Experimental Parasitology 123 (2009) 244–249

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Entamoeba invadens: Cloning and molecular characterization of chitinases

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ARTICLE INFO

Article history:
Received 26 March 2009
Received in revised form 2 June 2009
Accepted 24 July 2009
Available online 29 July 2009

Keywords:
Amebiasis
E. histolytica
E. invadens
Chitinase
Homology modeling

ABSTRACT

Entamoeba histolytica, the causative agent of amebiasis infects through its cyst form and this transmission may be blocked using encystation specific protein as drug target. In this study, we have characterized the enzyme chitinase which express specifically during encystation. The reptilian parasite *Entamoeba invadens*, used as a model for encystation study contain three chitinases. We report the molecular cloning, over-expression and biochemical characterization of all three *E. invadens* chitinase. Cloned chitinases were over-expressed in bacterial system and purified by affinity chromatography. Their enzymatic profiles and substrate cleaving patterns were characterized. All of them showed binding affinity towards insoluble chitin though two of them lack the chitin binding domain. All the chitinases cleaved and released dimmers from the insoluble substrate and act as an exochitinase. Homology modeling was also done to understand the substrate binding and cleavage pattern.

1. Introduction

Entamoeba histolytica,(*Eh*) the main cause behind the amebiasis and diarrhea throughout the tropical continents is responsible for more than 40 million infection and 100,000 death per anum (Walsh, 1986), standing next to *Plasmodium*, one of the most pathogenic protozoa. Life cycle of this parasite encircle between two stages, cyst and trophozoite which help the parasite to accommodate within harsh environment and host body respectively. Till today metronidazole remains as a single drug of choice, though several reports of metronidazole resistant strains of different protozoa have been published over past years (Megraud and Doermann, 1998; Koivisto et al., 2004). The transformation stages are significant and potential point to inhibit the further propagation (Laughlin and Temesvari, 2005). Few stage specific molecules are reported and classified as attractive drug targets (Eichinger, 1997; Jarroll and Keriman, 2003).

Chitinase is one of such encystation specific molecule, and may be used as a potential drug target as reported in other protozoan parasites (Shahabuddin et al., 1993; Tsai et al., 2001). *Entamoeba* chitinase, being a stage specific enzyme found to express only during encystation (de la Vega et al., 1997). *E. invadens* (*Ei*) has been used as encystation model system for *Eh*, because *Eh* does not encyst well *in vitro*. *Ei* exist within some reptiles as a commensal, but is pathogenic in a subset of hosts, where it causes a similar type of colon pathology to that of *Eh* in humans (Donaldson et al., 1975). Genome sequencing have established that *Ei* has 74% identity in genic regions and 50% in intergenic regions of *Eh*, made the former an ideal material for comparative gene identification and gene model refinement study (Wang et al., 2003).

Transcription of chitin synthase begins within 8 h of *Ei* encystation (Campos-Gongoraa et al., 2004), which coincides, with the accumulation of vesicles containing microfibrilar structure (Chavez-Munguia et al., 2003). Onset of encystation was also found to initiates the chitinase expression that might be involved in chitin wall synthesis by producing tailor-made chitin fibril along with other cyst wall lectins (Ghosh et al., 1999; Van Dellen et al., 2006). During excystation, quadruplet trophozoites emerged from the chitin walled cyst by dissolving the wall (Chavez-Munguia et al., 2003), indicating the need of chitin breaking enzymes during excystation also. Transfected *Eh* and cysts were reported to have chitinase filled vacuoles in their cytoplasm (Ghosh et al., 1999) and probably some of the chitinase filled vesicles are stored within the cleft between the cyst wall and the cell membrane (Chavez-Munguia et al., 2003). Allosamidine, a typical secondary metabolite of *Streptomyces*, known as family-18 chitinase inhibitor, (Sakuda et al., 1986; Sakuda, 1996) inhibit the encystation of *Ei* (Villagomez-Castro et al., 1992). Several methylxanthine derivatives were also identified as chitinase inhibitor (Rao et al., 2005). Genome analysis of *Eh* predicted a single chitinase (de la Vega et al., 1997), while its reptilian counterpart *Ei* reported to have three chitinases, which were characterized partially (Villagomez-Castro and Lopez-Romero, 1996).

Chitinolytic system comprised of multiple chitinase is common among many prokaryotes and in few eukaryotes (Rast et al., 1991; Schickler et al., 1998). Multiple chitinase controlled by differential expression observed to play an array of roles from offensive to

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doi:10.1016/j.exppara.2009.07.008

defensive one. Occurrence of multiple chitinase may be attributed to gene duplication, though in most cases they are controlled by different promoters. Multiple chitinase system is very rare among human parasites, but present in some non-human parasites.

Here in this study we are reporting the molecular cloning and biochemical characterization of all three recombinant chitinase of *Ei*, as an initiating effort to understand the importance of these enzymes in protozoan parasite system, and their impact on host parasite interaction.

2. Methods and material

All the chemicals were of molecular biology grade and purchased from Sigma (USA), Merck (Germany), and HiMedia (India) unless it is mentioned in the text.

2.1. Culture of Entamoeba

Axenic culture of *Ei* (IP1 strain) was maintained in Diamond's TYIS-33 medium (Diamond, 1986), at 25 °C in 50 ml culture flask.

2.2. Cloning of Ei chitinases

Presence of three chitinase genes was predicted in *Ei* genome (Wang et al., 2003). From completed *Ei* genome project and the submitted sequence at GenBank, we obtained the sequences of three *Ei* chitinase (Chit1-Acc. number **AAB52724**, Chit2-Acc. number **ABC59330**, Chit3-Acc. number **ABC59331**), and gene specific primers were designed using Oligo perfect software (Invitrogen), excluding the N-terminal signal sequence in every case (Primer sequences are given in Table 1).

