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Short Paper

Determination of aspartic protease gene dosage in the *Onchocerca volvulus* genome

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Summary

Aspartic proteases are a relatively small group of enzymes which express in various nematodes including *Onchocerca volvulus*. An estimation of the gene copy number corresponding to the OV7A clone, which contains a cDNA insert encoding approximately two-thirds of the entire coding sequence of aspartic protease of *O. volvulus*, was made by slot blot analysis in a closely related species *O. gibsoni* genome. Nylon membrane was loaded with serial dilutions of genomic DNA alongside the OV7A plasmid DNA before hybridizing the membrane to that ^{32}P-labeled cDNA insert. To prepare the initial probe, OV7A cDNA insert was amplified using gene-specific primers. By comparing the signal intensity of slot blot hybridization of known amounts of genomic DNA and plasmid DNA containing the cDNA insert under similar conditions, the abundance of sequence homologues to the ^{32}P-labeled cDNA insert in the genome was calculated. For confirmation, southern blot analysis was performed by digesting genomic DNA with a panel of different restriction enzymes. Hybridizing patterns of the same probe revealed a single band except when predicted internal restriction sites were affected. It was confirmed that Onchocerca contains a single copy of the gene corresponding to this cDNA insert per haploid genome.

Key words: Nematode, Aspartic protease, Parasite, *Onchocerca volvulus*

Introduction

Human onchocerciasis, commonly known as river blindness, is a complex disease caused by the filaria *Onchocerca volvulus*. Onchocerciasis is a major cause of skin pathology and eye lesions including blindness, and has its highest prevalence in tropical Africa (WHO Expert Committee on Onchocerciasis. Third Report World Health Organization, 1987). Both adult worms and microfilaria are responsible for the disease process; however, the main symptoms are produced by the migration of microfilaria through subcutaneous tissues and skin of the host. This process is apparently facilitated by the secretion of proteases (McKerrow *et al*., 1990). The proteolytic activity of the third stage larvae from a variety of gastro-intestinal parasites indicated that an organism's mobility is not the only factor necessary for the host tissues invasion; the secretions of proteolytic enzymes are critical (Knox and Jones, 1990).

Aspartic proteases are a relatively small group of enzymes which includes the mammalian pepsins, chymosins, cathepsins, and renins (Barrett *et al*., 1998). Various organisms express mRNAs which are homologous to the aspartic protease. They are thought to be involved in the regulation of physiological activities by processing or degradation of proteins and peptides. With regard to parasites, aspartic proteases play key roles in the degradation of hemoglobin obtained from ingested or parasitized erythrocytes. Aspartic proteases function as digestive enzymes in some pathogens including *Plasmodium* (Goldberg *et al*., 1991) and *Schistosoma* (Brindley *et al*.,

2001), in which they play a key role in hemoglobin proteolysis. In particular, aspartic proteases termed plasmepsins play a key role in hemoglobin proteolysis in the digestive vacuole of the intraerythrocytic stages of the malaria parasite, *Plasmodium falciparum*, and represent potential targets for the development of next generation anti-malarial drugs (Banerjee *et al*., 2002). Our previous studies led to the identification of cDNA encoding *O. volvulus* aspartic protease (*Ov*-APR-1), clearly most closely related to mammalian lysosomal cathepsin D in primary sequence comparisons (Jolodar *et al*., 2004a). Although most aspartic proteases are lysosomal, both schistosome cathepsin D and a pepsin-like aspartic protease from the sheep parasitic nematode *Haemonchus contortus* (Longbottom *et al*., 1997) are expressed in the gut, further indicating digestive roles.

In this study, the relative abundance of the aspartic protease gene was quantified by comparison of the hybridization signal intensity between plasmid DNA containing partial cloned fragment and known amounts of genomic DNA.

Materials and Methods

Adult worm collection

For southern blot experiments DNA was used from *O. gibsoni*, since phylogenetic studies deduced from rRNA sequence comparisons revealed the close genetic relationship between the species *O. volvulus* and *O. gibsoni* (Gill *et al*., 1988) and because of difficulties in obtaining *O. volvulus*. Uncalcified nodules were excised carefully so as not to damage female worms which can be easily broken. Intact worms were transferred to a Petri dish containing Dulbecco's modified eagle medium (DMEM; Gibco). The collected worms were frozen immediately on dry ice and then transferred to -70°C for long-term storage. In addition to worm collection by dissection, worms are also released from the fibrous capsules by enzymatic digestion (Schultz-Key *et al*., 1977).

