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ORIGINAL PAPER

Immunodominant antigens in *Naegleria fowleri* excretory–secretory proteins were potential pathogenic factors

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Abstract *Naegleria fowleri*, a ubiquitous pathogenic free-living amoeba, is the most virulent species and causes primary amoebic meningoencephalitis in laboratory animals and humans. The parasite secretes various inducing molecules as biological responses, which are thought to be involved in pathophysiological and immunological events during infection. To investigate what molecules of *N. fowleri* excretory–secretory proteins (ESPs) are related with amoebic pathogenicity, *N. fowleri* ESPs fractionated by two-dimensional electrophoresis were reacted with *N. fowleri* infection or immune sera. To identify immunodominant ESPs, six major protein spots were selected and analyzed by N-terminal sequencing. Finally, six proteins, 58, 40, 24, 21, 18, and 16 kDa of molecular weight, were partially cloned and matched with reference proteins as follow: 58 kDa of exendin-3 precursor, 40 kDa of secretory lipase, 24 kDa of cathepsin B-like proteases and cysteine protease, 21 kDa of cathepsin B, 18 kDa of peroxiredoxin, and 16 kDa of thrombin receptor, respectively. These results suggest that *N. fowleri* ESPs contained important proteins, which may play an important role in the pathogenicity of *N. fowleri*.

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Introduction

Naegleria fowleri is a pathogenic free-living amoeba which causes primary amoebic meningoencephalitis (PAME) in experimental animals and humans. The organisms are commonly found in soil and in warm bodies of fresh water such as lakes, rivers, hot springs, and unchlorinated swimming pools and in warm water discharge pools from industrial plants (Culbertson 1970). PAME is a fulminant infection that typically leads to death with 1 to 2 weeks from the onset of symptoms. The trophozoites, active stages of *N. fowleri*, infect the brain through the olfactory bulb by penetrating nasal epithelium (Ma et al. 1990). Cloning of pathogenicity-related agents of PAME by *N. fowleri* is important in the exploration to determine the mechanisms of parasite–host interaction. The factors that determine the pathogenicity of *N. fowleri* have not been fully established. The pathogenicity-related agents of the *N. fowleri* depend on a contact mechanism and non-contact mechanism with host cell. In a contact mechanism of *N. fowleri* infection, Nfa1 protein had been characterized (Shin et al. 2001a; Jeong et al. 2004). In non-contact mechanism, excretory–secretory proteins (ESPs) of parasites are involved, and their pathogenicity may be related to the properties of ESP components. In fact, it was the amount of evidence that several studies have shown that ESPs contributed to parasitic disease. In secreted factors of helminthes, which affect the function of host cells, *Echinococcus ganulosus*

secretes a protein, which inhibits neurtophil chemotaxis (Shepherd et al. 1991).

Darcy et al. (1988) reported that excreted/secreted antigens of *Toxoplasma gondii* could play a major role in the immune response and demonstrated the key role played by excreted/secreted antigens in the protective immune response. Thus, they suggested that these antigens should be of value for the development of new strategies for immunization against toxoplasmosis. It is apparent that the ability of *Candida albicans* to transport proteins onto the cell surface via the secretion pathway and to secrete degradative enzyme out of the cell is required for virulence and pathogenesis (Naglik et al. 2004).

At present, the role of ESPs in immunity against *N. fowleri* infection and the mechanisms by which *N. fowleri* produce cell damage were poorly understood. Several attempts have been made to induce protective immunity against experimental *N. fowleri* amoebae in mice, but the study of immunity in experimental and natural infections has been limited to the analysis of serum antibody responses (Bush and John 1988).

In a preliminary study, ESPs of *N. fowleri* showed the in vitro cytotoxicity against target Chinese hamster ovary (CHO) cells. In the present study, we would like to explain pathogenecity-related agents, which contribute to *N. fowleri* diseases and found pathogenicity-related proteins from *N. fowleri* ESPs using proteomics-based approaches such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional electrophoresis (2-DE), and Western blot analysis with anti-ESP/infection sera obtained from infected BALB/c mice. Also, this study pursued the possible role of ESPs on the penetrating of *N. fowleri* into host cells.

Materials and methods

Parasite and ESPs

N. fowleri trophozoites (ATCC no. 30215) were axenically cultured at 37°C in Nelson's medium containing 10% fetal bovine serum (Willaert 1971). For ESP collection, *N. fowleri* trophozoites (1×10^8) were incubated at 37°C for 1 h with phosphate-buffered saline (PBS). After centrifugation at 1,300 rpm for 5 min, supernatants were saved as ESPs. The protein concentration of ESPs was adjusted to 1.0 mg/ml per the batches.

