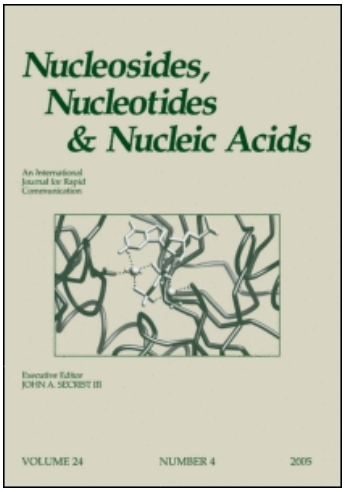

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AN EXTRACELLULLAR NUCLEASE FROM *BACILLUS FIRMUS* VKPACU-1: SPECIFICITY AND MODE OF ACTION

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□ *An extracellular nuclease from* Bacillus firmus *VKPACU-1 was multifunctional enzyme, this nuclease hydrolyzed poly U rapidly and more preferentially than the other homopolyribonucleotides. Hydrolysis of RNA this enzyme released mononucleotides in the order 5′ UMP > 5′ AMP > 5′ GMP where as in hydrolysis of DNA the mononucleotides in the order of 5′ dAMP > 5′ dGMP > 5′ dTMP and oligonucleotides. Uridylic linkages in RNA and adenylic linkages in DNA were preferentially cleaved by the nuclease. Nuclease produced oligonucleotides having only 3' hydroxyl and 5' phosphate termini. Present nuclease hydrolyzed RNA and DNA released oligonucleotides as major end products and mononucleotides, suggesting an endo mode of action.*

Keywords Extracellular nuclease; *Bacillus firmus*; multifunctional enzyme; endo mode of action

INTRODUCTION

Nucleases are ubiquitous phosphodiesterase enzymes that cleave phosphodiester bonds within nucleic acid molecules. Nucleases are extensive application in probes for the determination of nucleic acid structure.[1] Their ability to recognize and hydrolyze a wide variety of substrates with different modes of attack, they primarily cleave the internucleotide phosphodiester linkage. The end products of hydrolysis of DNA and RNA by single strand specific nucleases are oligonucleotides or mononucleotides with 5′ or 3′phosphoryl termini. Enzymes may attack either from the 3′ end or from the 5′ end of the nucleic acid molecule. Nucleases are classified based on the type of attack into three groups. Endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain, in contrast to exonucleases, which cleave phosphodiester bonds at the end of a polynucleotide chain. Restriction endonucleases cleave DNA at specific sites

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and are divided into three categories, type I, type II, and type III according to their mechanism of action. 5′ Nucleotides have been widely used in pharmaceutical and food industries.[2] They can be used to synthesize the antivirus and anticancer medicaments.[3] The nucleotide derivates have important uses in the illness of human's central nervous system and circulatory system. 5′ Nucleotides are known to exhibit enhancing flavor properties in food. When 5′ phosphodiesterase is added during the production of bakers yeast, the enzyme hydrolyses the yeast RNA efficiently into 5′GMP. An interesting feature of 5′GMP is that it acts synergistically with monosodium glutamate and thus can largely replace monosodium glutamate in various food products.[3] This article describes the detailed analysis of the hydrolytic products of DNA and RNA to determine the specificity and mode of action.

MATERIALS AND METHODS

Cultivation and Purification of Extracellular Nuclease from Test Strain **Bacillus firmus** *VKPACU-1*

The *Bacillus firmus* VKPACU-1 was grown in the optimized medium at 35°C for 32 hours.[4] After the incubation, the cells were harvested by centrifugation at 8000 g for 30 minutes at 4°C. The cell free culture filtrate was precipitated using 80% ammonium sulfate. The resulting precipitate was centrifuged and the pellet was dissolved in 30 mM Tris–HCl (pH 7.0) and dialyzed against the same buffer. The dialyzed enzyme was used for further purification studies. In the present study, all the purification steps were carried out at 4°C.

