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Fractionation of DNases Specific to *Haemonchus contortus* Intestine by Phenyl Sepharose Column

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Multiple DNases were identified from *Haemonchus contortus* intestine based on previous studies. The DNases detected at 34, 36 and 38.5 kDa had diverse characteristics. Some of them had characteristics similar to those of mammalians and others had unusual characteristics. This study was carried out to fractionate worm intestinal DNases from other proteins using phenyl Sepharose chromatographic methods. All DNases detected from *Haemonchus contortus* intestine were fractionated in the flowthrough of phenyl Sepharose, indicating the worm DNases are hydrophilic. The DNases were enriched five-fold in the flowthrough fraction while additional steps are required for isolation of the worm DNases. Thus, fractionation with phenyl Sepharose could be used as a good initial step to enrich and separate DNases from other proteins.

Key words: DNases, *Haemonchus contortus*, intestine, chromatography, phenyl Sepharose

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A blood-sucking parasitic nematode, *Haemonchus* (*H.*) *contortus*, dwells in abomasum of ruminants (Jasmer and McGuire, 1991; Karanu *et al.*, 1993). The parasite is highly pathogenic in young animals and is the primary cause of anemia. Anthelmintics are commonly used to control gastrointestinal nematodes (Jasmer *et al.*, 2000; Kwak and Jasmer, 2003). However, development of anthelmintic resistance in the various parasites has been reported worldwide (Jasmer *et al.*, 2000; Coles *et al.*, 2006) and is especially acute with *H. contortus* (Lacey, 1988; Lubega and Prichard, 1990). Therefore, it is necessary to find new targets that are critical for worm survival.

Rationale for the nematode intestine as a target for control strategies was strengthened by experiments using fenbendazole (FBZ) anthelmintics (Jasmer *et al.*, 2000). It was shown that cytotoxic effects could be induced by FBZ in intestinal cells of *H. contortus*. These effects included genomic DNA fragmentation (DF) in intestinal cell nuclei, with 200 bp increment and 3′-hydroxyls, which resembles programmed cell death (Saraste and Pulkki, 2000; Wu *et al.*, 2000; Shiokawa and Tanuma, 2001). The pathologic effects induced by FBZ treatment are significant for several reasons. The results provide the first demonstration that DF can be induced in nematode cells by an ectopic treatment (Jasmer *et al.*, 2000). Since DF reflects nuclear genome fragmentation, the cytotoxic effect is expected to cause intestinal cells to die. Induction of DF in the polyploid nuclei of intestinal cells has added significance (Jasmer *et al.*, 2000). The DNA content of these nuclei is acquired through several life cycle stages. Therefore, replacement of these nuclei is unlikely to occur during a single life cycle stage. This consideration along with the apparent inability of nematodes to regenerate tissue indicates that DF induced by FBZ reflects irreversible intestinal pathology. This pathology is expected to be lethal to the parasite, given the function of the intestine in nutrient acquisition. Therefore, elucidating the mechanisms that regulate and mediate inducible DF in intestinal cells is of interest. Knowledge on the mechanisms responsible might lead to alternative or improved methods to induce this anthelmintic effect.

Since cellular mediators to produce genome fragmentation are DNases (Hedgecock *et al.*, 1983; Wu *et al.*, 2000; Parrish *et al.*, 2001; Kwak and Jasmer, 2003; Parrish and Xue, 2003), worm DNases have been investigated as candidates for the genome fragmentation induced by FBZ treatment (Jasmer

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et al., 2000; Kwak and Jasmer, 2003). Of the variety of DNases that exist in eucaryotic cells, only endonucleases have been implicated in oligonucleosomal cleavage of chromosomal DNA. These DNases can be separated into two classes, neutral and acidic DNases. Neutral DNases require neutral pH and divalent cations [*e.g.* DNase I (Ribeiro and Carson, 1993), DNase γ (Shiokawa and Tanuma, 1998) and caspase-activated DNase (Enari *et al.*, 1998)]. Acidic DNases require acidic pH but no divalent cations [*e.g.* DNase II (Barry and Eastman, 1993) and related enzymes (Shiokawa and Tanuma, 1999)].