Production of anti-ESPs and infection sera

Anti-ESPs and infection sera were prepared according to a method described in a previous paper (Shin et al. 2001b). For the production of infection sera, *N. fowleri* trophozoites (1×10^5) were infected intranasally into 6-week-old female BALB/c mice (purchased from the Korea Institute of Science and Technology, Daegeon, Korea) under anesthesia. Blood were collected from mice showing the behavior of death due to PAME. For the production of anti-ESP sera, ESPs (50 μg/mouse) were mixed with an equal volume of Freund's complete adjuvant (Sigma, St Louis, MO, USA) and injected intraperitoneally into a mouse, and then the mouse received biweekly booster injections with ESPs (25 μg/mouse) containing equal volume of incomplete Freund's adjuvant (Sigma Chemical Co., St. Louis, USA) for another 4 weeks. After third booster injection, ESPs (5 μg/mouse) without adjuvant were injected intravenously. Four days later, anti-ESP polyclonal sera were collected from mice blood by centrifugation at 12,000 rpm and 4°C for 10 min. An enzyme-linked immunosorbent assay (ELISA) was performed with ESPs (5 μg/mouse) and rabbit antimouse whole immunoglobulin (1:10,000 dilution) conjugated with alkaline phosphate (Sigma Chemical Co.). Western blot analysis against ESP protein was performed according to a method described in a previous paper (Laemli 1970). These sera were stored −20°C until use.

In vitro cytotoxicity of *N. fowleri* ESPs on CHO cells

CHO cells are useful in observing in vitro cytotoxicity of amoeba (Song et al. 2008). CHO cells were cultured as monolayer using 24-well culture flask (Nunc A/S, Roskilde, Denmark) in Earle's minimal essential medium (EMEM; Gibco BRL, Gaithersburg, MD, USA) at 37°C. In control group, 3×10^4 CHO cells were cultured only in 500 μl of EMEM, 3×10^4 CHO cells cultured with various concentration *N. fowleri* ESPs in experimental groups. The total volume per well was 500 μl with EMEM. CHO cells were observed at cultured intervals using an inverted microscope. To measure in vitro cytotoxicity of ESPs, lactate dehydrogenase (LDH) released from lysed cells was assayed as previously described (Kim et al. 2008). For LDH assay, 50 μl of reacted supernatant in each well was transferred on 96-well assay plates (Nunc A/S, Roskilde, Denmark). After 50 μl of the reconstituted substrate mix, buffer in CytoTox96® Non-radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA) for LDH release assay was added, the plate was incubated 30 min at room temperature, and then 50 μl of stop solution was added. The reactants were read at 490 nm with ELISA reader. The formula of in vitro cytotoxicity was as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

SDS–polyacrylamide gel electrophoresis and 2-DE

ESP samples were analyzed by 15% SDS-PAGE using reducing sample buffer (62.2 mM Tris pH6.8; 10% glycerol; 10% 2-mercaptoethanol; 3% SDS and 0.1% bromophenol blue). ESP samples (100 μg) were applied for analytical run (pattern comparison). Rehydration, isoelectric focusing (IEF), and equilibration were performed as previously described (Lee et al. 2006). The ESP samples, containing 100 μg for 2-DE SYPRO Ruby-stained gels, were diluted in 125 μl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.2% IPG buffer of the respective pH gradient and bromophenol blue). The samples were allowed to mix gently for 1 h at room temperature before centrifugation at 18,000×*g* over 30 min to remove all particulate material. The supernatants were applied to the IPG strips by in-gel rehydration at 20°C for at least 12 h, after which IEF was run for a total of 20,000 V/h. Following IEF, the strips were reduced in equilibration buffer (6 M urea, 0.05 M Tris pH8.8, 2% SDS, and 20% glycerol) containing 2% DTT over 15 min and then alkylated in equilibration buffer containing 2.5% iodoacetamide for 10 min. The second dimension was performed on 4–20% gradient SDS-polyacrylamide gels (Amersham Biosciences, Uppsala, Sweden). Running proceeded at 80 V/gel for the first 15 min and then at 130 V/gel. After running, 2-DE gels were either stained or transferred onto polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA, USA) for N-terminal sequencing and Western blot analysis.

