v/v for 5 min,

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10–20% v/v for 7 min followed by 20–100% v/v for 5 min) was used at 25 ± 1 °C and at a flow rate of 0.8 ml/min. Twenty microliters of the standard or the sample solution was injected onto the column and the nucleosides were detected, at 255 nm, using Waters 2487 Dual λ Absorbance Detector. The amount occupied under each peak was computed on the basis of the total area occupied by each peak of the standard and the sample. The nucleosides eluted in the order of dC, dG, dT, and dA with retention times of ca. 11.14, 11.86, 16.05, and 19.03 min, respectively (data not shown).

5′ *termini.* An aliquot (500 μl) of the digested sample, obtained after treatment with calf intestinal alkaline phosphatase, was incubated overnight with 0.06 U of snake venom phosphodiesterase at 37 °C. The nucleosides liberated from the 5′ termini were then analyzed as described above.

Determination of size of the hydrolytic products of DNA. The total reaction mixture of 1 ml containing 500 μg of either sonicated and heat denatured DNA or native DNA, in 30 mM Tris–HCl buffer, pH 7.0 (containing 2 mM of either Mg^{2+}, Mn^{2+} or Co^{2+}), was incubated with 5 U of purified nuclease Rsn at 37 °C for 1 h. Subsequently, 5 U of the enzyme was added at an interval of 1 h up to 3 h and incubated for 24 h. The products were then resolved by successive chromatography on DEAE-cellulose (carbonate) and DEAE-cellulose (chloride) as described by Tomlison and Tener [4]. The fractions were pooled, freed of residual ammonium carbonate, and lyophilized.

Determination of the fragment length. The lyophilized samples obtained from the above step were then reconstituted in 500 μl of calf intestinal alkaline phosphatase buffer (10 mM Tris–HCl buffer, pH 7.9, containing 50 mM NaCl, 1 mM DTT, and 10 mM Mg^{2+}). The amounts of terminal phosphate and total phosphate were then determined by incubating the samples (100 μl each) with 0.05 U of calf intestinal alkaline phosphatase alone and together with 0.05 U of snake venom phosphodiesterase, respectively, at 37 °C for 24 h followed by estimating the inorganic phosphate according to Chen et al. [5]. The ratio of total phosphate to terminal phosphate was then determined to assign the fragment length of the oligonucleotides.

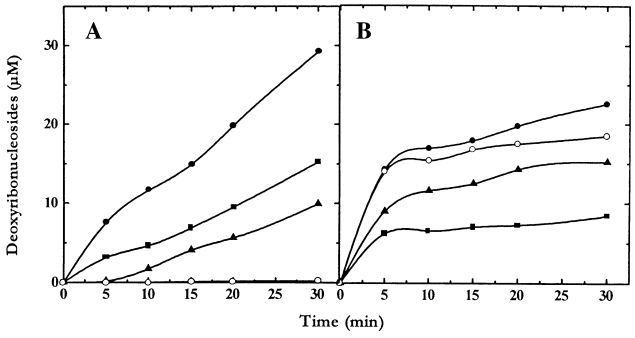

Fig. 1. Time course analysis of the 3′ and 5′ termini of the hydrolytic products of ssDNA. (A) 3′ termini: the control and nuclease Rsn treated samples were dephosphorylated with calf intestine alkaline phosphatase, treated with spleen phosphodiesterase, and the nucleosides released were then analyzed by HPLC. dT (●), dG (■), dC (▲), and dA (○). (B) 5′ termini: the control and nuclease Rsn treated samples were dephosphorylated with calf intestine alkaline phosphatase, treated with snake venom phosphodiesterase and the nucleosides released were then analyzed by HPLC. dT (●), dA (○), dC (▲), and dG (■). For details refer to Materials and methods.

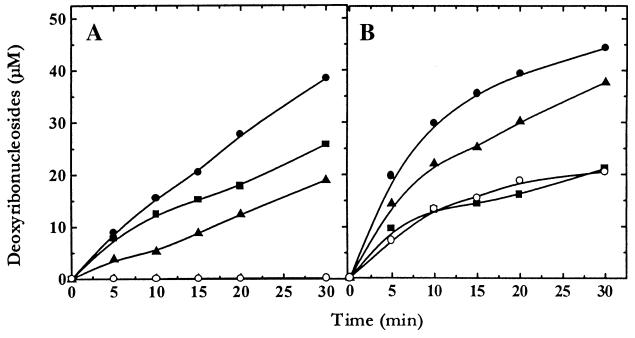

Fig. 2. Time course analysis of the 3′ and 5′ termini of the hydrolytic products of dsDNA. (A) 3′ termini: the control and nuclease Rsn treated dsDNA samples were dephosphorylated with calf intestine alkaline phosphatase, treated with spleen phosphodiesterase and the nucleosides released were then analyzed by HPLC. dT (●), dG (■), dC (▲), and dA (○). (B) 5′ termini: the control and nuclease Rsn treated dsDNA samples were dephosphorylated with calf intestinal alkaline phosphatase, treated with snake venom phosphodiestrease and the nucleosides liberated were analyzed by HPLC. dT (●), dA (○), dC (▲), and dG (■). For details refer to Materials and methods.

