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Purification and Characterization of Extracellular Nuclease from Bacillus Firmus VKPACU-1

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Abstract: ***An extracellular nuclease from Bacillus firmus VKPACU-1 was purified by ammonium sulfate precipitation, phenyl-sepharose followed by gel filtration chromatography. The overall yield was 4.09%. Molecular weight of the purified enzyme was determined by SDS/PAGE, it was 17.1 kDa. The specific pH (6.5) and temperature (35°C) was optimized for the nuclease activity. Stability of the enzyme was pH 6.5 to 8.0 and temperature upto 40°C. Without NaCl concentration showed maximum nuclease activity. Enzyme was not affected by dithiothreitol, β-mercaptoethanol and PMSF. The enzyme was inhibited by Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+}, inorganic phosphate, pyrophosphate, Triton X-100, Tween-80 and metal chelator EDTA. It was highly stable to high concentrations of organic solvents and Urea, SDS and guanidine hydrochloride. This enzyme showed the substrate specificity is in the order of RNA, ssDNA and dsDNA. This may be the first report on the extracellular nuclease from B. firmus VKPACU1.***

Keywords: Purification, Characterization, Extracellular nuclease, Bacillus firmus

I. INTRODUCTION

Nucleases are important analytical enzymes and have extensive applications in the determination of nucleic acid structure. Nucleases are ubiquitous phosphodiesterase enzymes that cleave phosphodiester bonds within nucleic acid molecules. [3]. Nucleases are divided into two groups: sugar specific nucleases (deoxyribonucleases and ribonucleases) and sugar non-specific nucleases. The non-specific sugar nucleases were characterized by their ability to hydrolyze both DNA and RNA [26]. Sugar non-specific nucleases have been extensively used in molecular biology researches, the rapid sequencing of RNA, the removal of nucleic acids during protein purification and the use as antiviral agents [19]. They have also been implicated in recombination, repair, replication and also the restriction of invading pathogens by degrading the incoming nucleic acids [8, 35]. An important role of the extracellular nucleases is scavenging of nucleosides and phosphate for growth [28]. Nucleases have been isolated from a wide variety of sources, including microbes, plants and animals [15].

Nucleases are important analytical tools used widely in biomedical and biotechnological applications. Their application depends on the specificity and mode of action of a particular enzyme. Nucleases with novel and unusual properties may facilitate the development of advanced technologies in many areas of biotechnology and biomedicine. Kang et al. [23] introduced the concept in purification of other RNA or DNA binding proteins by applying RNase or DNase directly to the cell extracts for removing the potential contaminations with host nucleic acids.

Many of these enzymes are intracellular, but microbial enzymes are extracellular. Nucleases are one among the hydrolytic enzymes which are produced even in a commercial scale; the existing producers could not accomplish the present demand. The scientific community has dipped into the stress of discovering an organism which could be an able producer of needed bioproducts higher than the existing ones. As part of a search for new enzymes with properties and enzymatic specificities permitting their use as nucleic acid reagents, microorganisms capable of degrading nucleic acids were isolated from natural sources. In the present investigations, an extracellular nuclease producing Bacillus firmus was isolated from contaminated sites and the purification and characterization of the nuclease was carried out.

II. MATERIALS AND METHODS

A. Determination of DNase and RNase activity

The assay for DNase activity was carried out essentially according to Apte et al. [2]. The RNase activity of the test isolate was determined by the method of Ho et al. [18]

B. Protein determination

Protein concentration was measured by the method of Bradford [6] using BSA as the standard. During chromatographic purification, protein concentration was estimated by observing the absorbance at 280 nm.

C. Optimization, Extraction and purification of extracellular nuclease from test strain VKPACU1

Optimization, extraction and purification of extracellular nuclease from VKPACU1 were reported by Ashokkumar et al. [4].

D. Influence of pH, Temperature and NaCl on Activity and Stability of the Enzyme

In the present study, the assay mixture was incubated in various pH from 3.5 to 12.0 using different buffers used at a concentration of 30 mM to study the effect of pH on the activity of purified enzyme and the activity was determined. An aliquot of enzyme (50 µg) was incubated with substrates in respective buffers at 35°C for 1 h and nuclease activity was determined by the method described previously. To study the pH stability, the purified nuclease was pre-incubated in the above mentioned buffers for 1 h. The residual activity was measured by the standard assay method described previously. To study the effect of temperature on the activity of the purified enzyme, the reaction mixture containing 50 µg of purified enzyme and substrates in 30 mM Tris-HCl (pH 7.0) was incubated at different temperatures *i.e*., 4, 10, 20, 30, 40, 50 and 60°C for 1 h and the activity was determined as described earlier. To study the thermal stability of the enzyme at different temperatures, purified enzyme was pre-incubated at different temperatures ranging from 4 to 60°C for 1 h and then it was brought to 35°C, equilibrated for 10 min and added with the three substrates separately. The reaction was carried out at 35°C and residual activities were measured by the standard assay procedure described earlier. To study the effect of NaCl on the enzyme activity, the purified enzyme was incubated with substrate in 30 mM Tris-HCl (pH 7.0) containing different concentrations of NaCl ranging from 0 to 300 mM at 35°C for 1 h. After 1 h incubation, the enzyme activity was measured by the method specified previously.

E. Effect of Metal ions on Activity of Purified Enzyme

Purified nuclease was incubated with RNA, ssDNA and dsDNA substrates were separately in 30 mM Tris-HCl (pH 7.0) containing different metal ions, Mn^{2+}, Mg^{2+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+} at a final concentration of 2 mM for 1 h at 35°C and the residual activity was measured by standard assay described in earlier . The activity without metal ions was considered to be the control (100%).

F. Effect of thiol Reagents, Protein Denaturants and Detergents on Purified Enzyme

To investigate the effect of thiol reagents, protein denaturants and detergents on enzyme activity, thiol reagents β-mercaptoethanol and dithiothretiol at 1 to 10 mM concentration were added to the enzyme and substrate mixture in Tris-HCl buffer (30 mM; pH 7.0). The detergents (Triton X-100 and Tween 80) were added at a final concentration of 1 to 3% (v/v). The protein denaturants SDS (0.02 to 0.1 w/v), Urea (1 to 5 M) and Guanidine HCl (0.05 to 0.5 M) were also added separately to the reaction mixture and were incubated at 35°C for 1 h. The relative residual activity was measured for each of the agents by standard assay as described earlier.