Western blot analysis

ESP proteins were separated by SDS-PAGE and transferred to PVDF membrane for 2 h at 250 mA post 2-DE. Following transfer, the membranes were incubated in PBST containing 5% skimmed milk overnight at 4°C. After washing with 0.05% PBST three times, primary anti-ESP (1:500 dilution) or infection (1:500 dilution) sera were applied to the membranes overnight at 4°C. After washing with 0.05% PBST, the antimouse Ig G secondary antibody conjugated with alkaline phosphatase (Sigma Chemical Co.) was applied to the PVDF membrane at a dilution of 1:1,000 in PBS for 2 h. After washing with 0.05% PBST three times, the membranes were developed using AP conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA).

N-Terminal sequencing analysis and data searching

Selected ESP spots were requested for N-terminal sequencing to Korea Basic Science Institute (Seoul, Korea). Unidentified proteins separated by 2-DE PAGE were electrotransferred onto a PVDF membrane, and selected protein spots were excised and subjected to N-terminal sequencing using a Procise 492 cLC Model 610A Protein sequencer (Applied Biosystems, Hong Kong, China). Amino acid sequences obtained were searched against either the Protein DataBank (PDB) by BLAST. Settings for querying short sequences for nearly exact matches of peptides were used.

Results

In vitro cytotoxicity *N. fowleri* ESPs on CHO cells

To evaluate the cytotoxicity of *N. fowleri* ESPs on CHO target cells, LDH release assay was carried out. When CHO cells were treated with ESPs of *N. fowleri*, the cytotoxicity was gradually increased by a time- and dose-dependent manner (Fig. 1). It suggested that *N. fowleri* ESPs affected target cells as cytotoxic agents.

Protein profiles and Western blot analysis of *N. fowleri* ESPs

SDS-PAGE (15%) analysis of *N. fowleri* ESPs revealed different protein profiles patterns in comparison with *N. fowleri* lysate. The *N. fowleri* ESP bands corresponding to 91, 73, 60, 58, 49, 45, 40, 36, 28, 24, 20, 21, 18, and 16 kDa of molecular weight were observed as major proteins by Coomassie brilliant blue (Fig. 2a). To investigate the antigenicity of the *N. fowleri* ESPs, Western blot analysis was performed with anti-ESPs or infection sera. The results of Western blot analysis with anti-ESPs, 58, 48, 40, 30, 26, 24, 21, 18, and 16 kDa bands, showed immunodominance (Fig. 2b). In the Western blot analysis with infection sera, two major immunodominant bands, 24 and 18 kDa, were detected (Fig. 2c).

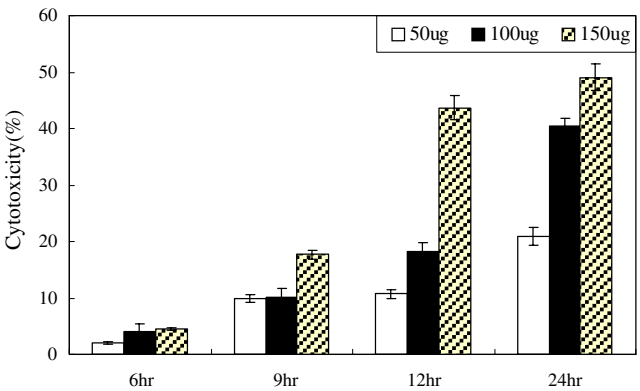

Fig. 1 In vitro cytotoxicity ESPs from *N. fowleri* on CHO target cells. CHO cells were cultured and treated with 50, 100, and 150 μg of *N. fowleri* ESPs for 6, 9, 12, and 24 h. Values are mean±standard error of three experiments in triplicate