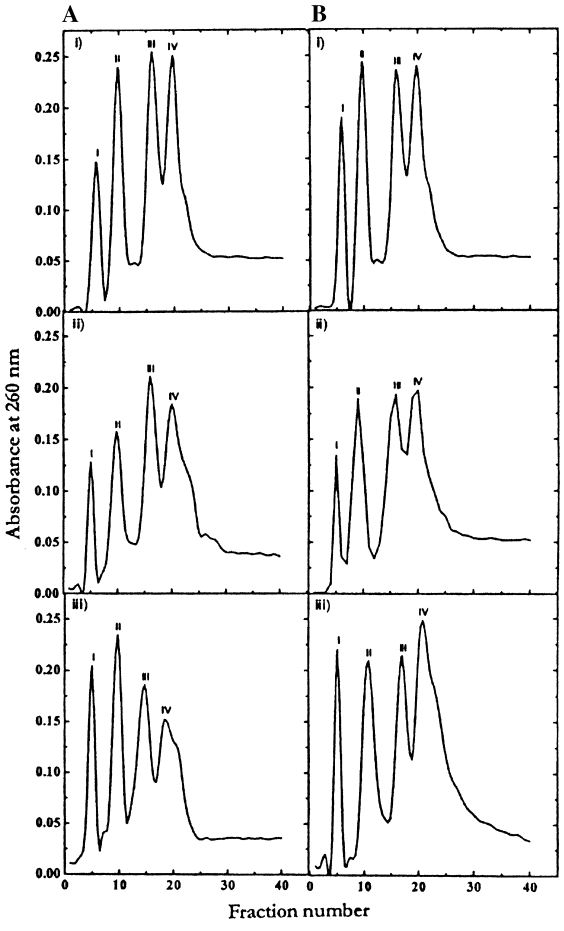

Fig. 3. Chromatographic profiles of the hydrolytic products of DNA. Nuclease Rsn digested DNA samples, in presence of Mg^{2+} (i), Mn^{2+} (ii), and Co^{2+} (iii), were resolved on DEAE-cellulose. The elution of the bound oligonucleotides was carried out with a linear gradient (0–1 M) of NaCl in 2.5 mM Tris–HCl buffer, pH 7.8, containing 7 M urea. ssDNA (A) and dsDNA (B).

Table 1
Analysis of the fragment size of DNA[a]

Sample	Peak I		Peak II		Peak III		Peak IV	
	(%)	Ratio[b]	(%)	Ratio[b]	(%)	Ratio[b]	(%)	Ratio[b]
Mg^{2+}								
ssDNA	11.0	1.25	23.0	2.44	28.0	2.82	38.0	3.80
dsDNA	11.0	1.20	22.0	1.80	1.0	3.17	36.0	3.89
Mn^{2+}								
ssDNA	7.0	1.30	20.0	1.80	28.0	2.86	45.0	3.78
dsDNA	7.0	1.25	22.0	1.80	31.0	3.17	36.0	4.39
Co^{2+}								
ssDNA	12.0	0.67	22.0	2.17	31.0	2.88	35.0	4.12
dsDNA	11.0	0.85	22.0	2.00	33.0	3.36	35.0	3.68

[a] Fragments obtained after DEAE-cellulose chromatography were analyzed for their terminal as well as total phosphate content as described under Materials and methods.
[b] Ratio of total phosphate to terminal phosphate concentration.

Results and discussion

The time course of the 3′ terminal nucleoside analysis, of the hydrolytic products of ssDNA, showed the presence of nucleosides in the order of $dT > dG > dC$ with very little, if any, of dA. From the initial stages of hydrolysis, the amount of deoxythymidine was higher than other bases (Fig. 1A), indicating that the enzyme prefers thymidylic acid linkages. Moreover, the time course of the 5′ terminal analysis revealed the presence of nucleosides in the order of $dT \cong dA > dC > dG$ (Fig. 1B). The presence of deoxythymidine and deoxyadenosine at the 5′ termini and deoxythymidine as the major product at the 3′ termini indicates the high preference of the enzyme for dTpdT and dTpdA type of linkages. The 3′ terminal base analysis of dsDNA too showed a similar trend, i.e., $dT > dG > dC$ with negligible amount of dA (Fig. 2A). However, the 5′ terminal analysis revealed the presence of nucleosides in the order of $dT > dC > dA \cong dG$ (Fig. 2B). In spite of the difference in the order of appearance of the nucleosides at the 5′ termini, the preference for dTpdT linkages remained unaltered. The 3′ and 5′ terminal analysis, of the hydrolytic products of ss- and dsDNA, indicates that nuclease Rsn does not show any strict base preference and cleaves DNA in a non-specific manner. Additionally, very low amounts of dA at the 3′ terminal of the hydrolytic products of both ss- and dsDNA suggest that dApdX bonds are resistant to cleavage. Like nuclease Rsn, non-specific cleavage of DNA has also been observed with endonucleases from *Serratia marcescens* [6] and yeast mitochondria [7].

Separation of the oligonucleotides, obtained after exhaustive digestion of ss- and dsDNA by nuclease Rsn, in presence of Mg^{2+}, Mn^{2+} or Co^{2+}, on DEAE-cellulose urea column gave four fractions (Fig. 3). Subsequent analysis of the individual fractions, for terminal as well as total phosphate, revealed that fractions I, II, III, and IV correspond to mono-, di-, tri-, and tetranucleotides. The relative percentages of the individual peaks showed the predominance of tetranucleotides (35–45%) and trinucleotides (28–33%) followed by dinucleotides (20–23%). The formation of low amounts of mononucleotides (7–12%) (Table 1), is consistent with our earlier observation on the exhaustive digestion of ss- and dsDNA by nuclease Rsn [2]. In this respect, nuclease Rsn is similar to nucleases from *S. marcescens* [6], yeast mitochondria [7], and *Aspergillus nidulans* [8] which produced di-, tri-, and tetranucleotides as the major end products of DNA hydrolysis with little (<3%) or no mononucleotides.

In conclusion, the present studies show that nuclease Rsn is a non-specific endonuclease. Since it is similar to pancreatic DNase with respect to metal ion requirement and mode of action, it can be used in conjunction with Mg^{2+} requiring enzymes like DNA polymerase, in nick translation reaction or techniques where limited digestion of DNA is required.

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