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Molecular Cloning, Purification, and Characterization of an Extracellular Nuclease from *Aeromonas hydrophila* ATCC14715

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Abstracts A gene encoding an extracellular nuclease was cloned from *Aeromonas hydrophila* strain ATCC14715. The gene was overexpressed and the enzyme was purified by fusing to maltose binding protein. It was shown that the protein possessed DNase activity on both single-stranded and double-stranded DNAs. It exhibited both endo- and exonuclease activities. It was also shown that the protein had an RNase activity. Possible roles of this extracellular enzyme in the *A. hydrophila* life cycle are discussed.

Key words: *Aeromonas hydrophila*, extracellular nuclease, DNase, RNase

Aeromonas hydrophila is a facultatively anaerobic bacterium belonging to *Aeromonadaceae* [10]. This is an opportunistic pathogen in both cold and warm animals including humans. It produces toxins like enterotoxin [9] and hemolysin [16]. It is also known to secret amylase [8, 15], β-lactamase [23, 31], chitinase [28, 30], glycerophospholipid-cholesterol acyltransferase [19], lipase [4], serine protease [32], as well as DNase [6, 10, 11] extracellularly. Most of these enzymes are involved in the acquirement of nutrient carbon sources and some are involved in pathogenesis.

Extracellular DNase is of special interest in the degradation of nucleic acid and the subsequent production of phosphates. Accordingly, the degradation rate of nucleic acid and intracellular uptake rate of phosphates are influenced by the concentration of phosphate present in the extracellular environment [1, 2, 3, 5]. Its role in the transfer of genetic material across the cell membrane and the bacterial colonization in the small intestine have also been implicated [6].

Two different extracellular DNases, 25 kDa [7, 12] and 114 kDa [11], were identified from *A. hydrophila*. The 25 kDa DNase was also shown to be a sugar-nonspecific nuclease possessing RNase activity [13]. However, the biochemical characterization of its enzymatic activity using purified protein has never been reported. In this study, the 25 kDa nuclease from *A. hydrophila* was cloned, expressed, purified, and characterized.

A. hydrophila ATCC14715 strain was used for the cloning of 25 kDa nuclease. From the chromosomal DNA of this strain, the following two primers were used for PCR so as to obtain the open reading frame of the gene (but not the putative signal sequence [7]): forward-5'ATGCGATATC-GGATATTCATGTTTCGCCTGTGC-3', reverse-5'ATGC-GTCGACGGGGTTATTGGCACTGCTTTTCAATG3'. The underlined sequence denotes the *Xmn*I and *Sal*I restriction sites, respectively. The PCR product was digested by the two enzymes and ligated in pMAL-c2 vector (New England Labs) cleaved with the same two *E. coli* enzymes. The resulting plasmid was used to transform JM105 (*sbcB15*) which lacked exonuclease I activity.

A fresh 2-l culture of this strain was induced with IPTG at a final concentration of 0.1 mM and subjected to centrifugation to obtain 3 g of wet bacterial pellet. A crude extract was prepared by using a French press and the lysate was diluted 1:5 with buffer containing 10 mM Tris-Cl, pH 7.6, 1 mM EDTA, and 10 mM NaCl. The diluted crude extract was chromatographed on amylose resin and eluted with excess maltose. The eluate was dialyzed against a buffer containing 10 mM Tris-Cl, pH 7.6, 1 mM EDTA, and 10 mM NaCl. The purified protein was analyzed on SDS-PAGE [26, 27].

[^{3}H] Thymine-labeled phage T7 DNA was prepared according to the procedure of Joseph and Kolodner [18]. The specific activity of the labeled DNA was 11.2 cpm/pmol. Single-stranded DNA was produced by heat denaturation of duplex DNA at 100°C for 10 min and then promptly chilled on ice.

The plasmid DNA used for both endo- and exonuclease activity assays were prepared as follows. A 100-ml culture of *E. coli* harboring pIN101 was grown and the plasmid was isolated using the Midi-prep kit from Promega. The

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plasmid was subjected to EtBr-CsCl (0.5 g/ml) gradient ultracentrifugation in a Beckman Tla100.3 rotor at 40,000 rpm for 20 h. The resulting 3 bands corresponding to closed circular, open circular, and linear forms of plasmids were isolated using a syringe.