Enzyme Assay

Determination of DNase Activity

The assay for DNase activity was carried out essentially according to Apte et al.[5]

Determination of RNase Activity

The RNase activity of the test isolate was determined by the method of Ho et al.[6]

Action of Nuclease on Homopolyribonucleotides and RNA[7]

The activity of the purified enzyme was tested against RNA and homopolyribonucleotides such as poly A, poly G, poly C, poly U, Poly(G).Poly(C), and Poly(A).Poly(U), at 0.25% (w/v) substrate concentration. The activity against these substrates was measured by estimating the acid soluble nucleotides spectrophotometrically at 260 nm as described earlier.

HPLC Analysis of the Reaction Products of Purified Nuclease[8]

Action on ssDNA and dsDNA. The total reaction mixture of 5 mL containing 2.5 mg of either sonicated and heat denatured DNA or native DNA, in 30 mM Tris-HCl buffer pH 7.0, was incubated with 5 U of purified enzyme at 37°C. Subsequently, 1 U of the enzyme was added at an interval of 1 hour up to 3 hours and incubated for 24 hours. Aliquots (1 mL) were removed after 24 hours and the reaction was terminated by the addition of 2 mL of chilled absolute ethanol. The mixture was left overnight at −20°C and the undigested DNA was removed by centrifugation (6000 ×g, 15 minutes). The supernatant was lyophilized, reconstituted in 100 μL of Milli Q water and subjected to HPLC.

Determination of Phosphoryl Termini of the Reaction Products

The hydrolytic products of DNA, obtained following the action of nuclease, were lyophilized, reconstituted in 100 μl of 30 mM Tris-HCl buffer pH 7.0 and incubated with either 0.05 U of snake venom phosphodiesterase or 0.1 U of spleen phosphodiesterase for 6 hours at 37°C. After the incubation period, the reaction was terminated by the addition of two volumes of chilled absolute ethanol. The samples were concentrated by lyophilization, reconstituted in Milli Q water and subjected to HPLC.

Separation of the Reaction Products

The resulted products of the reaction was separated by high performance liquid chromatography (HPLC; Agilent 1100 Hewlett Packard 1100 series) having Nucleosil C18 column. The mobile phase comprising of a linear gradient of acetonitrile, in 100 mM triethylammonium acetate pH 6.2 (0–15% v/v for 20 minutes followed by 15–100% v/v for 5 minutes), was used with a flow rate of 1.0 mL/min at 25 ± 1°C. An amount of 20 μL of the standard or the sample solution was injected onto the column and the nucleotides were detected, at 255 nm, using an Agilent 1100 Hewlett Packard 1100 HPLC-G1315A Diode Array Detector. The amount occupied by each peak was computed on the basis of total area occupied by each peak of the standard and sample. The nucleotides eluted in the order of 5′dCMP, 5′dTMP, 5′dGMP and 5′dAMP with retention times of 4.43, 10.33, 12.53, and 15.17, respectively.

Action on RNA[9]. The total reaction mixture of 2 mL containing 2.5 mg of RNA, in 200 mM Tris-HCl buffer pH 7.0, was incubated with 1 U of purified enzyme at 37°C. Subsequently, 1 U of the enzyme was added at an interval of 1 hour upto 3 hours and incubated for 24 hours. After the incubation period, an aliquot (1 mL) was removed and the reaction was terminated by the addition of 3 mL of chilled absolute ethanol. The mixture was left overnight at -20°C and the undigested RNA was removed by centrifugation

(6000 ×g, 15 minutes). The supernatant was lyophilized, reconstituted in 200 μL of Milli Q water and subjected to HPLC.