Previous research identified 34, 36 and 38.5 kDa DNases in *H. contortus* intestine with diverse characteristics based on pH, metal ion dependence and 3′-ends (Kwak and Jasmer, 2003). Some of them had characteristics similar to classical DNases of mammalians and others had characteristics distinct from the prototype of classical DNases. Since individual characteristics of DNases could not be determined in unfractionated intestinal lysates, in this research, phenyl Sepharose chromatography was used to further fractionate the worm intestinal DNases from other proteins and evaluate enrichment of the DNases.

Materials and Methods

Preparation of *H. contortus* intestinal proteins

H. contortus (Beltsville isolate, USA) intestinal lysates were gifted from Prof. Douglas Jasmer at Washington State University and methods for preparing samples are described previously (Karanu *et al.*, 1993). Protein concentration was determined by the bicinchoninic acid assay (Pierce, USA; Kwak and Jasmer, 2003).

Phenyl Sepharose chromatography

To fractionate worm intestinal DNases, phenyl Sepharose chromatographic methods (Amersham, USA) were applied. Worm intestinal lysates were pre-equilibrated to a binding buffer (25 mM Tris-HCl, pH 7.5, 1 M $(NH_4)_2SO_4$). Elution was conducted with a step gradient (1.0, 0.5, 0.3, 0.2, 0.1 or 0.0 M) of $(NH_4)_2SO_4$. Each fraction was concentrated using Centricon (YM-10, Millipore, USA) and desalted (100 times dilution) using Tris buffer (20 mM, pH 7.0). Then, protein concentration was determined using the bicinchoninic acid assay (Pierce, USA; Kwak and Jasmer, 2003).

Zymogram analysis

DNases in fractions were analyzed in zymogram activity gels to determine M_rs and biochemical characteristics (Kwak and Jasmer, 2003). Proteins were electrophoretically separated on non-reducing SDS-PAGE (10%), which contained salmon sperm DNA (200 μg/mL, Sigma) as a substrate for DNases. Electrophoresis, SDS removal and gel incubation were done as described (Kwak and Jasmer, 2003). DNase activity was determined in an incubation buffer (250 mL; 40 mM Tris-HCl and 0.02% sodium azide) at pH 5.0 or 7.0 in the presence of 2 mM $CaCl_2$/$MgCl_2$ for 88-136 h. DNase positions on gel remained unstained following incubation with ethidium bromide (1 μg/mL) and UV illumination. The M_rs of DNases were estimated based on Rainbow molecular mass standards, which ranged from 14.3 to 220 kDa (Amersham, USA).

Plasmid digestion assay

DNase activity in each fraction was evaluated using a Bluescript plasmid DNA as described (Kwak and Jasmer, 2003). Briefly, each fraction (400 ng protein) was incubated with plasmid DNA (400 ng) in a 20 μL reaction under conditions of pH 5.0 or 7.0 in the presence of 2 mM $CaCl_2$/$MgCl_2$ or 10 mM EDTA. Incubation was done at 37°C for 8 h. Degradation of plasmid DNA was determined by eletrophoretic separation on agarose gels (0.8%) and ethidium bromide (500 ng/mL) staining.

Determination of DNA 3′–ends produced by DNase

Fragments of 3′-ends (3′-OH or 3′-P) produced by DNase activity in the original intestinal lysates or flowthrough fraction were determined by a 3′-end labeling assay (Kwak and Jasemr, 2003). The assay uses terminal deoxynucleotidyl transferase (TdT) that adds nucleotides to 3′-OH. Aliquots (20 ng) of DNase digested plasmid DNA were incubated with TdT (6 U, Roche, USA) and biotin-dideoxyadenosine triphosphate (ddATP, 250 pmol, Perkin-Elmer, USA) in a 10 μL reaction at 37°C for 1 h. Then, electrophoresis was done as described above and the DNA fragments were transferred onto positively charged nylon membranes (Roche). Detection of labeled biotin-ddATP was done with streptavidin-conjugated horseradish peroxidase (1:1,000 dilution, Kirkegaard and Perry Laboratories, USA) and an enhanced chemiluminescence system (Amersham). X-ray films were used for recording signals.