Genomic DNA extraction

Purified *O. gibsoni* worm tissue (0.5-1.0 g) was prepared as described by Jolodar *et al*. (2004b) with some modifications. Cell lysate was incubated with DNase-free RNase (20 mg/ml) in the presence of protease K (50 mg/ml) until the material was completely dissolved. After phenol/chloroform extraction, NaCl to a final concentration 0.1 M and two volumes of ice cold absolute ethanol were added to the aqueous phase. The DNA pellet was desalted by rinsing with 70% ethanol then dissolved in TE buffer (10 mM Tris-HCl, 1 mM, EDTA, pH = 7.4).

Southern transfer procedure and slot blot

For southern blots, 4 µg of genomic DNA was digested with restriction enzymes, separated through 1% agarose gels by electrophoresis, and blotted onto the positively charged nylon membranes by capillary blotting. The membranes were transferred in denaturing solution (0.2 M NaOH/1.5 M NaCl) for 7 min before being soaked into neutralizing buffer (0.5 M Tris-HCl pH = 7.4/ 1.5 M NaCl/ 1 mM EDTA) for 3 min. In the case of slot blotting, both genomic DNA and DNA from plasmid pUC18 containing the OV7A insert were transferred onto the nylon membranes using a schleicher and schuell slot blot apparatus. The membranes were washed with 2 × SSPE (saline sodium phosphate EDTA buffer) and air-dried prior to cross-linking to the membrane by UV irradiation. Membranes were then incubated with a ^{32}P-labeled probe in prehybridization buffer overnight. After incubation, the membranes were washed at high stringency prior to subjecting to X-ray film with intensifying screens overnight.

PCR amplification

For preparing the initial probe, the insert of OV7A clone encoding approximately two-thirds of the entire coding sequence between nucleotide positions 273 and 969 of *O. volvulus* aspartic protease gene (GenBank entry U81605) was amplified using gene-specific primers. The clone was originally identified as a cDNA, and has previously been characterized in more detail (Jolodar and Miller, 1997; Jolodar and Miller, 1998). In order to amplify the insert, forward (APF: 5'-CATAAATATGAATTTG GTTCACG)

and reverse (APR: 5' -TATAG CCTAACATCCATTGGCAC) primers were designed. PCR amplification was performed using plasmid DNA clone OV7A as the template with initial denaturation for 5 min at 95°C, followed by 35 cycles of 45 sec at 94°C, 1 min at 59°C, and 1 min at 72°C and, finally, 5 min of incubation at 72°C in 50 µl PCR SuperMix (Life Technologies, Hamburg, Germany) containing 20 pmol of each primer, deoxynucleotides (each at 220 µM), 1.5 mM $MgCl_2$, 10 ng plasmid DNA and 1 U Taq polymerase. PCR with specific primers using OV7A clone as the template resulted in the amplification of a 696 bp fragment of *O. volvulus* aspartic protease.

Random oligonucleotide primer labeling

25-200 ng of gel purified insert DNA was denatured by heating to 95°C for 5 min. Denatured DNA was chilled on ice and combined with 4 µl of dNTP stock (0.25 mM dCTP, dGTP and dTTP), 5 µl (0.37 mM) of random hexanucleotide solution, 5 µl of 10 × Klenow buffer, 5 µl of (50 µCi) [α-^{32}P]dATP and 2 U of Klenow enzyme in a total volume of 50 µl. The solution was incubated at 37°C for 15 min. Unincorporated dNTPs were removed from labeling reactions by column chromatography. The bottom of a Pasteur pipette was plugged with a small amount of sterile glass wool and the pipette filled with a suspension of BioGel P-6 (Bio-Rad) in SSPE. After washing the column with 1 × SSPE buffer, the labeling reaction was applied to the top of the column in a volume of 50 µl after the addition of 5 µl of tracking dye. The column was eluted with 1 × SSPE.

Results

In order to characterize the genomic arrangement of aspartic protease gene, southern blotting analysis was performed by digesting DNA with a panel of different restriction enzymes. Four µg aliquots of *O. gibsoni* DNA were digested using *Sph*I, *Alu*I, *Hinf*I, *Sau3A*I and *Hind*III using three-fold excess restriction enzyme for 4 h. Patterns of ethidium bromide fluorescence revealed that restriction fragments generated from digestion with *Sph*I is distinct, meaning that the remaining samples appeared to be sheared over a wide range of molecular weight. The DNA was blotted to nylon membrane prior to hybridization with ^{32}P-labeled cloned insert of OV7A. Hybridizing patterns of ^{32}P-labeled probe revealed that fragments generated from digestion with *Hind*III, which has no site in the probe, yielded a distinct band at 5 kb, whereas in digestion with *Sph*I with one site in the probe, two predominant bands at 3.5 and 5 kb were apparent (Fig. 1A). Hybridization gave a major band of 1 kb in length to *Alu*I-digested DNA. Digestion with *Sau3A*1 with two sites in the probe gave three 2.5 and 0.8 and 0.7 kb. No major bands were detected upon digestion with *Hinf*I.