Determination of Phosphoryl Termini of the Reaction Products

The hydrolytic products of DNA, obtained following the action of nuclease, were lyophilized, reconstituted in 100 μl of 30 mM Tris-HCl buffer pH 7.0 and incubated with either 0.05 U of snake venom phosphodiesterase or 0.1 U of spleen phosphodiesterase for 6 hours at 37°C. After the incubation period, the reaction was terminated by the addition of two volumes of chilled absolute ethanol. The samples were concentrated by lyophilization, reconstituted in Milli Q water and subjected to HPLC.

Separation of the Reaction Products

HPLC (Agilent 1100 Hewlett Packard 1100 series) was carried out on a Symmetry C18 column (250 × 4.6 mm, 5 μm, Waters, USA) for the separation of the resulted reaction products. The mobile phase comprising of a linear gradient of acetonitrile in 100 mM triethylammonium acetate pH 6.2 (0–2.5% v/v for 20 minutes, 2.5–50% v/v for 10 minutes followed by 50–100% v/v for 5 minutes), was used at 25 ± 1°C at a flow rate of 0.8 mL/min. An amount of 10 μL of the standard or the sample solution was injected onto the column and the nucleotides were detected, at 254 nm, using Waters 2487 Dual Absorbance Detector. The amount occupied by each peak was computed on the basis of total area occupied by each peak of the standard and sample. The nucleotides eluted in the order of 5′CMP, 5′UMP, 5′GMP, and 5′AMP with retention times of 7.55, 9.16, 16.70, and 30.75 minutes, respectively.

RESULTS

Effect of Homopolyribonucleotides and RNA on the Activity of Purified Nuclease

The rate of hydrolysis of different polynucleotides such as RNA, Poly A, Poly G, Poly U, Poly C, Poly(G).Poly(C), and Poly(A).Poly(U) by purified nuclease was determined under standard assay conditions. The extracellular nuclease hydrolyzed poly U rapidly and more preferentially than the other substrates tested. RNA, poly A and poly G were cleaved at a lower rate which was approximately 48.2, 29.8, and 22.7% respectively when compared to the rate of hydrolysis recorded for poly U, Poly (A).Poly (U), and Poly (C) were cleaved with still lower rate of about for 12 and 15%, respectively, to the rate of hydrolysis of using poly U as substrate. Poly (G).Poly(C) was resistant to hydrolysis even after 60 minutes of incubation (Figure 1).

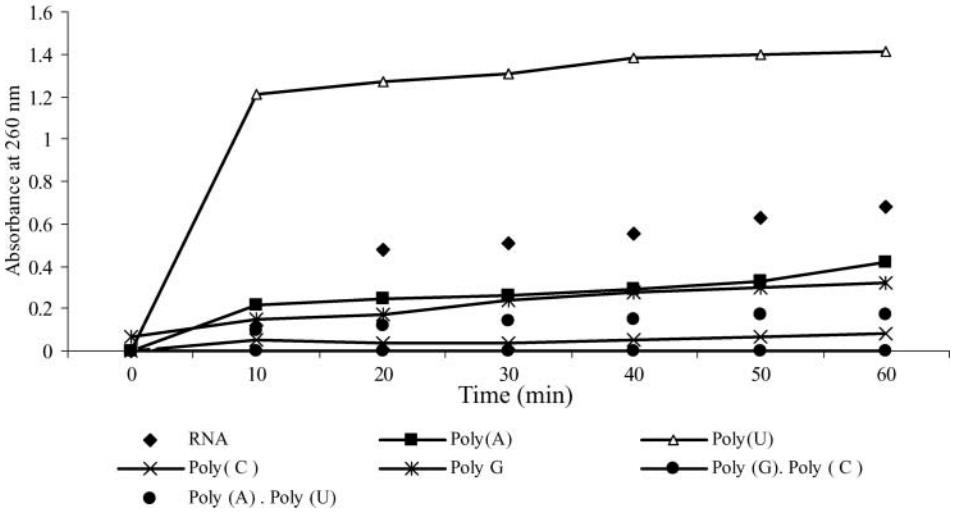

FIGURE 1 Effect of purified nuclease on homopolyribonucleotides and RNA.