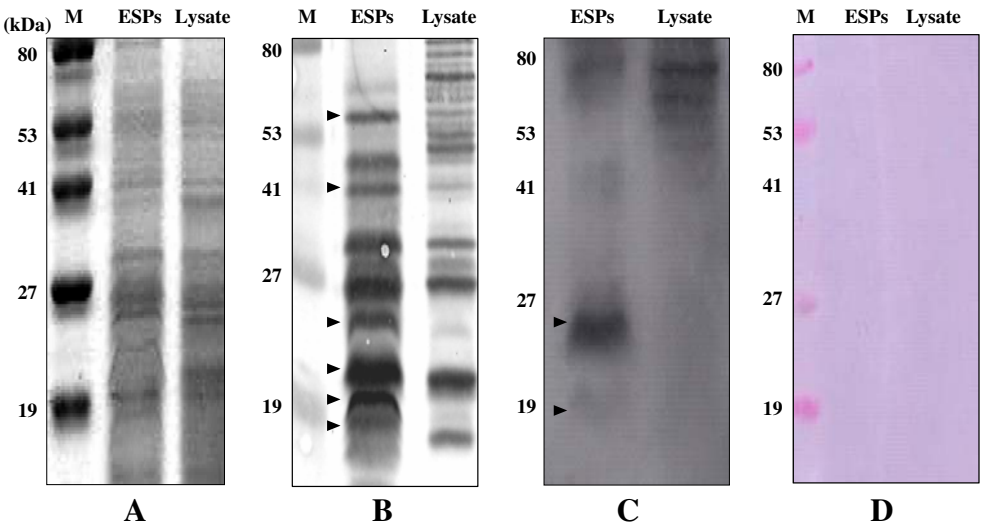

Fig. 2 SDS-PAGE band patterns and Western blots of *N. fowleri* ESPs and lysates. **a** *N. fowleri* ESPs and lysates were electrophoresed under reducing conditions and stained with Coomassie blue. **b**, **c**, and **d** Western blots analysis of the *N. fowleri* ESPs with anti-ESP or infection sera and normal mouse sera for negative control. *M* molecular size marker, *lane 1N. fowleri* ESPs, *lane 2N. fowleri* lysates. *Arrowheads* indicate major antigenic ESPs

Protein profiles and Western blot analysis of *N. fowleri* ESPs post 2-DE

To select immunodominant proteins in the *N. fowleri* ESPs, 2-DE and Western blot analysis with ESPs sera or infection sera were carried out (Fig. 3). Most spots are localized in the low p*I* region (data not shown), so we separated the ESPs with low p*I* IEF strip (p*I*4–7; Fig. 3a). In the results of Western blotting with the anti-ESPs sera, six antigenic spots, 58, 40, 30, 25, 24, 21, 18, 16, and 15 kDa, were