The DNase assay was performed in the following standard buffer: 50 mM Tris-Cl, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, and 1 mg/ml BSA. The purified nuclease and substrate DNA were added to the reaction mixture and incubated at 30°C for 1 h, and then the reaction was stopped with a solution containing 50 mM EDTA, 200 mM NaCl, and 1% SDS. One unit of enzyme released 1 nmol of nucleotides in 30 min at 30°C. The remaining substrate DNA was precipitated with 20% TCA, and the radioactivity was counted on a scintillation counter. RNA substrate for the RNase assay was prepared from *E. coli* using the total RNA isolation kit (RNeasy kit) from Qiagen. Formaldehyde gel electrophoresis was used to visualize the substrate RNA after treatment with the purified nuclease in the same conditions as applied to the DNase assay. The release of inorganic phosphate from salmon sperm DNA was measured using the phosphomolybdate complex method at 625 nm [29].

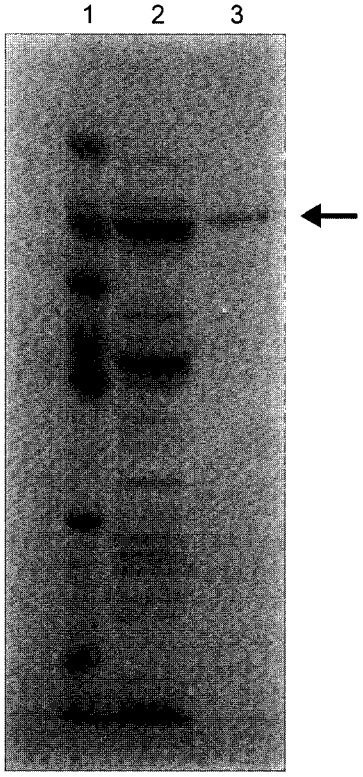

Fig. 1. SDS-PAGE analysis of the purified MBP-Nuc.
Lane 1: molecular weight marker (97, 66, 55, 43, 40, 31, 22 kDa from the top to the bottom); lane 2: crude extract of induced cell expressing MBP-Nuc; lane 3: affinity purified MBP-Nuc. The arrow indicates 65 kDa MBP-Nuc.

Table 1. Requirements for nuclease activity.

Condition	Relative activity (%)
Complete*	100
- Mg^{2+}	3
- bovine serum albumin	67
- dithiothreitol	78
+ 200 mM NaCl	23
+ 200 mM KCl	19
- Mg^{2+}+10 mM Mn^{2+}	85
- Mg^{2+}+10 mM Zn^{2+}	10
- Mg^{2+}+10 mM Cu^{2+}	35
- Mg^{2+}+10 mM Ca^{2+}	33

*Complete means standard assay condition. See text for details.

From PCR amplification with the two primers, a 0.6-kilo base pair fragment was obtained. After cloning of this fragment, it was named *nuc*, and then the whole gene sequence was analyzed. The open reading frame was composed of 684 nucleotides and encoded 228 amino acids (GenBank accession number AF004392). BLAST analysis of the amino acid sequence revealed 87% and 85% sequence homology with *A. hydrophila* Dns and DnsH, respectively [7, 12]. It was also shown to have sequence homology with *Vibrio cholerae* Dns (62%), *Vibrio vulnificus* Vvn (61%), *Escherichia coli* EndA (57%), suggesting a common evolutionary origin of these Gram-negative bacteria [14, 17, 34].

A 208 amino-acid-long mature portion of the protein (a 20 amino-acid putative leader sequence was deleted) was expressed as fused to the maltose binding protein (MBP) [25]. A low concentration of IPTG was used for induction, as this protein was toxic to the host *E. coli*. After affinity chromatography, purified MBP-Nuc was analyzed on a SDS-PAGE (Fig. 1). The purity was more than 97%, as determined by a densitometer. An attempt was made to cleave the fusion protein with factor Xa. The fusion protein was not cleaved by this method (data not shown), suggesting that the cleavage site was not accessible to the protease. The MBP was previously reported to have no influence on nuclease activity, nor to possess any nuclease activity [25]. Thus, any possibility of interfering nuclease activity from MBP was excluded.

The requirements for nuclease activity of MBP-Nuc are shown in Table 1. It needed divalent cations such as Mg^{2+} and Mn^{2+}. Other divalent cations were not effective. Many

Table 2. Relative activities of MBP-Nuc on different DNA substrates.