Specificity and Mode of Action of the Purified Nuclease

HPLC analysis of the hydrolytic products of RNA showed that the nuclease liberated 5′ mononucleotides in the order of 5′UMP > 5′AMP >5′GMP. Interestingly, throughout the RNA hydrolysis, 5′CMP could not be detected in the hydrolyzed products. The HPLC analysis also showed the oligonucleotides as major end products which were further analyzed for determining the site specific activity of the purified nuclease by hydrolyzing with snake venom phosphodiesterase and spleen phosphodiesterase. HPLC analysis of the snake venom phosphodiesterase treated oligonucleotides showed high levels of 5′CMP. This observation coupled, with the inability of purified nuclease to hydrolyze 5′CMP revealed the resistance of cytidylic acid linkages to cleavage by the nuclease. Analysis of the hydrolytic products of RNA revealed the presence of high proportion of oligonucleotides than mononucleotides during the initial phase of hydrolysis. The presence of higher amount of 5′UMP than other nucleotides during initial stages of hydrolysis of RNA and the rapid hydrolysis of poly U indicated the preference of the enzyme to cleave uridylic acid linkages in the substrates (Figure 2a). On the other hand, during the hydrolysis of ssDNA and dsDNA the nuclease liberated 5′ mononucleotides in the order of 5′dAMP > 5′dGMP >5′dTMP. After treating the hydrolytic products with snake venom phosphodiesterase, the proportion of nucleotides eluted were in the order of 5′dCMP > 5′dTMP >5′dGMP and 5′dAMP. These results also indicate the resistance of cytidylic acid linkages to cleavage by the present nuclease. The presence of high proportion 5′dAMP than the other nucleotides recorded during the initial