Determination of 3′ ends (3′-OH or 3′-P) was done by treating calf intestinal alkaline phosphatase (ALP, Roche), which converts 3′-P to 3′-OH (Kwak and Jasmer, 2003). Plasmid DNA fragments (400 ng) digested by DNase were treated with ALP (4 U) in a 50 μL reaction at 37°C for 1 h. ALP-treated or untreated samples (20 ng) were incubated as described above under the same conditions. Signals on X-ray films were quantitated using Chemilmage 4000 (Alpha Innotech, USA). Ratios of signals in ALP treated or untreated samples were calculated. A ratio of 1.0 was obtained for DNase I, which is known to produce 3′-ends with hydroxyls,

while a ratio of 34.0 was obtained for DNase II, which produces 3′-ends with phosphates (Kwak and Jasmer, 2003).

Results

Chromatography with phenyl Sepharose

Phenyl Sepharose chromatography was used to separate *H. contortus* intestinal DNases from other proteins by their hydrophobic interaction. Figure 1 summarizes results for phenyl Sepharose fractionation. Zymograms show that fractionation with phenyl Sepharose resolved all the DNases detected at 34, 36 and 38.5 kDa in the flowthrough fraction at both pH 5.0 and 7.0. This shows that *H. contortus* intestinal DNases have little hydrophobic interaction, indicating the worm intestinal DNases are hydrophilic.

Plasmid digestion assay

DNase activities in phenyl Sepharose fractions (flowthrough, 0.5, 0.3, 0.2, 0.1, or 0.0 M $(NH_4)_2SO_4$) were tested using plasmid digestion assay (Figure 2). Whole intestinal lysates had activity at pH 5.0 and 7.0 as described previously (Kwak and Jasmer, 2003). In addition, inhibition of plasmid degradation by EDTA occurred partially at pH 5.0, but most activity, if not all, was inhibited at pH 7.0 in the presence of EDTA. The highest activity was detected in the flowthrough fraction at both pH 5.0 and 7.0. The activity in the flowthrough fraction was partially inhibited by EDTA at pH 5.0, but most if not all activity was inhibited at pH 7.0 by EDTA. Relatively low activities were eluted in different concentrations (0.5, 0.3, 0.2 or 0.1 M) of ammonium sulfate. In the 0.5 M fraction, weak activity at pH 5.0 was not detectably inhibited by EDTA, but the activity at pH 7.0 was sensitive to EDTA. In the 0.3, 0.2 and 0.1 M fractions, relatively weak activities were detected at pH 5.0 and 7.0, but most activities were sensitive to EDTA at both pHs. In contrast, DNase activity was not recognizably detected in the 0.0 M fraction.