G. Effect of Different Organic Solvents on Stability of Purified Enzyme

An aliquot (50 µg) of purified nuclease in 2 ml of Tris-HCl buffer (30 mM; pH 7.0) was mixed with different concentrations of organic solvents (ethylene glycol, chloroform, formamide, dimethyl sulfoxide with different concentration of 10 to 50% (v/v), Glyoxal at 5 to 20 mM, formaldehyde 2.5 to 10% and then added substrates. The reaction mixtures were incubated at 35°C for 1 h and the residual nuclease activity was quantified by standard procedure described earlier.

H. Effect of Enzyme Inhibitors on the Activity of Purified Nuclease

An aliquot of purified nuclease (50 µg) in 2 ml of Tris-HCl (30 mM; pH 7.0) was mixed with different concentrations of enzyme inhibitors such as Phenyl methyl sulfonyl fluoride (PMSF) at 2 and 5 mM and 10 mM and Ethylenediaminetetraaceticacid (EDTA) at 1 mM, incubated for 1 h and then added with substrates. The reaction mixtures were incubated at 35°C for 1 h and the residual nuclease activity was quantified by standard procedure described earlier.

I. Substrate Specificity of The Enzyme

The preference of the purified enzyme for different substrates was evaluated with various substrates like dsDNA, ssDNA and RNA. The activity against these substrates was measured as described previously.

J. N-Terminal Analysis

Purified enzyme (100 µg) was extensively dialyzed against Milli Q water and subjected to SDS-PAGE at pH 8.3 (10% (w/v)) according to Laemmli [24]. Subsequently, the protein was electroblotted onto a PVDF membrane, in 10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer pH 11.0 (containing 10 % (v/v) methanol), under a constant current of 250 mA for 40 min as described by LeGendre et al. [25]. After the electro-transfer, the PVDF membrane was washed several times with Milli Q

water and stained with Coomassie Brilliant Blue R-250. The N-terminal amino acid sequence was determined by subjecting the blot to Edman degradation on an automated protein sequencer (Applied BioSystems, USA).

K. MALDI TOF Mass Spectrometry

Microcrystalline matrix surfaces were made on the probe tips of the mass spectrophotometer according to the sample preparation procedure of Jimenez et al. [22]. Mass spectra were obtained on an Applied Biosystems 4700 Proteomics Analyzer (Life Technologies, USA) and subjected to database search by using MASCOT (http://www.matrixscience.com) and allowing up to 2 missed trypsin cleavage and monoisotopic mass tolerance of 1.2 Da. The calibrated peptide mass was searched with 200 ppm mass accuracy.

III. RESULTS

A. Enzyme purification And Molecular Weight Determination

Extracellular nuclease produced by the *B. firmus* VKPACU-1 was purified by ammonium sulphate precipitation, Phenyl Sepharose chromatography and Sephadex G-100 gel filtration chromatography. The active fractions resulting from gel filtration were pooled and checked for its purity on SDS-PAGE. The purified fraction showed a homogenized band in 10% SDS-PAGE. The molecular weight was calculated to be about 17.1 kDa in SDS-PAGE using standard molecular weight markers. Based on the above purification steps, the enzyme was purified to 16.71 fold with a final recovery of 4.09%. The specific activity of purified nuclease (RNase) was recorded to be 2089.13 U/mg protein [4].

B. Effect of Temperature on the Activity and Thermal Stability of the Purified Nuclease

The purified nuclease showed highest activity at 35°C with all the three substrates tested and the maximum activity of 2247 U/mg protein was recorded with RNA as the substrate, followed by 1327.5 U/mg protein with ssDNA and 687 U/mg protein with dsDNA. The nuclease activity was completely lost at 4 and 60°C (Fig. 1). The thermo stability of the purified nuclease was found to be an unaffected during incubation at 4 to 40°C for 1 h. Pre-incubation of purified enzyme at temperatures higher than 40°C resulted in reduction of activity. The residual activity was recorded as 48, 26 and 15% with RNA, ssDNA and dsDNA at 50°C respectively. Incubation at 60°C resulted in total loss of activity with all the three substrates tested (Fig.2).

C. Effect of pH on the activity and stability of the purified enzyme

The purified nuclease was active at a broad pH range of 4.5 - 11.5 and the maximum activity was recorded at pH 6.5 for all the three substrates tested. The purified enzyme preferred RNA as the best substrate with which it produced an activity of 2172 U/mg protein, followed by 1354 U/mg protein with ssDNA and 672 U/mg protein with dsDNA. Above and below of pH 6.5, the enzyme activity was decreased gradually (Fig. 3). The stability of the purified enzyme at various pH, the maximum residual activity of 100% was recorded both at pH 6.5 and 7.0. The dsDNA degrading ability of the nuclease remained stable at pH 6.5 and the ability to degrade ssDNA and RNA were found to be highly stable between pH 6.5 to 8.0. Over 30% residual activity was recorded at pH 10.0 while at pH 8.5 and 9.0 the enzyme retained over 40% residual nuclease (RNase) activity (Fig. 4).

D. Effect of sodium chloride concentration on the activity of purified nuclease

The purified nuclease showed maximum activity in the absence of NaCl and the hydrolyzing activity of nuclease was recorded to be very high with RNA, followed by ssDNA and the preference of the enzyme was very low with dsDNA. At 50 mM NaCl the enzyme retained 80% of its original activity with RNA while 66% and upto 50% of the maximum activity with ssDNA and dsDNA respectively. Further increase in NaCl concentration resulted in the loss of activity. (Table 1).

E. Effect of metal ions on the activity of purified enzyme

Among the metal ions tested in the present study, only two metal ions enhanced the nuclease activity. Mn^{2+} ions enhanced the residual activity it was recorded as 146 and 134% on ssDNA and RNA respectively. Fe^{2+} enhanced the RNA hydrolyzing activity about 8% while, only 2% residual activity was recorded with ssDNA, whereas 15% inhibition was observed with dsDNA as substrate. The dsDNase activity of purified nuclease was slightly inhibited (3% and 10%) in the presence Mn^{2+} and Fe^{2+} while other metals like Mg^{2+}, Ca^{2+}, Ba^{2+} and Zn^{2+} (60 % inhibition) and complete inhibition of activity was exhibited by Cu^{2+} and Co^{2+} (Fig. 5).