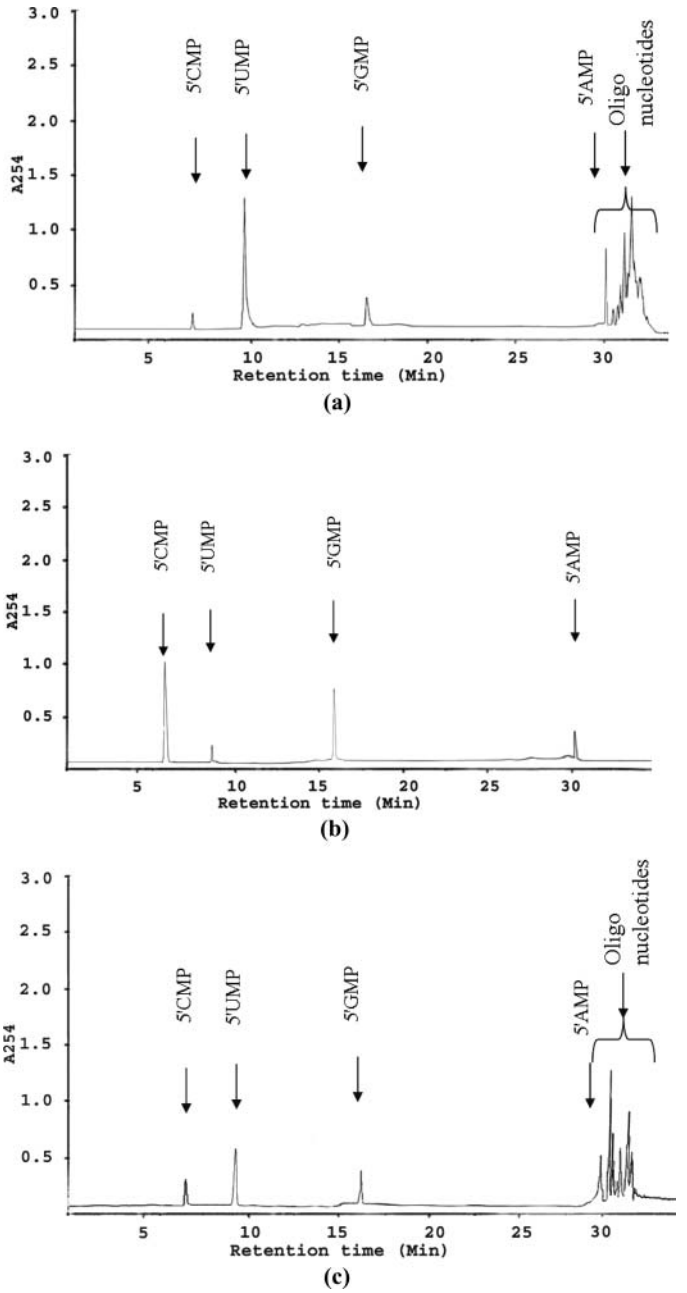

FIGURE 2 HPLC analysis of the hydrolytic products of RNA. a) Hydrolytic products of RNA treated with purified nuclease; b) oligonucleotides of RNA resulted from purified nuclease action treated with snake venom phosphodiesterase; c) oligonucleotides of RNA resulted from purified nuclease action treated with spleen phosphodiesterase.

stages of hydrolysis of ssDNA and dsDNA suggests that the nuclease preferred adenylic acid linkages in the DNA substrates for cleavage (Figures 3a and 4a).

The digestion of oligonucleotides with snake venom phosphodiesterase liberated more 5′ mononucleotides as exhibited by HPLC analysis of phosphodiesterase treated samples (Figures 2b, 3b, and 4b) from both DNA and RNA and, hence, it can be assumed that the enzyme performed the lysis action at 3' hydroxyl and 5′ phosphate termini. Further, the failure of spleen phosphodiesterase to digest the oligonucleotides resulted from the digestion of nuclease also suggest that the nuclease produced oligonucleotides having only 3' hydroxyl and 5′ phosphate termini as the spleen phosphodiesterase can act only on oligonucleotides having free 5′ OH termini.

DISCUSSION

The action of nuclease on homoribopolynucleotides and RNA was in the order of poly U> RNA > poly A whereas, poly A.poly U, poly C, and poly G were resistant to cleavage suggesting that the purified nuclease rapidly hydrolyze the poly U than the other homoribopolynucleotides. Similar to this observation, mung bean nuclease also showed higher activity on poly (U) than poly (A) at pH 5.0.[10] But the Bh1 nuclease hydrolyzed homoribopolynucleotides in the order of poly A > poly U > RNA > poly A >poly U whereas, poly C and poly G were resistant to cleavage.[11] But the RNase from *Bizonia sp* rapidly hydrolyzed poly U and RNA.[6] The low rate of hydrolysis of the double stranded polymer poly A. poly U suggested the preference of the enzyme for single-stranded nucleic acids, like the ribonuclease from *Basidiobolus haptosporus*.[12]

HPLC analysis of hydrolytic products of nuclease showed the oligonucleotides as the major end products of hydrolysis in addition to low amount of mononucleotides suggesting that the enzyme worked on endo mode of action. In addition to exo mode of action, the extracellular nucleases from wheat chloroplasts,[13] wheat chloroplast stromal protein,[14] rye germ ribosomes,[15] nucleoplasm of rye germ nuclei,[16] barley,[17] and yeast,[18] hydrolyzed ssDNA and RNA endonucleolytically. In contrast, nuclease from *Flammulina velutipes* exhibited endonucleolytic activity on ss and dsDNA while RNA and linear polynucleotides were degraded exonucleolytically.[19] Further, nuclease from *Serratia marcescens*,[20] BAL 31 nuclease,[21] as well as *Ustilago maydis* nuclease,[22] cleaved ssDNA endonucleolytically while they exhibited exonucleolytic action on dsDNA. However, the present nuclease differs from these enzymes in that it cleaved the substrates ssDNA, dsDNA, and RNA only endonucleolytically. The end products of hydrolysis of DNA and RNA by single strand specific nucleases are 5′or 3′ mononucleotides and/or oligonucleotides containing 5′ or 3′ phosphoryl termini. However,