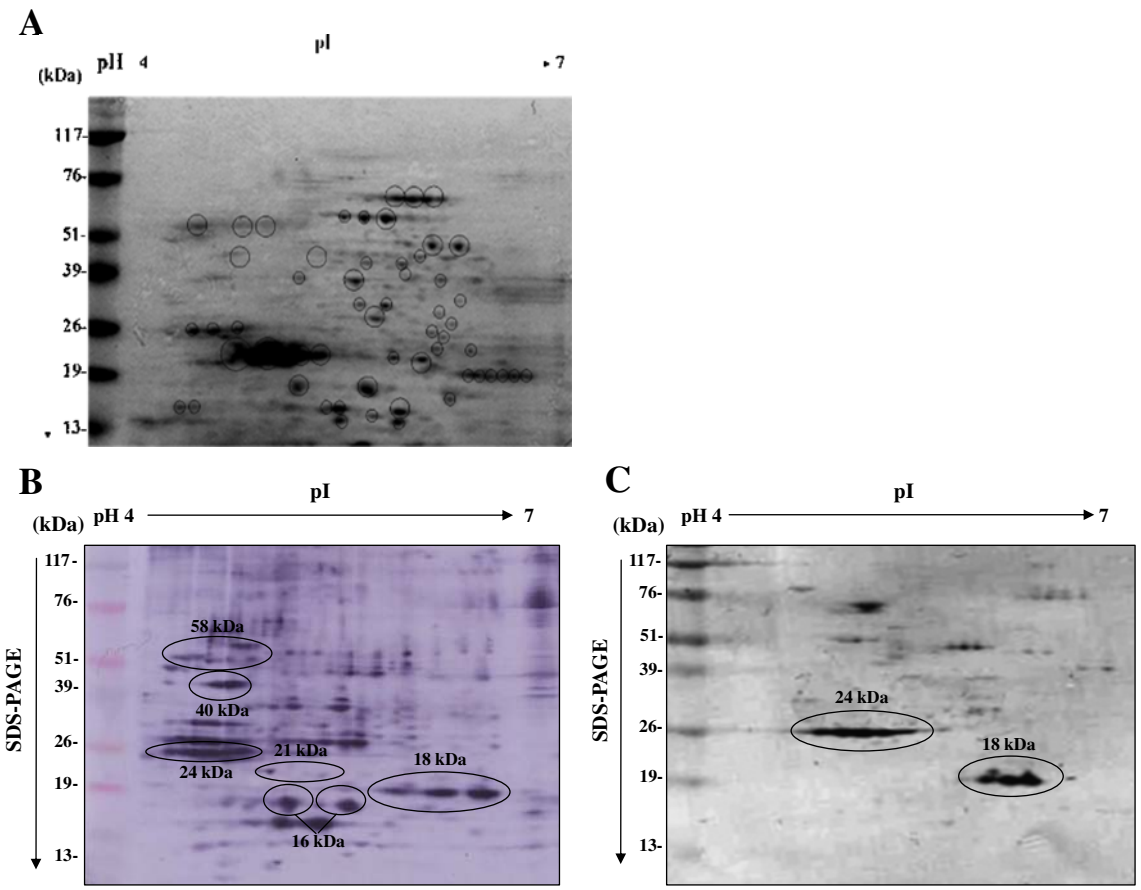

Fig. 3 Protein profiles by 2-DE and Western blots of *N. fowleri* ESPs. *N. fowleri* ESPs were analyzed by 2-DE and Western blotted with anti-ESPs sera and infection sera. **a** Protein spots after 2-DE. **b**, **c** Western blots analysis of the *N. fowleri* ESPs with anti-ESP sera or infection sera

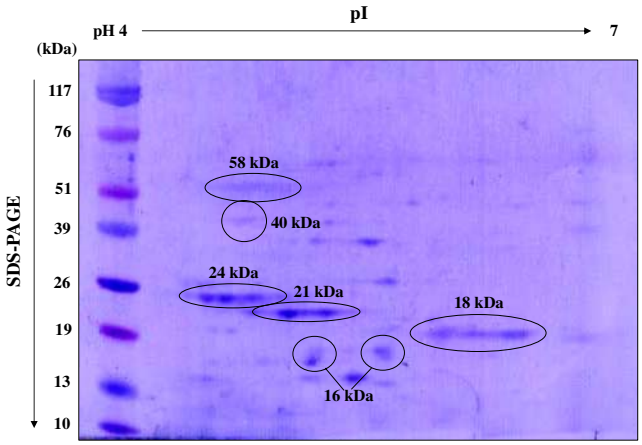

Fig. 4 Protein spots of *N. fowleri* ESPs post 2-DE. ESPs spots of *N. fowleri* were electrotransferred to PVDF membrane post 2-DE for N-terminal sequencing

immunodominant (Fig. 3b). In the case of the infection sera, two antigenic spots, 24 and 18 kDa, were immunodominant (Fig. 3c).

N-Terminal sequencing of candidate antigen-related proteins

Immunodominant 2 proteins (24 and 18 kDa), which commonly reacted with the two kinds of antibodies, and four proteins (58, 40, 21, and 16 kDa), which reacted only anti-ESPs sera, were requested for N-terminal sequencing (Fig. 4). As results of search with the PDB by BLAST, 58 and 40 kDa of protein were matched as secreted exendin-3 precursor protein and as secretory lipase. In turns, 24 and 21 kDa of protein were matched as cathepsin B-like protease and as cathepsin B. In addition, 18 and 16 kDa protein were matched as peroxiredoxins and as thrombin receptor (Table 1).

Discussion

To understand the pathogenesis of PAME induced by *N. fowleri* infection, the identification of pathogenecity-related agents of *N. fowleri* is important. Previous studies reported that in relation with pathogenicity, parasites secreted or excreted various proteins (Shin et al. 2001a). Parasites employ a variety of strategies to evade and/or modify host immune responses. ESPs released by parasitic helminths have been shown to both induce host antiparasite immune responses as well as modify the function of host immune cells (Lightowlers and Rickard 1988). In this present study, the distinct patterns of the ESPs were observed by 2-DE and Western blotting with anti-ESP or infection sera. Despite the absence of a *N. fowleri* genome sequencing project, this global investigation of the components from *N. fowleri* ESPs by a proteomic approach has been shown that it was possible to identify a number of the prominent proteins found using 2-DE.

The results revealed that *N. fowleri* ESPs contained various pathogenic proteins, which function in organism entering into host cell and various dominant antigenic proteins, such as secreted effector protein, cathepsin B-like protease, cysteine protease, secretory lipase, peroxiredoxins, and thrombin receptor.