F. Effect of thiol reagents on the activity of purified nuclease

The thiol reagents exhibited mixed influence on the activity of the purified nuclease. No inhibitory activity was recorded at 1 mM concentration of dithiothretiol irrespective of the substrates (RNA, ssDNA and dsDNA) tested. Marginal inhibition of the enzyme

activity of about 1.3% was recorded with 2 mM concentration of DTT with RNA as the substrate. The inhibition was slightly higher for ssDNA (11.7%) and dsDNA (12.1%). The three activities of nuclease were completely inhibited (95%) by 10 mM concentration of DTT (Table 2). The other reducing agent β-mercaptoethanol was not inhibited at 1 and 2 mM concentration and a gradual decrease in activity was recorded with concentrations of above 2 mM and approximately 20% of the activity was retained at 10 mM concentration.

G. Effect of protein denaturants on the activity of purified nuclease

The nucleic acid hydrolyzing ability of the purified nuclease was analyzed with three protein denaturing agents such as Guanidine hydrochloride, Urea and SDS. RNA and ssDNase hydrolyzing activity of the nuclease showed high stability in presence of low concentrations of protein denaturants and retained 98, 95 and 92% and 95, 93 and 95% of its initial activity in presence of 0.05 mM guanidine hydrochloride, 1 M Urea and 0.02 % (w/v) SDS respectively. Among the three protein denaturing agents SDS exhibited high inhibitory effect on activity than the Urea and Guanidine HCl. The RNA and ssDNase hydrolyzing activity of the nuclease was severely inhibited at 0.08 and 0.1% level of SDS. Though guanidine HCl and Urea also exhibited inhibitory effect on RNA lysing activity of the nuclease, over 75.5 and 61% residual was recorded with 0.2M guanidine HCl and 5 M Urea respectively (Table 3).

The ssDNase activity of the purified enzyme also showed higher residual activity in the presence of Urea (Table 3). Nearly 93% activity retained in the presence of 1 M Urea which was maintained at 90, 78 and 68% in the presence of 2, 3 and 4 M concentration of Urea respectively. ssDNase activity of enzyme was recorded as 95, 90 and 87.6% with 0.05, 0.1 and 0.15 M of guanidine HCl respectively. The enzyme retaining a residual activity of 44.6% in the highest concentration tested (0.5 M).

The dsDNase activity of purified nuclease was slightly inhibited at lower concentrations of SDS, guanidine HCl and Urea; the activity was severely affected at higher concentrations. The enzyme showed 76.8% residual dsDNase activity at 0.04% SDS and 21% residual activity were recorded at 0.08% concentration. Over 75% residual activity was recorded in the presence of 3 M of Urea while more than 52% activity was retained at 5 M concentration. Though the enzyme exhibited 46% residual dsDNase activity in the presence of 0.05 M concentration, only 5% of the initial activity was retained with 0.5 M concentration of guanidine HCl (Table 3).

H. Effect of detergents on the activity of purified nuclease

The detergents namely Triton X-100 and Tween 80 showed significant inhibitory effect on the activity of purified nuclease. Triton X-100 at 1% concentration, about 20, 23 and 25% of ssDNA, dsDNA and RNA lysing activity of the purified nuclease was recorded and complete inhibition of enzyme activity was noticed at 3% concentration. At 1% concentration of Tween 80 retained 42.5, 37.7 and 45.6% of its ssDNA, dsDNA and RNA hydrolyzing activity respectively. About 30% ssDNA and RNA hydrolyzing activity and 25% dsDNA hydrolyzing activity was recorded in the presence of 2% Tween 80 which thus exhibited the stability of the purified nuclease in the presence of detergents (Table 4).

I. Effect of organic solvents on the activity of purified nuclease

The effect of organic solvent has been evaluated using several solvents such as Ethylene glycol, DMSO, Formamide, Chloroform, Glyoxal and Formaldehyde at different concentrations. Ethylene glycol, DMSO, Formamide and Chloroform at 10% concentration enhanced the RNA hydrolyzing by 5, 17, 22 and 53% respectively. Further, Chloroform at 20 and 30% also enhanced the RNA hydrolyzing activity about 136 and 125.5% respectively. RNase activity of the nuclease was marginally inhibited at 20% concentration of the above solvents and residual activity preserved was about 91.4, 97 and 97.4% with ethylene glycol, DMSO and formamide respectively. Significant amount of RNase activity was preserved even at 50% of these solvents. RNase activity was not seriously affected by Glyoxal at 5 and 10 mM concentrations and the enzyme retained over 90% of its activity. Formaldehyde though moderately inhibited the RNase activity of the enzyme, more than 60% and 45% of nuclease activity at 5% and 7.5% concentration of Formaldehyde respectively (Table 5).

The ssDNase activity of the nuclease was not affected by Ethylene glycol, DMSO, Formamide at 10% concentration and Glyoxal at 5 mM concentration and the enzyme retained 91, 97.94 and 98% of its initial activity respectively. The nuclease preserved over 60% of its activity at 30% concentration of Ethylene glycol and DMSO and it was over 80% in 30% Formamide (Table 5). At 10 mM Glyoxal, the enzyme retained over 89% of its activity and more than 54% of its activity at 15 mM concentration. The nuclease retained 78% of its initial activity with 2.5% Formaldehyde and the enzyme was capable of maintaining 59% of its activity at 5% while 42% ssDNase lysing activity was recorded in the presence of 7.5% Formaldehyde.

For dsDNA lysing activity, Ethylene glycol, DMSO, Formamide and Chloroform at 10% showed no significant effect since the nuclease retained 93, 95, 93 and 98% of its original activity at this concentration. With 5 mM Glyoxal the enzyme retained 97% of its activity and more than 52% of the dsDNase activity of nuclease was preserved in the presence of 15 mM concentration. Though

the enzyme exhibited 72% of its activity at 2.5%, more than 54% residual activity was recorded in the presence of 5% Formaldehyde (Table 5). The results thus showed the high stability of all the three types of activities of nuclease in the presence of solvents.

J. Effect of enzyme Inhibitors on the Activity of Purified Nuclease

The purified enzyme retained more than 90% of its initial activity even at 5 mM concentration of PMSF and over 84% of its activity was recorded with 10 mM concentration indicating the inability of serine residue inhibitor (Table 6). But the metal chelator EDTA completely inhibited the activity of the nuclease even at 1 mM concentration indicating the presence of metal ions in the active site of the enzyme.