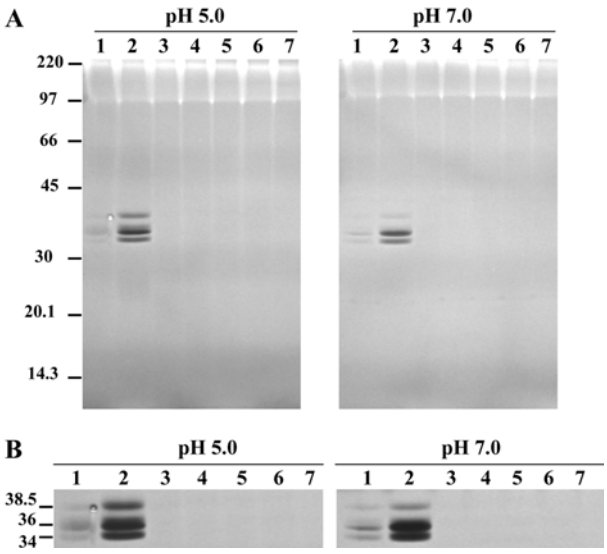

Figure 1. DNase activities from the intestine of *Haemonchus contortus* adult worms. (A) Zymograms of DNases from the intestine of *H. contortus* in phenyl Sepharose fractions. Samples (100 μg of protein), including original intestinal lysates (lane 1), flowthrough (lane 2), 0.5 M (lane 3), 0.3 M (lane 4), 0.2 M (lane 5), 0.1 M (lane 6) or 0.0 M (lane 7) of $(NH_4)_2SO_4$ fractions, were separated by SDS-PAGE (10% gel containing 200 μg/mL salmon sperm DNA) and incubated in buffers (pH 5.0 or 7.0) containing 2 mM $CaCl_2$/$MgCl_2$ for 88 h. M_rs of DNases were identified by staining the gel with ethidium bromide. Molecular markers are indicated in kDa on the left. (B) Selected enlargement and longer incubation (136 h) of panel A to show individual DNases better. M_rs of DNases are indicated in kDa on the left.

Determination of 3′-ends produced by DNase

3′-End labeling assay was done to assess 3′-ends (3′-OH or 3′-P) of plasmid DNA fragments produced by DNase activity in the flowthrough fraction of phenyl Sepharose (Figure 3). Ratios of 3′-end labeling produced by DNA fragments treated or untreated with ALP were compared to those for classical neutral DNase I or acidic DNase II (Kwak and Jasmer, 2003). The worm intestinal lysates before fractionation produced ratios of 2.9 and 1.4 at pH 5.0 and 7.0, respectively. These

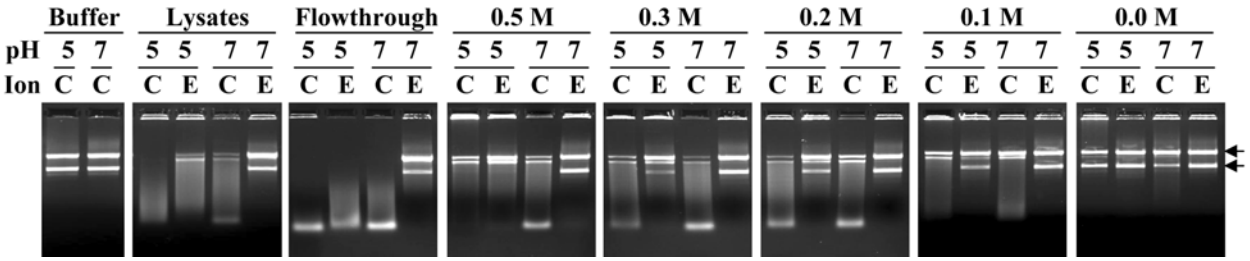

Figure 2. Plasmid digestion assays for DNase activities in phenyl Sepharose fractions of *Haemonchus contortus* intestine. Samples (400 ng protein), including unfractionated intestinal lysates, flowthrough, 0.5, 0.3, 0.2, 0.1 or 0.0 M $(NH_4)_2SO_4$ fractions of phenyl Sepharose, were incubated with plasmid DNA (400 ng) at 37°C for 8 h in buffers (pH 5.0 or 7.0) with 2 mM $CaCl_2$/$MgCl_2$ (C) or 10 mM EDTA (E). Buffers without sample were incubated for 8 h as a control. The DNA fragments produced were analyzed by staining agarose gel (0.8%) with ethidium bromide. Arrows on the right refer to estimated sizes of two forms of uncut plasmid DNA at 4 kb (upper) and 2.5 kb (lower), respectively.