Christel and DeNardo (2006) have recently demonstrated that release of exendin-4, an exendin-3 analog, is controlled by mechanical action in glia monsters, *Heloderma suspectum*, and suggest that exendin-4 is released from the salivary glands in response to mechanical stimulation and not detected in food either by smell, taste, or distention of gut. In this present study, 58 kDa secreted exendin-3 precursor protein of *N. fowleri* ESPs may play an important role in the digestion and absorption-related events of uptake molecules through food-cup structure, but further study is needed to elucidate the functional role of exendin-3 precursor protein. Both cathepsin B and cathepsin B-like

Table 1 Identification of *N. fowleri* ESPs identified by N-terminal sequencing

Protein MW (kDa)	Species	Accession no. (NCBI)	N-Terminal sequence in data base	Matching protein	Matched protein peptide (%)
58	*Heloderma horidium*	P20394	FTSDLSKQMEEEAV	Exendin-3 precursor	14 (100)
40	*Polynucleobacter* spp.	YP001155982	PVVPLNDY	Secretory lipase	7 (87)
24	*Ancylostoma ceylanicum*	AAC46877	APAQSFDART	Cathepsin B-like protease	7 (87)
21	*Streblomastix strix*	ABC97349	AFDSRTKWPBN	Cathepsin B	7 (77)
18	*Aedes aegypti*	EAT34216	IQKPAPQFSG	Peroxiredoxins	8 (80)
16	*Cricetulu longicaudatus*	AAA86747	SLPLNILAFV	Thrombin receptor	9 (75)

protease are cysteine proteases, which are secreted into the hosts, and each of these has been proposed to facilitate degradation of ingested host proteins including hemoglobin (Brindley et al. 1997; Klinkert et al. 1989; Caffrey and Ruppel 1997; Weinbauer et al. 2001). Kong et al. (1994) showed that 27-kDa cysteine proteases of *Spirometra mansoni* has been found to be most important in tissue invasion and nutrient uptake, and its biochemical and structural nature has well been characterized. The cysteine proteases also modulate host immune response by cleaving immunoglobulins or by provoking IgE antibody responses. The roles and developmental modulation of cysteine proteases have been known in many parasites (Chung et al. 1997; Skuce et al. 1999). In this study, two cysteine proteases secreted by *N. fowleri*, 21 and 24 kDa of molecular weight, have been shown to be major enzymes in the *N. fowleri* ESPs. We suggest that the functional roles of the cysteine protease secreted by *N. fowleri* may be involved in an invasion to the blood–brain barrier of host.

Peroxiredoxins are ubiquitous peroxidases that reduce reactive oxygen and nitrogen species such as hydrogen peroxide, alkyl hydroperoxides, and peroxinitrate using an active-site cysteine residue. The reducing equivalents required for these reactions are provided by thioredoxin, glutaredoxin, alkylhydroperoxide reductase, glutathione, or cyclophilin. Donnelly et al. (2005) reported that thioredoxin peroxidase secreted by *Fasciola hepatica* induces the alternative activation of macrophages. Hence peroxiredoxins contained within the ESPs may be inhibited macrophages, which activated by a specific signal dependent pathway.

Bacterial lipases have been implicated in a variety of pathogenic processes, including the inhibition of phagocyte function (Aragon et al. 2002). Thrombin is concentrated at vascular injury sites within fibrin clots. Fibrin-binding thrombin is protected against inhibitors and gradually released into a wound area, in order to regulate tissue repair (Coughlin 1999). Thrombin interaction with cells is mediated by thrombin binding to a seven transmembrane domain, G-protein-coupled cell surface receptor, which belongs to the so-called protease activated receptors family (Cocks and Moffatt 2000). We suggest that the role of thrombin receptor in *N. fowleri* ESPs may be to activate inflammation, but the exact role of secreted thrombin receptor of *N. fowleri* would be elucidated with further studies.

In this study, we identified several candidate proteins, which should be related with pathogenicity of *N. fowleri*. These results suggest that antigenic proteases in *N. fowleri* ESPs may play a part in host cell invasion by *N. fowleri* and the cleaving ability of host immunoglobulins, which may contribute as one of the immune evasion mechanisms for parasite survival in the host. Peroxiredoxins, cysteine protease, and thrombin receptor secreted by *N. fowleri* have been implicated in host cell invasion and facilitated immunoresponse suppression of hosts. Finally, *N. fowleri* ESPs appear to play a vital role, with the proteolytic activity being preferentially expressed during the period in which the parasite actively invades or migrates through the tissue/organs of the host. Also the above several protein findings prompted us to observe what other composing proteins exist in *N. fowleri* ESPs and to confirm what other antigenic proteins are secreted and what functions exist in ESPs.

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