For confirmation, another approach was pursued, enabling the copy number assessment of the gene by analysis of plasmid DNA clone OV7A versus genomic DNA digests on slot blots. Six concentrations of digested genomic and plasmid DNA, 5 to 0.5 µg and 500 to 0.05 pg, respectively, were applied to agarose gels. Following electrophoresis, southern blots were prepared and hybridized with the same probe (Fig. 1B).

Discussion

Southern blot analysis of *O. gibsoni* genomic DNA using OV7A probe showed a single band, except when predicted internal restriction sites were affected. In some of the digests the less intense bands may be due to polymorphism. Most likely, the organization of the gene which encodes OV7A insert is not complex because *Hind*III, which do not cut within the cDNA insert, only produce a single hybridizing band when a digest of genomic DNA is probed with the OV7A insert (Fig. 1). Given that two *Sau3A*I and no *Hind*III sites are present in the cDNA sequence, these results suggest the presence of a single copy gene.

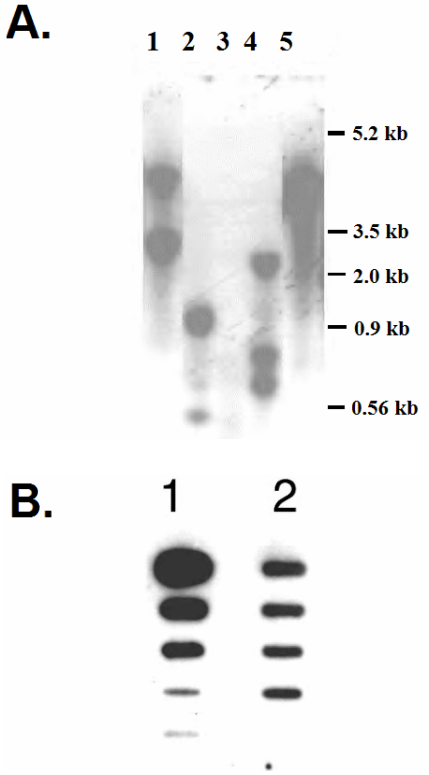

Fig. 1: Southern transfer analysis of *Onchocerca gibsoni.* (A) Genomic DNA was digested with *Sph*I (Lane 1), *Alu*I (Lane 2), *Hinf*I (Lane 3), *Sau3A*I (Lane 4), and *Hind*III (Lane 5) and hybridized with ^{32}P-labeled OV7A insert. The size of the DNA markers is indicated (in kb). (B) Determination of aspartic protease sequence copy number in genomic DNA. Nylon membrane was spotted with dilutions of DNA from plasmid pUC18 containing the OV7A insert (Lane 1) and genomic DNA of adult worms (Lane 2). The membrane was hybridized to a ^{32}P-labeled OV7A insert at moderate stringency. Loading: OV7A insert DNA (Lane 1): 500, 50, 5, 0.5, 0.05 pg; genomic DNA (Lane 2): 5, 3, 2, 1, 0.5 µg

The copy number of aspartic protease was estimated by comparing the intensity of slot blot hybridizations of known amounts of genomic DNA and OV7A plasmid DNA containing the cDNA insert under similar conditions. As shown in Fig. 1B, hybridizing signal intensity obtained with 5 µg of genomic DNA corresponded to approximately 5 pg of OV7A plasmid DNA. Since plasmid OV7A contains 696 bp of *O. volvulus* cDNA insert, it can be estimated that the insert represents about 1/5 of the total clone. This suggests that 2×10^{-5}% of the genome contains sequence homologues of the cloned fragment. Assuming that the haploid *O. gibsoni* genome size is similar to that of *O. volvulus* (1.5×10^8 bp) (Donelson *et al*., 1988), this implies a single copy of the gene is present per haploid genome. Coupled with simple patterns of southern blot hybridization, this interpretation is consistent with the gene titration experiments. Although the results suggest that there is likely to be a single copy of the gene encoding OV7A in *O. volvulus*, the genomic structure of aspartic protease of *O. volvulus* remains to be elucidated.

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