K. Substrate Specificity of Purified Nuclease

The purified nuclease was tested against the various substrates such as RNA, ssDNA and dsDNA. The purified enzyme was also incubated with other substrates such as 3'AMP, 5'AMP, bis (p-nitrophenyl) phosphate and p-nitophenyl phosphate. The enzyme showed maximum activity of 2395 U/mg protein with RNA and 2077 U/mg protein with ssDNA (Table 7). The enzyme exhibited low preference towards dsDNA with which the nuclease exhibited an activity of only 652 U/mg protein. Not only the bis (*p*-nitrophenyl) phosphate and p-nitophenyl phosphate was resistant to hydrolysis, but the monomeric substrates such as 3'AMP and 5'AMP were also not hydrolysed by the purified nuclease.

L. N-Terminal Sequence Analysis of Purified Nuclease

The identity of the nuclease was studied further by analyzing the amino acid sequences in the N-terminal end of the protein. The N-terminal 17 amino acids of purified nuclease were IFKTDRFPVMWKLLDNK. BLAST-P analysis of the N-terminal sequence of the nuclease revealed highest homology (82%) with exonuclease from *Bacillus* sp (NCBI Accession B14905) and nuclease sbc CD subunit C from Lysinibacillus sphaericus which showed 76% homology. Further, N-terminal sequences of exonuclease from several strains of Bacillus cereus have also showed homology upto 64%. The N-terminal sequences prominently aligned with sequences of nucleases from majority of *Bacillus* sp and thus confirming the identity of the purified enzyme.

M. MALDI TOF analysis of purified Nuclease

The MALDI TOF MS MS/MS spectrum of the purified nuclease exhibited several short peptides along with the noises. These peptides varied in size from 7 to 23 aminoacids with their calculated masses ranging from 883.02 to 2776.91 daltons and the observed masses varied from 882.01 to 2775.90 daltons (Table 8). The data derived from protein blast of NCBI revealed that the amino acid sequences of nine peptides selected randomly had very close homology with exonuclease protein family of *Bacillus cereus* strain ATCC 10987. Thus the purified enzyme was confirmed to be an exonuclease of *Bacillus* origin with multiple functions.

IV. DISCUSSION

Nucleases are important analytical enzymes which have extensive applications in the determination of nucleic acid structure and in the preparation of mono and oligonucleotides [12]. In the present study the purification of an extracellular nuclease was carried out by the test strain grown in the optimized medium [5]. The enzyme was concentrated by ammonium sulphate precipitation and purified by hydrophobic and gel filtration chromatography [4].

Optimum temperature for the hydrolysis of ssDNA and RNA by the nuclease was 35˚C and similar observations were made for the nuclease from Anabaena nucleases [27] and yeast [13]. In contrast, nucleases from tea leaves exhibited very high optimum temperature (60 - 70°C) [21]. The purified nuclease showed high stability from temperatures 4 - 40°C for 1 h and higher temperatures like 50 and 60°C proved to be destructive. The single strand specific extracellular nuclease from Rhizopus stolonifer lost more than 50% of its residual activity when incubated at 37˚C for 1 h [32]. The uracil specific ribonuclease from a Bizionia species was shown to be active in the temperature ranging from 25-50˚C, retaining 60% activity and the enzyme preserved more than 50% activity after 1 h preincubation at 50˚C [34]. From this study, the nuclease is proved to be different from other nucleases showing hydrolyzing activity on both DNA and RNA and being stable even at 50˚C.

The optimum pH of a nuclease is an important property that determines the potential of the enzyme as an analytical and biotechnological tool. Single strand specific nucleases have pH optima ranging from 4 - 9. In this study, the nuclease showed maximum ssDNase activity at pH 7.0 and the activity was marginally reduced at pH 6.5 but dsDNase activity was maximum at pH 6.5. RNase from Pleurotus tuber regium and Pleurotus eryngii mushroom showed the optimum activity at pH 6.2 [29, 38]. In case of exo-deoxy ribonuclease from Streptomyces coelicolor recExoSc exonuclease was active in a broad pH from 6.5 to 10.5 with a

neutral maximum at pH 7.5 [7]. Nuclease from Neurospora crassa (mitochondria) also exhibited different pH optima for the hydrolysis of ssDNA (pH 6.5 - 7.5) and dsDNA (5.5 - 6.5) [11] and this observation strongly supported the present study. The purified enzyme from this study showed high stability between pH 6.5 and 8.0 (100%) and retained a significant amount of activity (50 - 65%) at pH 6.0 and 8.5 for the hydrolysis of ssDNA and RNA, but the dsDNA hydrolyzing activity was highly stable in pH 6.5 and over 80 and 65% residual activity was recorded when incubated at 6.0 and 7.0 for 1 h at 35°C. Thus the present nuclease differs from other nucleases in pH stability by showing 100% single strand hydrolyzing activity between 6.5 and 8.0 and the dsDNA hydrolyzing activity between pH 6.0 and 8.0. Similar observations were also made for an extracellular nuclease from Basidiobolus haptosporus (Bh1), which showed high stability and retained a significant amount (65%) of its activity between pH 6.0 and 9.0 for 12 h at 37°C [14].

Salt concentration is known to affect the activity of single strand specific nucleases. The present study showed that highest hydrolyzing activity on both DNA and RNA in the absence of sodium chloride**.** Addition of even 50 mM NaCl the 20, 40 and 50% of activity was reduced in RNA, ssDNA and dsDNA as a substrate. Similar observations have been made in the case of Uracil specific ribonuclease from Bizionia sp which strongly supported the present observation [34]. Further Desai and Shankar [14] also have reported the highest activity of Bh1 nucleases from Basidiobolus haptosporus. The high sensitivity of the dsDNase activity towards low salt concentrations can be correlated to the suppression of localized melting by electrostatic stabilization of the DNA, especially the stabilization of AT rich regions in dsDNA [37].

Metal ions play crucial roles in the structure and function of many biological molecules [33]. Most of the single strand specific nucleases are either metalloenzymes or metal requiring enzymes. In the present study Mn^{2+} and Fe^{2+} ions enhanced the ssDNA and RNA hydrolyzing activity**.** It is interesting to note that none of the other metal ions tested were able to show any enhancement of nuclease activity. The inhibition exerted by other metal ions was observed on all the three types of activity. Similarly 5-6 fold stimulation has been recorded in the dsDNase Stn β activity when 2 mM concentration of Mn^{2+} included in the assay mixture by Patil et al. [30] who have failed to record any effect by Mg^{2+}, $Ca^{2+}$and Co^{2+}. The present nuclease showed very high RNA hydrolyzing activity than the DNA hydrolysis. The presence of Mn^{2+} and Mg^{2+} was proved to be essential for both dsDNA and ssDNA hydrolyzing activity of C1 nuclease from Cunninghamella echinulata var. echinulata [18]. This observation indicated that the single stranded substrate hydrolyzing activity was highly favored in the presence of both Mn^{2+} and Fe^{2+}. Thus, Mn^{2+} and Fe^{2+} ions showed structural integrity and modulating the specificity for substrate structure enhanced the activity of the present nuclease particularly the single strand substrate hydrolyzing activity than the dsDNA hydrolyzing activity.