Ei genomic DNA was isolated using genomic DNA isolation kit (Promega) following the manufactures protocol. A typical PCR reaction mixture contained 20 pmol of each forward and reverse primer, and 0.5 U of the Expand Hi fidelity *Taq* polymerase (Roche) in a final volume of 50 μl. Reaction mixtures were incubated in a thermocycler, at 95 °C for 5 min, followed by 35 cycles at 92 °C for 30 s, 55 °C for 30 s, 68 °C for 90 s and then by a final extension at 72 °C for 5 min. Amplified products were checked in 0.8% agarose gel and were purified using gel purification kit (Qiagen) following the manufacturer's protocol. All the amplified chitinases were cloned in pGEM-T vector (Promega, USA) following the manufacturer's instruction. In brief, ligation was carried out at 16 °C for 16 h. Ligated products were transformed to *E. coli,* strain DH5α. Recombinant colonies were confirmed by restriction digestion and sequencing. Finally all three *Ei* chitinase genes were cloned in bacterial expression vector pQE30 (Qiagen, Germany) for overexpression. Expression was done in *E. coli* M15 strain and was optimized at different IPTG concentrations and temperature varying the time of post induction incubation.

Table 1
Sense and antisense primer sequence used for EiChit1, EiChit2 and EiChit3 amplification. BamHI in all the sense primers and XhoI in all the antisense primers are in italics and underlined.

EiChit1	Sense-5′ *GGATCC* ATG TGT GAA GGA CTC GAC AAC GG 3′
	Antisense-5′ *CTCGAG* TTA GCA ACC GAT CAA GCT CTT TC 3′
EiChit2	Sense-5′*GGATCC* ATG AAG GTG GTT GGG TAC TAT AC 3′
	Antisense-5′ *CTCGAG* TCA ATC AGA TGC TAA AAC AC 3′
EiChit3	Sense-5′ *GGATCC* ATG AAA GTG ATT GGA TAC 3′
	Antisense-5′ *CTCGAG* TTA GTT TTT CAA CTC ATC GAT C 3′

2.3. Expression and purification of Ei chitinases in bacterial system

To determine the solubility of expressed chitinases within the bacterial cell, the induced cell pellets were washed and sonicated in 1× PBS. The sonicated lysate were then centrifuged to separate the membrane bound and cytoplasmic fraction. Both the supernatant and pellet fractions were boiled with 1× sample loading buffer, and verified in 12% denaturing SDS–PAGE. Further confirmations of expressed protein were done by Western blot using Anti-His monoclonal antibody (Sigma). Expressed proteins were purified by affinity chromatography using Ni–NTA agarose (Qiagen) as per the manufacturer's instruction. Briefly the Ni–NTA beads were washed and equilibritated with 1× PBS in column; washing of column was done by wash buffer (50 mM Sodium dihydrogen phosphate, 300 mM sodium chloride, 20 mM Imidazole) (pH 8). 250 mM Imidazole was used for elution, and protein homogeneity was checked in 12% SDS–PAGE. The eluted fractions were further concentrated using 30 k Microsep Centrifugal device (Pall life sciences, Germany) and concentrations were measured using Bradford method.

2.4. Enzymatic characterization

Enzymatic activities of expressed different chitinases were measured using soluble artificial substrate 4-methylumbelliferyl-tri-*N*-acetylchitotrioside (Sigma). The substrate was dissolved in pure DMSO to prepare 1 mM stock. In a typical reaction volume of 300 μl, 200 mM of sodium phosphate buffer (pH 7.2) and substrate (1–10 μM) was added, with varying amount of purified enzyme and reaction was carried out for 15–30 min at 25 °C. 100 μl of 200 mM sodium carbonate solution was used to stop the reaction. DMSO without substrate was used as negative control. The concentration of the fluorescent 4-methylumbelliferone (4MU) product generated from these substrates was determined using 4MU as standard. One unit of chitinase activity was defined as the amount of enzyme required to release 1 nmol of 4-methylumbelliferone (4-MU) per second at optimum temperature. Optimum pH was determined using 200 mM sodium phosphate buffer for 5–8 pH range, and 200 mM sodium acetate buffer for pH 3–5 range. Optimum temperature was determined by repeating the experiments between 5 °C and 45 °C temperature. Fluorescence of the products was measured by a Varian spectro-fluorometer, model Cary–Eclipse equipped with a Xenon lamp (excitation at 360 nm and emission at 450 nm).

2.5. Estimation of substrate binding specificity

Substrate binding specificity and affinity of different *Ei* chitinases were determined by incubating the purified enzyme, with the chitin beads (New England Biolabs), prepared colloidal chitin (Hackman, 1962; Jeuniaux, 1966), and crab shell chitin powder (Sigma). Briefly the substrates were washed with 1× PBS and incubated with purified enzyme for 2 h with occasional shaking, at room temperature. The supernatant was removed by centrifugation at 10,000 rpm for 5 min, and the pellets were washed twice with 1× PBS. The supernatant and pellet were boiled with 1× sample dye and were checked by 12% SDS–PAGE. BSA (fraction five) was used as a negative control under same condition to nullify the adsorption probability.

2.6. Substrate cleavage pattern

Colloidal chitin was prepared following the previously published protocol (Hackman, 1962; Jeuniaux, 1966). The colloidal material was freeze dried and used as a substrate to understand the substrate cleavage pattern. The reaction mixture included