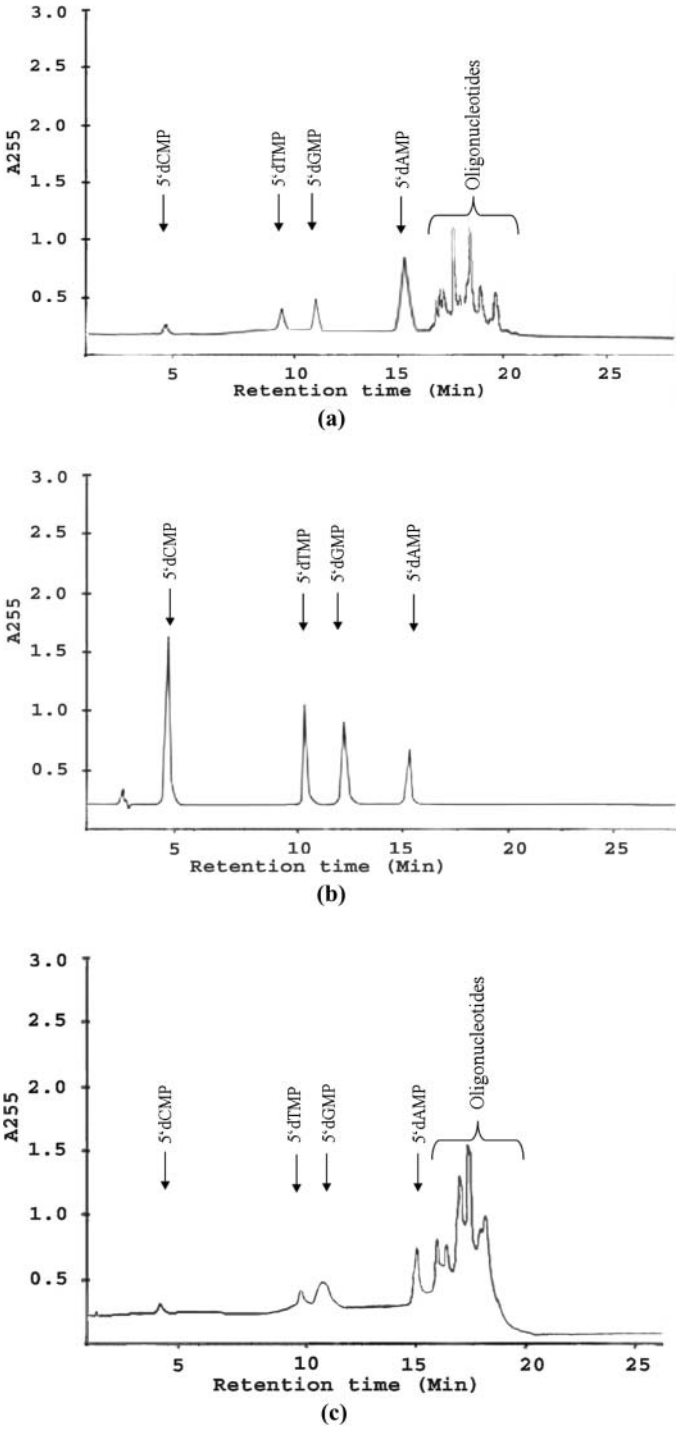

FIGURE 3 HPLC analysis of the hydrolytic products of ssDNA. a) Hydrolytic products of ssDNA treated with purified nuclease; b) oligonucleotides of ssDNA resulted from purified nuclease action treated with snake venom phosphodiesterase; c) oligonucleotides of ssDNA resulted from purified nuclease action treated with spleen phosphodiesterase.