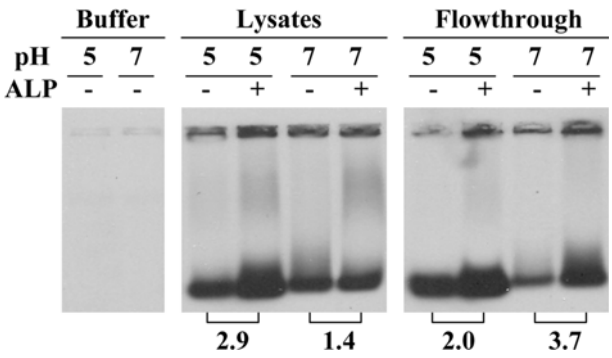

Figure 3. 3'-End labeling of plasmid DNA fragments produced by DNase activities from unfractionated lysates and phenyl Sepharose flowthrough fraction of *Haemonchus contortus* intestine. Digested plasmid DNA (see Figure 2) was treated with (+) or without (-) alkaline phosphatease (ALP) at 37°C for 1 h, and then subjected to 3'-end labeling reactions. DNA (20 ng) separated on agarose gel (0.8%) was transferred to nylon membranes. Labeled ends were detected by an enhanced chemiluminescence detection system. Buffer without sample was evaluated as a control. The ratio of signal obtained with (+) or without (-) ALP was determined with densitometric quantitation.

Table 1. Enrichment of *Haemonchus contortus* intestinal DNases by phenyl Sepharose

Purification step	Protein amount (μg)	Yield (%)
Lysates	6000	100
Phenyl flowthrough	1,102.2	18
0.5 M	1,871.5	31
0.3 M	740.7	12
0.2 M	312.8	5
0.1 M	266.4	4
0.0 M	250.5	4

ratios at both pHs indicate the worm DNase activities produce a mixture of fragments with 3′-OH and 3′-P with higher ratios at pH 5.0, which agrees with previous measurements (Kwak and Jasmer, 2003). Due to low activity in other fractions, only ratios for the flowthrough fraction were considered here since activity was highly enriched in this fraction. The DNase activity in the flowthrough fraction produced the ratios of 2.0 and 3.7 at pH 5.0 and 7.0, respectively. Again, these ratios suggest the DNase activity in this fraction leaves plasmid ends with a mixture of 3′-OH and 3′-P.

Enrichment of DNase by phenyl Sepharose

Whole intestinal lysates (6 mg protein) were applied to phenyl Sepharose column (Table 1). The amount of unbound protein in flowthrough fraction was 1,102.2 μg (18%), which is 5.4 fold enrichment of total protein. The most abundant protein (1,871.5 μg, 31%) was eluted in the 0.5 M fraction. The fractions eluted with 0.2, 0.1 and 0.0 M ammonium sulfate had proteins of 312.8 (5%), 266.4 (4%) and 250.5 μg (4%), respectively. Seventy four percent of the total proteins were recovered by phenyl Sepharose column and 26% of proteins were lost during the procedure.

Discussion

Genome fragmentation was induced in intestinal cells of a blood-sucking parasite *H. contortus* by an ectopic treatment of anthelmintic fenbendazole, with characteristics of 200 bp increment and 3′-ends with hydroxyls (Jasmer *et al.*, 2000). Since cellular DNases in the intestine of *H. contortus* were considered as candidates for mediating genome fragmentation, the worm intestinal DNases have been investigated and research has identified multiple DNases from the worm intestine.

While production of 3′-OH in genome fragmentation was associated with the worm intestinal DNases, the property was not able to be attributed to a specific one (Jasmer *et al.*, 2000; Kwak and Jasmer, 2004). Thus, chromatographic fractionation for worm intestinal DNases was assessed to dissect their biochemical characteristics and to separate worm DNases from other proteins. Chromatographic methods applied included phenyl Sepharose in this study. Fractionation with phenyl Sepharose, which has hydrophobic interaction, resolved all three DNases (34, 36 and 38.5 kDa) in the flowthrough fraction. While other proteins were contaminated in the flowthrough fraction, the phenyl fractionation could be used as an initial step to enrich and separate DNases from other proteins.