Dithiothreitol (DTT) and β-mercaptoethanol (thiol reagents at 2 mM) showed about 3-10% inhibition of ssDNA and RNA hydrolyzing activity of purified nuclease but the dsDNA hydrolyzing activity was inhibited up to 12% by DTT. But a single strand specific nuclease from Rhizopus stolonifer showed no effect with thiol reagents at 5 mM concentaration [32]. In contrast, the activity of nuclease Bh1 was severely inhibited by Dithiothreitol and β-mercaptoethanol at 1 mM concentration [14] and GBSV1-NSN nuclease was slightly reduced [36]. It was found that the nuclease activity in this study was not inhibited by thiol reagents DTT or β-mercaptoethanol, suggesting that thiol groups are not essential for the activity of the enzyme.

However all the three substrates activity was inhibited at 1% concentration of TritonX- 100 and Tween-80. But the activity of P1 nuclease from Penicillium citrinum was not affected by Tween -80 at 0.1 and 0.2% concentration [31]. In the present investigation, the nuclease showed over 20% and 45% residual activity against 1% Triton X-100 and Tween-80 and proved to be superior to P1 nuclease. But stimulation of nuclease activity by Triton X-100 and Tween-20 has been reported for nuclease NSN from bacteriophage GBSV1 [36]**.**

The nuclease of the present study showed high stability against protein denaturants and the nuclease retained over 90% of both the RNase and ssDNase activity at 0.02% SDS, 2 M Urea and 1 mM Guanidine HCl which points to the stability of hydrolyzing activity of the nuclease and dsDNase activity was inhibited severely by 0.05 M Guanidine HCl. The present nuclease which showed around 50% ssDNA hydrolyzing activity with 500 mM of Guanidine HCl was slightly inferior to Bh1 nuclease which retained around 80% of its original activity in the presence of same concentration of Guanidine HCl and retained <80% of its activity in the presence of 4 M urea [14], while the present nuclease retained 70% of its activity at this concentration due to the high stability against protein denaturants. However, the present nuclease can be very well applied in the protein based pharmaceutical formulation processes.

In the present study, RNase activity was recorded to be enhanced by many solvents including chloroform (10-30%), ethylene glycol, DMSO and formamide (all at 10%) but retained above 90% of residual ssDNase and dsDNase activity. However, nuclease Bh1 retained its full activity in 50% (v/v) formamide but was stable only in the presence of low concentrations (10%v/v) of dimethylformamide and dimethylsulfoxide [14]. The nucleases from Streptomyces glaucescens [1] and Streptomyces antibioticus [9] exhibited more than two-fold stimulation of their activity in the presence of dimethylsulfoxide. Though, S1 nuclease showed high stability in 2% formaldehyde [20]. The present nuclease was superior to S1 since being stable even in the presence of 5% formaldehyde. This observation is of importance as the enzyme can be used as a single strand specific nuclease to study DNA

structure in the presence of organic solvents. Due to the high stability (~90%) at 10 mM glyoxal, the nuclease could also be used to obtain thermal melting profiles in the presence of formamide [10]. Isolation of single strand specific nucleases exhibiting high stability in the presence of organic solvents had added a new dimension to these studies as they can be used as probes for the determination of the secondary structure of DNA in the presence of various organic solvents.

The purified nuclease showed high activity on RNA and ssDNA but very low activity on dsDNA suggesting that the nuclease strongly preferred single stranded substrates for its activity where as nuclease Stn β from Streptomyces thermonitrificans showed high activity on dsDNA and ssDNA but very low activity on RNA [30] and the nuclease Rsn preferred the ssDNA for its activity [16]. The inability of the enzyme to hydrolyse the bis (p-nitrophenyl) phosphate indicated that the enzyme is not a typical phosphodiesterase. More over the resistance of p-nitrophenyl phosphate to cleavage points towards the absence of nonspecific phosphatase activity by the present nuclease [14]. The incapability of the present nuclease to release phosphate from 3′ and 5′AMP proved the absence of phosphomonoesterase activity of the enzyme [32]. The present nuclease showed its uniqueness by differing with the nuclease S1, mung bean and tobacco nucleases which preferred ssDNA and 3′AMP and RNA [17].

The partial N-terminal sequences of the present enzyme showed homology of about 82% with the exonuclease from Bacillus sp (B14905). Further the present nuclease from Bacillus firmus VKPACU-1 also showed 64% similarity with the exonuclease of Bacillus cereus (03BB108) and thus confirmed that the present enzyme is an exonuclease from a species of Bacillus**.** The single strand specific nucleases from Lentinus sp, Aspergillus sp, Penicillum sp and Basidiobolus haptosporus have been reported to have tryptophan as the first residue in N-terminal region where as the present nuclease possess an isoleusine at the corresponding position in N- terminal region and infact almost all the residues differ in their position showing no homology with above nucleases.

MALDI TOF MS and MS/MS analysis of the present enzyme has authentically proved that the present nuclease secreted by Bacillus firmus VKPACU-1 has high homology with exonuclease from Bacillus cereus strain ATCC 10987. None of other sequences of protein in the database showed such a close homology with the present nuclease. As no information is available in the literature on the MALDI TOF MS and MS/MS analysis of nuclease, it is possible to state that this may be the first attempt on this line. MALDI TOF M and MS/MS analysis thus helped in the identity of the enzyme with acceptable authenticity.

The nuclease from this study differs from all other nucleases both in the amino acid sequences at the N-terminal region and catalytic activity. These differences were also reflected in the metal affinity where the above nucleases were shown to be activated in the presence of Mg^{2+} while the activity of present nuclease was enhanced only by Mn^{2+}.