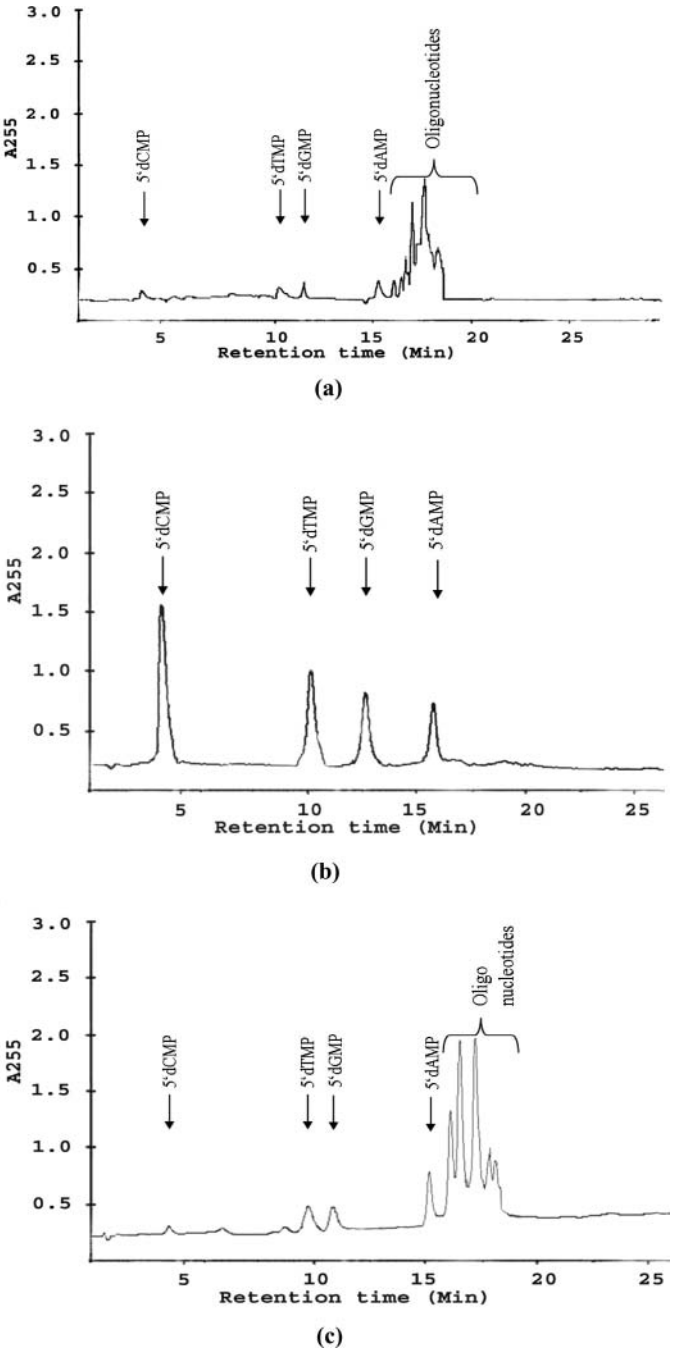

FIGURE 4 HPLC analysis of the hydrolytic products of dsDNA. a) Hydrolytic products of dsDNA treated with purified nuclease; b) oligonucleotides of dsDNA resulted from purified nuclease action treated with snake venom phosphodiesterase; c) oligonucleotides of dsDNA resulted from purified nuclease action treated with spleen phosphodiesterase.

the same enzyme does not produce both 5′ and 3′ phosphorylated end products.

The production of oligonucleotides, having 3′ hydroxyl and 5′-phosphate termini as the major end products of DNA and RNA hydrolysis recorded for the present nuclease, suggests that the mode of action of nuclease is similar to S1,[23] P1,[24] *N. crassa*,[25] Bh1,[26] mung bean nuclease,[27] Wheat seedling,[28] nucleases and nuclease Rsn[8] all of which produced 5′ mononucleotides as the end products of DNA and RNA hydrolysis.

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