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Isolation and characterization of endochitinase and exochitinase of *Setaria cervi*

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ABSTRACT

Chitin metabolism has been shown to have a role in the development of parasitic nematodes including filarial parasites and the enzymes associated with chitin metabolism have been considered as potential vaccine and drug target. Chitinases are members of the enzyme superfamily of glycoside hydrolases, which are characterized by the ability to hydrolyze glycosidic bonds in chitin chain by either an endolytic or an exolytic mechanism. In the present study, we have demonstrated the chitinase (exochitinase and endochitinase) activity in different stages of *Setaria cervi* (bovine filarial parasite) and have also purified and characterized the endochitinase from microfilarial stage of the parasite. The chitinase activity has been detected in adult and microfilarial stages of *S. cervi* using the fluorescent substrates. The *S. cervi* adult stage was found to have high activity of exochitinase (28.72 ± 0.25 nmol/min/mg) while microfilarial stage showed high activity of endochitinase (24.40 ± 0.25 nmol/min/mg). Native polyacrylamide gel electrophoresis, followed by staining of enzyme activity with fluorescent substrates, revealed single isoenzymic form of exochitinase in adults and endochitinase in microfilariae of *S. cervi*. The endochitinase from *S. cervi* microfilariae was purified employing chitin affinity matrix and DEAE-Sephacel ion-exchange chromatography. The enzyme was purified about 55 fold with an enzyme recovery of 22.33%. The purified enzyme exhibited a doublet of protein bands on SDS-PAGE at 65–70 kDa. The closantel (chitinase inhibitor) strongly inhibited the enzyme activity of *S. cervi* microfilariae endochitinase with a Ki value of 4.3 ± 0.18 μM.

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1. Introduction

Lymphatic filariasis, caused by infection with the filarial nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* is estimated to infect large numbers of people in tropical and subtropical areas worldwide. Control and treatment of filarial infections are difficult as no vaccines are available, vector control programs are threatened by insecticide resistance and the repertoire of effective drugs is very limited [1]. The parasite enzymes could be utilized as potential therapeutic drug targets if there are marked differences between the parasite and the host enzymes [2]. Therefore, identification of new molecular targets and the characterization of parasite specific proteins/enzyme are of considerable importance to improve the treatment and control strategies against filariasis.

Chitin (linear poly β-1,4-N-acetyl-D-glucosamine) is one of the most abundant polysaccharide in nature and can be found mainly in the cuticles, egg-shell [3–5], microfilarial sheath [6] and pharynx [7,8] of nematode parasites. The chitin metabolism has been proposed to be a parasite unique drug target, as the chitin has not been found in vertebrates [9,10]. In filarial parasites, the chitin metabolism has been implicated in the larval development and the enzymes associated with chitin metabolism might be good targets for the development of anthelmintic drugs and vaccines [11,12].

Chitinases (E.C. 3.2.2.14) are glycosyl hydrolases which degrade β-1,4-N-acetyl-D-glucosamine linkages in the chitin molecule and play important structural, metabolic, physiological and defensive roles [13]. Chitinases are further divided into endochitinase and exochitinase according to the hydrolysis mechanism and the manner in which they cleave the chitin chain [14,15]. The exochitinases have been divided further into two subcategories: Chitobiosidases (E.C. 3.2.1.29) which progressively release di-acetylchitobiose from the non-reducing end of the chitin and 1-4-β-glucosaminidases (E.C. 3.2.1.30) cleaving the oligomers of chitin thereby generating monomers of glucosamine [16]. Chitinases have been reported to be present in a number of protozoan [17,18] and helminth parasites [19–24]. In most of the filarial parasites, chitinases have been identified in the microfilarial and infective larval stages [25] except for *Onchocerca gibsoni* where the

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chitinase was reported in the adult female worms [9]. The *B. malayi* recombinant chitinase and DNA encoding *Onchocerca volvulus* chitinase induced partial protection against challenge infections in rodents [26,27].

Setaria cervi, the bovine filarial parasite resembles human filarial parasite in having microfilarial periodicity, antigenic pattern and sensitivity towards known anti-filarial [11,28,29]. It has been exploited as an experimental model for various immunological and biochemical studies because of the non-availability of human filarial parasite in sufficient amount. In the present study, chitinases (exochitinase and endochitinase) have been identified and characterized in different stages (adult, microfilariae and embryo) of the bovine filarial parasite *S. cervi*. The endochitinase from the microfilarial stage of *S. cervi* was partially purified and characterized.

2. Materials and methods

2.1. Chemicals and reagents

Chitinase fluorometric assay kit, closantel, chitin azure, 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide [4-MU-GlcNAc], 4-Methylumbelliferyl β D-N,N′-diacetylchitobioside [4-MU-$(GlcNAc)_2$] and 4-Methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside [4-MU-$(GlcNAc)_3$] were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other biochemicals and reagents used were of analytical grade.

2.2. Parasitic material

The adult worms of *S. cervi* (bovine filarial parasite) were collected from the peritoneal folds of freshly slaughtered Indian water buffaloes at a local abattoir and brought to the laboratory in normal saline. The adult worms were washed extensively with normal saline to remove all the adhering materials and either used immediately or stored at −70 °C for further use. The microfilariae and embryo of *S. cervi* were collected by the dissection of gravid females and incubation of the distal portion of the uteri for 3–4 h at 37 ± 1 °C in Ringer's salt solution containing glucose [30]. The microfilariae and embryo released in the medium were recovered by centrifugation and purified on two step Percoll gradient [31].