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Fig. 1 Effect of temperature on the activity of purified nuclease

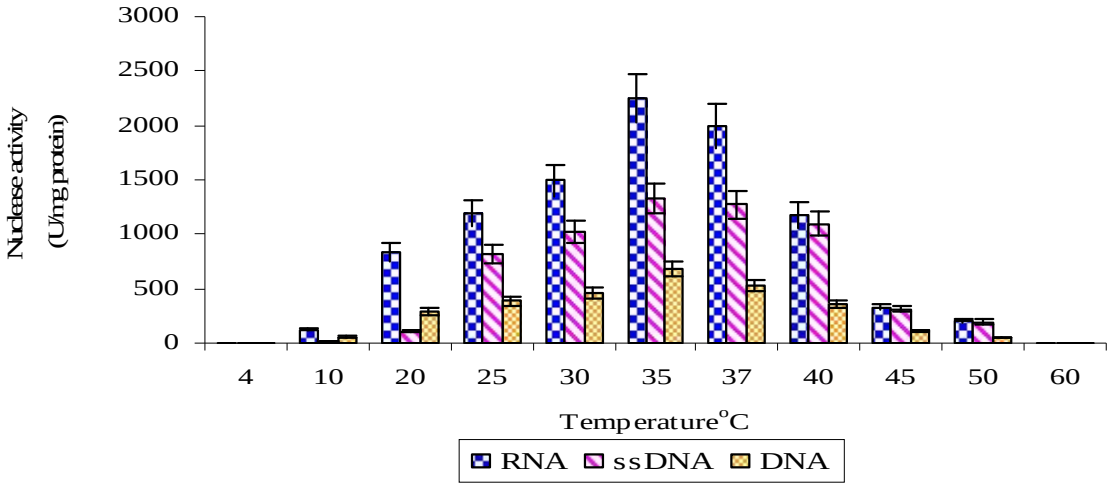


Fig. 2 Temperature stability of purified nuclease

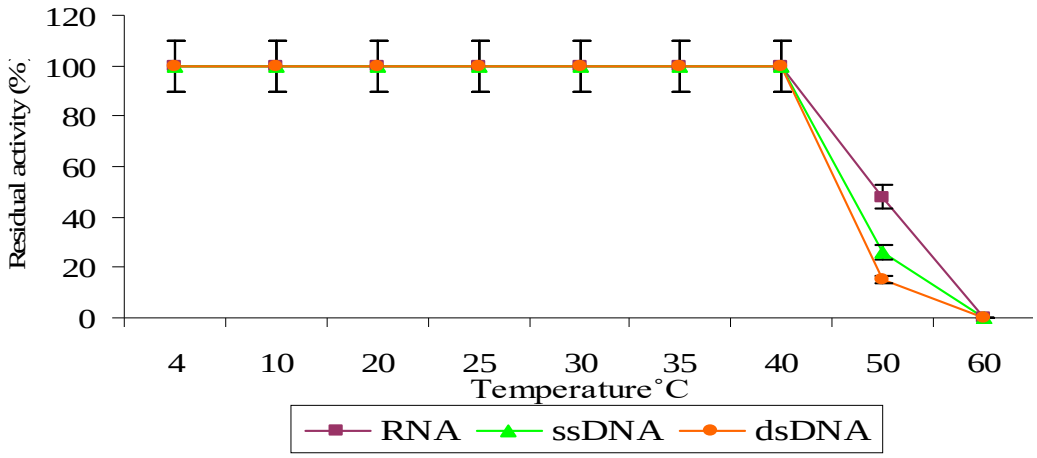


Fig. 3 Effect of pH on the activity of purified nuclease of *Bacillus firmus* VKPACU-1

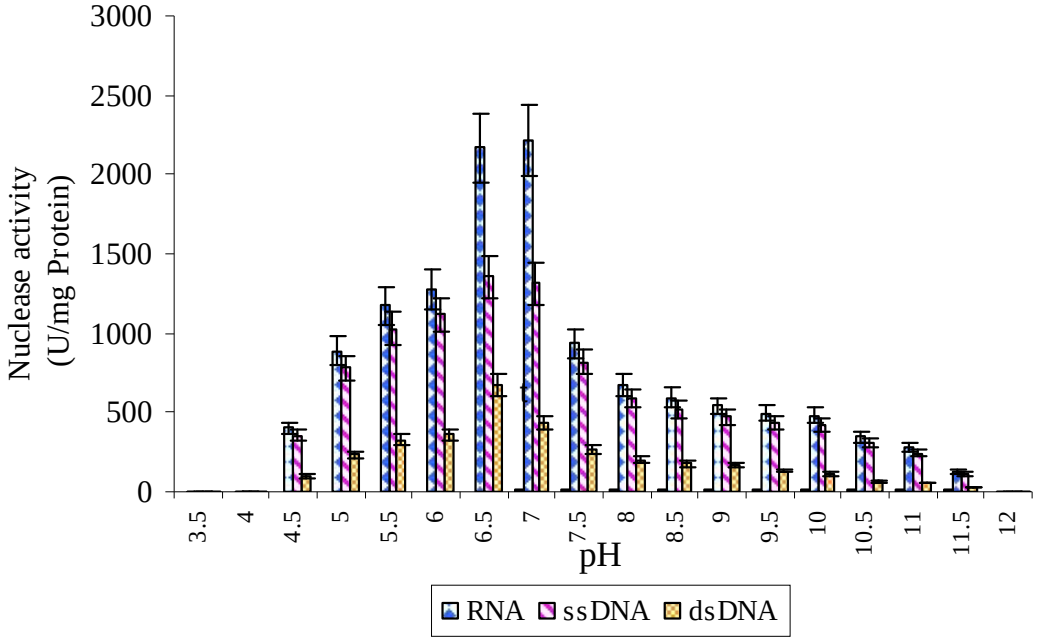


Fig. 4 Effect of pH on the stability of purified nuclease by *Bacillus firmus* VKPACU-1

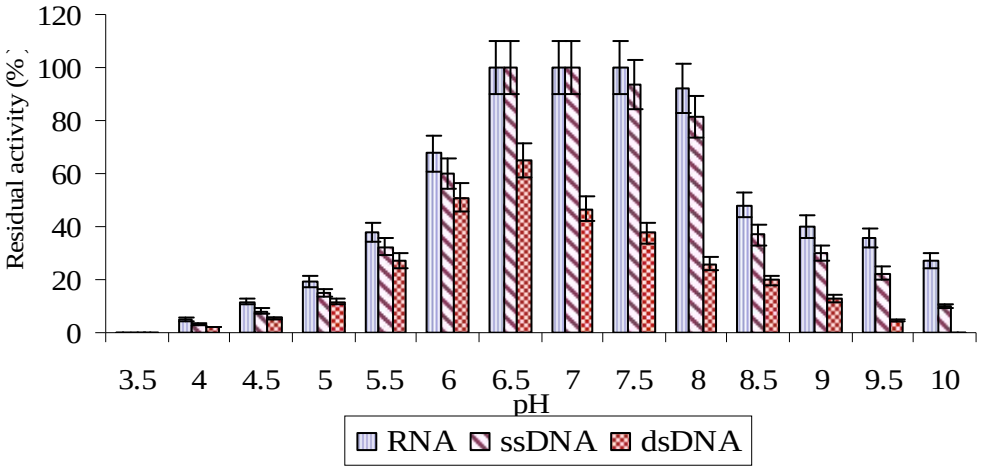


Fig. 5 Effect of metal ions on the activity of purified nuclease of *Bacillus firmus* VKPACU-1