Substrate	Relative activity
Double-stranded phage T7 DNA	100*
Single-stranded phage T7 DNA	76

*100% means the amount of nucleotide released from double-stranded DNA substrates after 30 min incubation with 10 units of purified enzyme.

nucleases have been reported to require divalent cations for their activity [14, 20, 21, 25]. The nuclease from *Vibrio* sp., which is closest to *A. hydrophila*, was also shown to require Mg^{2+} [20, 21]. A high concentration of salts inhibited the nuclease activity. The optimal pH was between 6.5 and 7.5, and the optimal temperature for MBP-Nuc was shown to be 30°C.

The substrate specificity for MBP-Nuc was tested. Bacteriophage T7 contains a linear double-stranded DNA genome. This DNA was isolated and used as the substrate. MBP-Nuc degraded both double-stranded and single-stranded DNA, with reduced activity on the single-stranded DNA (Table 2). *E. coli* EndA which has 57% amino acid sequence homology to Nuc was also reported to degrade both double- and single-stranded DNA [26]. Three different forms (linear, nicked circular, and closed circular) of a plasmid were used as substrates and all were degraded by MBP-Nuc (Fig. 2). Thus MBP-Nuc seemed to possess both endo- and exonuclease activity. Figure 3 shows the RNase activity of MBP-Nuc. An extracellular nuclease from *Serratia marcescens* was also shown to degrade RNA, as well as double- and single-stranded DNA [6]. MBP-Nuc possesses different characteristics in terms of substrate conformation compared to other microbial nucleases. Exonuclease I of *E. coli* prefers denatured DNA, while exonuclease III of *E. coli* prefers native DNA [21, 34]. The fact that MBP-Nuc degraded both DNA and RNA suggests that it is a sugar-nonspecific hydrolase. The same characteristics were reported for Vvn of *V. vulnificus* and NucM of *Erwinia chrysanthemi* [24, 33]. An intracellular nuclease possessing both endo- and exonuclease activity in prokaryote is rare.

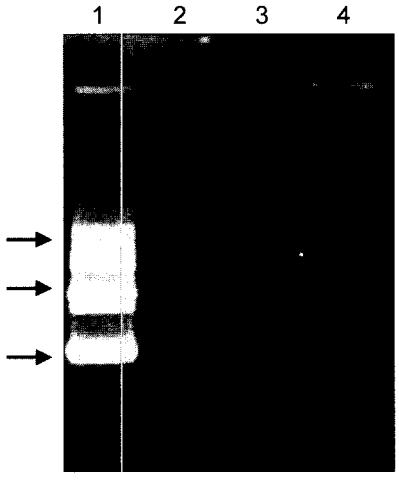

Fig. 2. Effect of substrate conformation on DNase activity.
An agarose gel electrophoresis was performed after incubation of DNAs. Arrows indicate closed circular, linear, and open circular plasmid DNAs from the bottom to the top. Lane 1: 3 conformations of plasmid DNA without any DNase; lane 2: plasmid DNAs incubated with 200 U of DNase I; lane 3: plasmid DNAs incubated with 100 U of MBP-Nuc; lane 4: plasmid DNAs incubated with 200 U of MBP-Nuc.

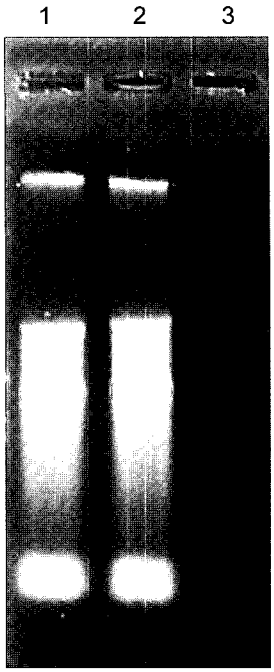

Fig. 3. RNase activity of MBP-Nuc.
Bacterial total RNAs were isolated and used as substrates. Intense bands corresponding to ribosomal RNAs are seen. An agarose gel electrophoresis was performed after incubation of RNAs. Lane 1: RNA without any nuclease; lane 2: RNA incubated with MBP; lane 3: RNA incubated with 100 U of MBP-Nuc.

This extracellular nuclease thus does not seem to be involved in intracellular nucleic acid metabolisms, such as recombination and repair. The role of this enzyme appears to be as a supplier of nucleotides and phosphates from the extracellular environment. This aspect is currently being studied.

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