Three M_rs of DNases at 34, 36 and 38.5 kDa were detected from the worm intestine. Their characteristics were compared to classical mammalian acidic or neutral DNases (Kwak and Jasmer, 2003). While classical neutral DNase I produces DNA fragements with 3′-OH and requires divalent cations under neutral pH conditions, classical acidic DNase II produces 3′-P and requires no divalent cations under acidic pH conditions. Some of the worm intestinal DNases had characteristics similar to those of classical acidic or neutral DNases and others had unique characteristics. For instance, the three intestinal DNases at 34, 36 and 38.5 kDa were highly sensitive to EDTA at both pH 5.0 and 7.0 (Kwak and Jasmer, 2003). Sensitivity to EDTA at pH 5.0 was unexpected and distinct from classical acidic DNase II since acidic DNase II is insensitive to EDTA under acidic conditions. However, the sensitivity to EDTA in all the DNases at 34, 36 and 38.5 kDa occurred at pH 7.0 exclusively, which are characteristics resembling classical neutral DNase I. Thus, these observations suggest the worm intestine have multiple DNases that differ in sensitivity to EDTA under acidic or neutral pH conditions.

DNases with unusual characteristics have been described

from other examples. DNase activity from excretory/secretory products of *Trichinella spiralis* had high sensitivity to EDTA under acidic pH conditions (Mak and Ko, 1999). DNAS1L2, which is a 35 kDa DNase described from humans, requires divalent cations and produces 3′-OH under acidic pH conditions (Shiokawa and Tanuma, 2001). While DNAS1L2 had amino acid similarity to classical neutral DNase I (Rodriguez *et al*., 1997), its characteristics were unexpectedly unique compared to classical neutral DNase I. These observations suggest DNases with unusual characteristics exist among lower and higher organisms. As another example, at least six DNases have been implicated in mediating DNA fragmentation in *Caenorhabditis elegans* by genetic screens and RNA interference analysis (Parrish and Xue, 2003). Among them, NUC-1 has characteristics of classical acidic DNase II (Wu *et al*., 2000), but characteristics of others have not been described to date. This raises the possibility of the existence of a DNase distinct from the classical prototype.

Between the methods used to detect DNase activities, plasmid digestion assay was more sensitive than zymogram. DNase activities in the fractions of 0.5, 0.3, 0.2 and 0.1 M ammonium sulfate were undetectable in zymogram, but weak activities in those fractions were detected in plasmid digestion assay. In addition, plasmid assay requires much smaller amount of worm samples (400 ng *vs.* 100 μg). While DNase activities detected in plasmid assay was not attributed to a specific DNase, zymogram was a useful tool to analyze DNases at the level of molecular mass.

3′-End labeling for DNA fragments produced by worm intestinal DNase activity showed 2.9 and 1.4 at pH 5.0 and 7.0, respectively. This suggests that ratios of 2.9 and 1.4 represent production of a mixture of 3′-OH and 3′-P based on the ratios of classical neutral DNase I (1.0) and acidic DNase II (34.0).

Separation of worm intestinal DNases was not successful using the methods applied. Sequential fractionation with phenyl Sepharose followed by DNA cellulose or diethyl-aminoethyl Sepharose could be a good combination for isolation of DNase. Limits of the amounts of intestinal samples and stability of DNase were obstacles for further fractionation of the intestinal DNases. Excretory/secretory products or whole worm materials could be used as alternative sources for intestinal DNases. While additional steps are required for the isolation of DNase, the most significant finding described here is the enrichment of the worm intestinal DNases by phenyl Sepharose. The methods established will be used for further isolation of DNase in the next step. Therefore, the research has laid a foundation to isolate individual DNases of *H. contortus* intestine in mediating DNA fragmentation.

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