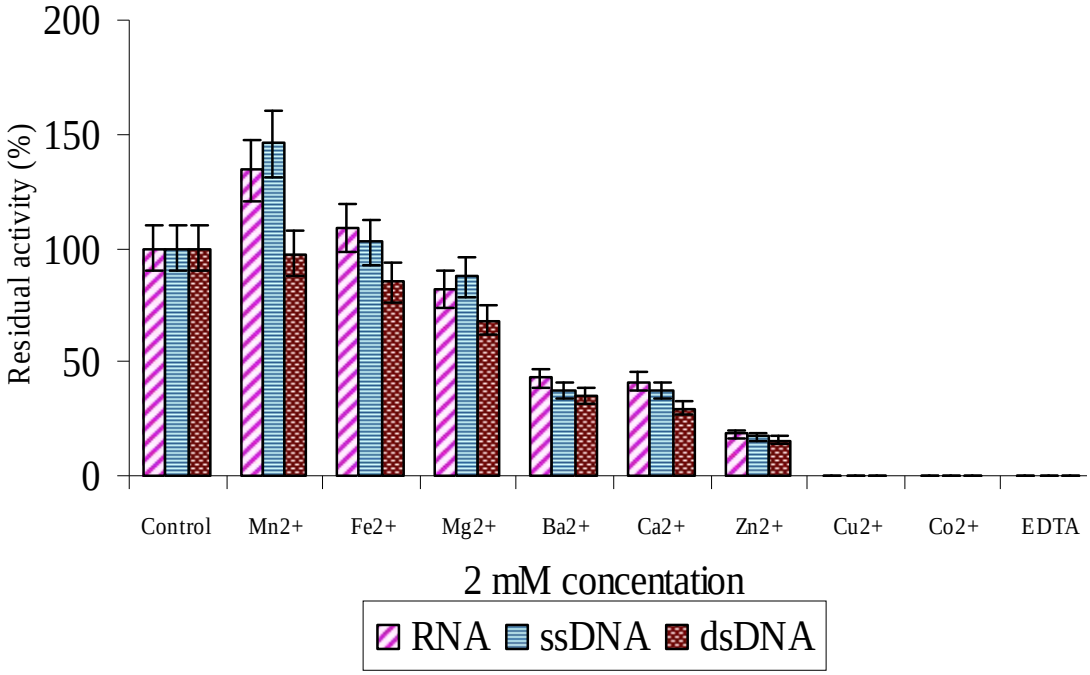


Table 1 Effect of NaCl concentration on the activity of purified nuclease

NaCl (mM)	Nuclease activity (U/mg protein)		
	RNA	ssDNA	dsDNA
0	2383 (100 %)	1285 (100 %)	694 (100 %)
50	1910 (80.15 %)	1191 (60 %)	389 (50.05%)
100	1062 (44.56 %)	912 (45.94 %)	329 (47.4 %)
150	711 (29.83 %)	493 (24.83 %)	205 (29.53 %)
200	494 (20.73 %)	364 (18.33 %)	128 (18.44 %)
250	400 (16.78 %)	209 (10.52 %)	102 (14.69 %)
300	216 (9.06 %)	125 (6.29 %)	0 (0%)

Values in parenthesis denote percent residual activity

Table 2 Effect of thiol reagents on the activity of purified nuclease

Reagent	Residual activity (%)		
DTT(mM)	RNA	ssDNA	dsDNA
0	100	100	100
1	100	100	100
2	98.7	89.3	87.9
4	91.3	83.3	78.3
6	81.6	77.5	70.5
8	37.8	32.5	35.8
10	7.2	5	5.8
β- mercaptoethanol (mM)	RNA	ssDNA	dsDNA
0	100	100	100
1	100	100	100
2	95.89	97.3	93.5
4	78.9	70.2	72.7
6	69	65.15	66.4
8	41.2	39.8	37.2
10	21.7	20.6	19.8

Table 3 Effect of protein denaturants on the activity of purified nuclease

Reagent	Residual nuclease activity (%)		
SDS (%)	RNA	ssDNA	dsDNA
0	100	100	100
0.02	92.5	95	93.5
0.04	78.91	75.8	76.85
0.06	45.8	43	41.5
0.08	23.6	20.68	21
0.1	13	9.6	11.5
Urea (M)	RNA	ssDNA	dsDNA
0	100	100	100
1	95	93.45	91.7
2	88.6	90	87.5
3	80.5	78.8	75.7
4	72.46	68.2	69.6
5	60.9	57.31	52.5
Guanidine HCl (M)	RNA	ssDNA	dsDNA
0	100	100	100
0.05	98	95	46
0.1	92	90	32
0.15	87.6	85.7	26
0.2	75.5	72.8	11.5
0.5	50.85	44.6	5

Table 4 Effect of different concentration of detergents on the activity of purified nuclease

Reagent	Residual nuclease activity (%)		
Triton X-100 (%)	RNA	ssDNA	dsDNA
0	100	100	100
1	25.8	20.2	23
2	9.28	8.9	7
3	0	0	0
Tween-80 (%)	RNA	ssDNA	dsDNA
0	100	100	100
1	45.6	42.5	37.7
2	30.2	29.7	24.5
3	5.9	4.8	3

Table 5 Effect of organic solvents on the activity of purified nuclease

Solvent	Residual nuclease activity (%)		
Ethylene glycol (%)	RNA	ssDNA	dsDNA
0	100	100	100
10	105	91.3	93.2
20	91.4	73.5	75.6
30	69.8	63	60.2
40	50	47.4	44.8
50	41.4	38.2	35.8
DMSO (%)	RNA	ssDNA	dsDNA
0	100	100	100
10	117	97.45	95.6
20	97	88.72	83.4
30	78	59.4	61.7
40	71	57.58	55
50	58.9	48.8	50.5
Formamide (%)	RNA	ssDNA	dsDNA
0	100	100	100
10	122	94.5	93.5
20	97.4	87.2	87.8
30	93.6	82.7	88.6
40	59	52.7	53
50	16.4	10	10
Chloroform (%)	RNA	ssDNA	dsDNA
0	100	100	100
10	153	123	98
20	136	116	95
30	125.5	103	88