2.3. Preparation of parasite extract

A 20% homogenate of *S. cervi* adult worms was prepared as described elsewhere [31] with slight modifications. Briefly, adult worms were cut into small pieces and ground in a pestle and mortar to a fine paste and then extraction was done with 0.15 M KCl containing 0.5% Triton X-100 under ice cold conditions for 1.5 h with intermittent vortexing. The microfilarial and embryo extracts were prepared by sonicating on ice using a Misonix ultrasonic sonicator (10 cycles of 20 s each with 10 s cooling between successive bursts at 20 kHz) and the extraction was done as described for adult worms. The enzyme extracts were centrifuged at 16,000 *g* and the soluble supernatants obtained from *S. cervi* adult (ScA), microfilariae (ScMf) and embryo (ScEmb) were used as enzyme source.

2.4. Fluorometric chitinase assays

The enzyme activities of exochitinase (N-acetylglucosaminidase and chitobiosidase) and endochitinase were measured in different stages (adult, microfilariae and embryo) of *S. cervi* using substrates for exochitinase [4-MU-GlcNAc, 4-MU-$(GlcNAc)_2$] and endochitinase [4-MU$(GlcNAc)_3$]. The stocks and the working solutions of the fluoroscent substrates: 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide [4-MU-GlcNAc], 4-Methylumbelliferyl β D-N,N′-diacetylchitobioside [4-MU-$(GlcNAc)_2$] and 4-Methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside [4-MU-$(GlcNAc)_3$] were prepared in DMSO following the instructions given in a chitinase fluorometric assay kit (Sigma, USA). The chitinase activity was determined in 96 well black colored microtiter plates (Nunc, USA).

2.4.1. Exochitinase activity

The exochitinase activity in *S. cervi* soluble protein extract was measured in a 96 well plate format using fluoroscent substrate 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide [4-MU-GlcNAc] for N-acetylglucosaminidase and 4-Methylumbelliferyl β D-N,N′-diacetylchitobioside [4-MU-$(GlcNAc)_2$] for chitobiosidase as described elsewhere [32] with slight modifications. Briefly, 50 μl of reaction mixture contained 50 mM sodium phosphate buffer (pH 6.0), 100 μM of 4-MU-GlcNAc or 4-MU-$(GlcNAc)_2$ and appropriate volume of enzyme extract followed by incubation at 37 °C for 30 min. The reaction was stopped by adding 100 μl of 0.4 M Na_2CO_3 and fluorescence was measured at 360 nm & 460 nm of excitation and emission wavelengths respectively using the fluorescence microplate reader (Biotek Inc., U.S.A.).

2.4.2. Endochitinase activity

The endochitinase activity was determined in a 96 well plate format using fluorescent substrate 4-Methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside [4-MU-$(GlcNAc)_3$] following the procedure of Robbins et al. [32] with certain modifications. Briefly, 50 μl of the reaction mixture (50 μl) contained 50 mM sodium phosphate buffer (pH 6.0), 100 μM of 4-MU-$(GlcNAc)_3$ and appropriate volume of enzyme extract was incubated at 37°C for 30 min and stopped by adding 100 μl of 0.4 M Na_2CO_3. The fluorescence was measured at 360 nm & 460 nm of excitation and emission wavelengths respectively employing the fluorescence microplate reader.

The exochitinase and endochitinase activities were calculated using the standard solution of 4-Methylumbelliferone (1.9 nmol/ml) and the results were expressed as nanomol of 4-Methylumbelliferone released from appropriate substrate at pH 6.0 respectively per minute at 37 °C.

$$\text{Units/ml} = \frac{(\text{FLUsample} - \text{FLUblank}) \times 1.9 \times 0.15 \times \text{DF}}{\text{FLUstd} \times \text{Time} \times \text{Venz}}$$

where:

FLUsample	fluorescence of the sample
FLUblank	fluorescence of the blank (containing only substrate working solution)
0.15	final reaction volume in milliliters after the addition of the stop solution
DF	enzyme dilution factor
FLUstd	fluorescence of the standard solution minus the fluorescence of standard blank
Time	minutes
Venz	volume of enzyme sample in milliliter.

2.4.3. Effect of substrate concentrations on chitinase activities of S. cervi adult and microfilariae

In order to study the effect of substrate concentrations on the enzyme activities of exochitinase and endochitinase, optimum enzyme concentrations were determined. The activities of exochitinase and endochitinase were measured in *S. cervi* adult and microfilarial stages using different enzyme concentrations and at a fixed concentration of substrates. The effect of substrates 4-MU-GlcNAc and 4-MU-$(GlcNAc)_3$ on the activities of exochitinase and endochitinase of *S. cervi* adult and microfilarial extracts was studied by measuring the enzyme activities at different concentrations (6.25–400 μM) of the substrates using the optimum enzyme concentration.

2.4.4. Chitin azure assay

The endochitinase activity of *S. cervi* microfilariae was also measured using chitin azure as a modified natural substrate by recording the

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