40	89	77.2	76
50	33.5	25.8	23.2
Glyoxal (mM)	RNA	ssDNA	dsDNA
0	100	100	100
5	98	98.5	97
10	91	89.2	87
15	54	54.7	52
20	19	17	13
Formaldehyde (%)	RNA	ssDNA	dsDNA
0	100	100	100
2.5	75	78	72
5	61	59	54
7.5	45	42	39
10	24	21	19

Table 6 Effect of inhibitors on the activity of purified nuclease

Inhibitors	Residual activity		
	RNA	ssDNA	dsDNA
Control	100	100	100
EDTA (1 mM)	0	0	0
PMSF (1 mM)	98.93	96.21	97.38
PMSF (5 mM)	93.84	91.05	91.83
PMSF (10 mM)	92.39	89.15.	84.31

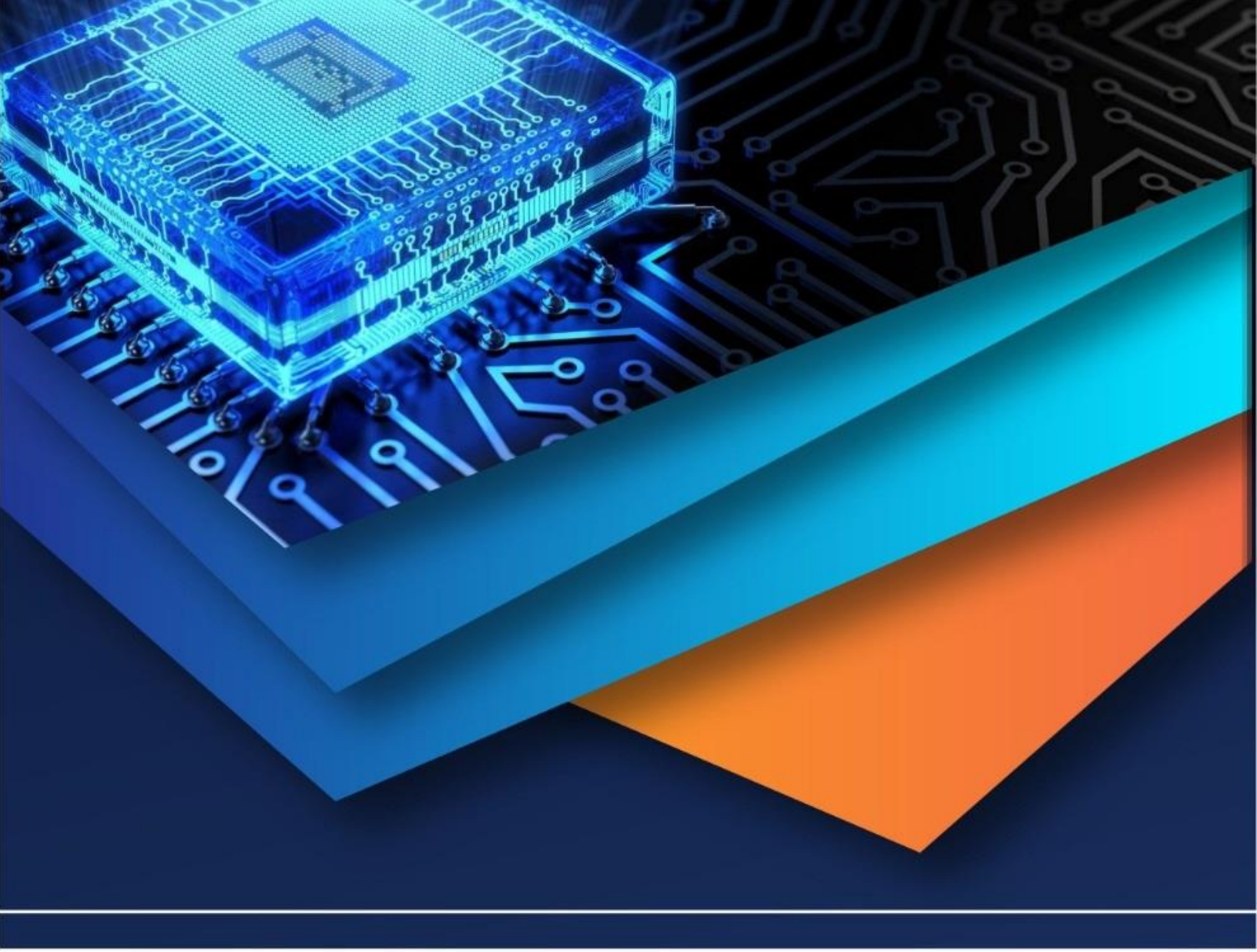
Table 7 Substrate specificity of the purified enzyme

Polymeric substrates	Nuclease activity (U/mg protein)
dsDNA	652.64
ssDNA	2077.41
RNA	2395
Monomeric substrates	
3'AMP	0
5'AMP	0
bis (p-nitrophenyl) phosphate	0
p-nitophenyl phosphate	0

Table 8 Mass and sequence similarity of the purified nuclease trypsin cleaved fragments to the known sequences in MASCOT database as observed by MALDI-TOF MS MS/MS analysis

Calc.Mass	Obsrv . Mass	± da[1]	± ppm[2]	Start Seq	End Seq	Sequence
883.0272	882.0197	-0.3959	-29	270	276	YLAEMEK
902.0610	901.0537	-0.4484	-30	77	83	FPQIIER
1224.3365	1223.3287	-0.2859	-29	190	200	HGELELVENGK
1444.5583	1443.5507	-0.1586	-34	21	34	SDDPSEIVDIGAVK
1456.6591	1455.6517	-0.1415	-37	163	176	ALADAENTANILLK
1501.7467	1500.7387	-0.0913	-1	42	55	VIGEFSELVKPGAR
1887.1653	1886.1577	0.1963	-34	84	99	FIQFIGEDSIFVTWGK
2047.4080	2046.4007	0.5176	-34	104	121	FLVHDCTLHGVDCPCMEK
2776.9120	2775.9047	0.6635	-40	220	242	NTERPFAWSTFESSDTWESITER

accuracy of the peptide mass observed
peptide mass accuracy in terms of C isotopes



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