77/04

Proteinases in *Naegleria Fowleri* (strain NF3), a pathogenic amoeba: a preliminary study

Nakisah Mat Amin
Department of Biological Sciences, Faculty of Sciences and Technology, Kolej Universiti Sains dan Teknologi Malaysia [KUSTEM], Mengabang Telipot, 21030 Kuala Terengganu, Terengganu, Malaysia

ABSTRACT

Naegleria fowleri is a free-living amoeba, known as a causative agent for a fatal disease of the central nervous system (CNS) in man such as Primary amoebic meningoencephalitis (PAM). Factors contributing to its pathogenicity and its distribution in the environment have been investigated by previous researchers. In case of its pathogenicity, several enzymes such as phospolipase A and sphingomyelinase, have been proposed to probably act as aggressors in promoting PAM but no study so far have been conducted to investigate the presence of proteinase enzyme in this amoeba although a 56kDa cystein proteinase enzyme has been identified in *Entamoeba histolytica* as an important contributing factor in the amoeba's virulence. In this preliminary study, a pathogenic amoeba, *Naegleria fowleri* (strain NF3) was examined for the presence of proteinases. Samples of enzymes in this amoeba were analysed by electrophoresis using SDS-PAGE-gelatin gels. The results showed that this amoeba possesses at least two high molecular weight proteinases on gelatin gels; their apparent molecular weights are ~128 kDa and ~170 kDa. Band of ~128 kDa enzyme is membrane-associated and its activity is higher at alkaline pH compared with lower pH; at lower pH, its activity is greatly stimulated by DTT. The ~170 kDa band enzyme appears to be inactivated at pH 8.0, at

lower ph its activity is higher and DTT-dependance. The activity of this enzyme is partially inhibited by inhibitor E-64 but markedly inhibited to antipain suggesting it belongs to the cysteine proteinase group.

INTRODUCTION

Naegleria fowleri, a free-living amoeba is known as a causative agent for diseases of the central nervous system (CNS) in man such as Primary amoebic meningoencephalitis (PAM), a fatal disease (Martinez, 1985). Factors contributing to its pathogenicity and its distribution in the environment have been investigated by previous researchers. In case of its pathogenicity, several enzymes such as phospholipase A and sphingomyelinase, have been proposed to probably act as aggressors in promoting PAM (Ferrante and Bates, 1988). No study so far have been conducted to investigate the presence of proteinase enzyme in this amoeba although a 56kDa-cystein proteinase enzyme has been identified in *Entamoeba histolytica* as an important contributing factor in the amoeba's virulence (Keene et al., 1990).

MATERIALS AND METHODS

In this preliminary study, a pathogenic *Naegleria fowleri* (strain NF3) grown in modified Chang medium, obtained from Prof Dr Huw Smith, Stobhill Hospital, Glasgow was examined for the presence of proteinase enzymes. Originally this medium was devised by Chang (1974) and described by De Jonckheere (1977) as Serum-Casein-Glucose-Yeast Extract Medium. This medium was made-up as follows: Casein digest (BBL), 10.0 g; $Na_2HPO_4.7H_2O$, 0.8 g; Yeast extract (DIFCO), 5.0 g; and glucose, 2.5 g, dissolved in 1000 ml distilled water. The pH the of medium was adjusted to 6.9 with 1 N

NaOH. The medium was autoclaved at 12 psi. for 15 min. The modified medium has 2.5 g liver digest (OXOID) added to the original recipe (Dr Simon Kilvington, Department of Infection, Immunity & Inflammation, School of Medicine, University of Leicester, Leicester, UK., personal communication). Before use, heat-inactivated fetal calf serum (GIBCO) was added to the autoclaved medium to a final concentration of 10% (v/v). Penicillin (1000 units) and streptomycin (1000 units) were added per 100 ml medium. The complete medium was stored at 4°C. The modified medium used to grow *N. fowleri* NF3 in this study obtained from Dr Andrew Campbell (Department of Bacteriology, Stobhill Hospital, Glasgow, personal communication) was as follows: Before use, 1.0 ml of 10% (w/v) liver digest in distilled water (PANMEDE), 5.0 ml of heat-inactivated foetal calf serum (GIBCO), penicillin (200 units) and streptomycin (200 units) were aseptically added to 95 ml of the original Chang's medium. The examination for proteinase activity in *N. fowleri* was done only in their trophozoites since this form has been reported to cause tissue destruction in diseases inflicted by this amoeba in man. Cell lysates of *Naegleria gruberi* (strain CCAP 1518/1A) was used for reference the position of the proteinase bands in *N. fowleri*.

In this study, the trophozoites of amoeba were harvested during log-phase growth (at an approximate density of 1-2 x 10^6 cells ml^{-1}) by centrifugation (500g, 10 min) and washed twice in phosphate buffered saline (PBS). The pellets formed were then subjected to freeze-thawed lysis alternately in liquid nitrogen and in water bath at 37^0C to obtain the samples of enzymes. After centrifugation in an Eppendoff bench centrifuge at 12,000 rpm for 5 min, two samples were obtained: 1) the supernatant sample and 2) the

remaining pellet which was resuspended in 0.25% (v/v) Triton-X. All samples were run on gelatin gels to detect the proteinase activities.

The electrophoretic analysis of samples was done using SDS-PAGE-gelatin gels following techniques of Lockwood et al., (1987). To characterise and classify the enzymes, the gelatin gels were incubated at 37^{o}C overnight in sodium phosphate buffer at different pH, with or without DTT and in two inhibitors, E-64 and antipain at concentrations recommended by Beynon and Salvesen (1989).

RESULTS AND DISCUSSION

Naegleria fowleri (strain NF3) in this study was observed to possess at least two high molecular weight proteinases on gelatin gels; their apparent molecular weights are ~128 kDa and ~170 kDa. (Figure 1). Band of ~128 kDa enzyme is membrane-associated since it was detected in the pellet sample, and its activity is higher at alkaline pH compared with lower pH; at lower pH, its activity is greatly stimulated by DTT. The ~170 kDa band enzyme appears to be inactivated at pH 8.0, at lower pH, its activity is higher and DTT-dependant. The activity of this enzyme is partially inhibited by inhibitor E-64 but markedly inhibited by antipain suggesting it belongs to the cysteine proteinase group.

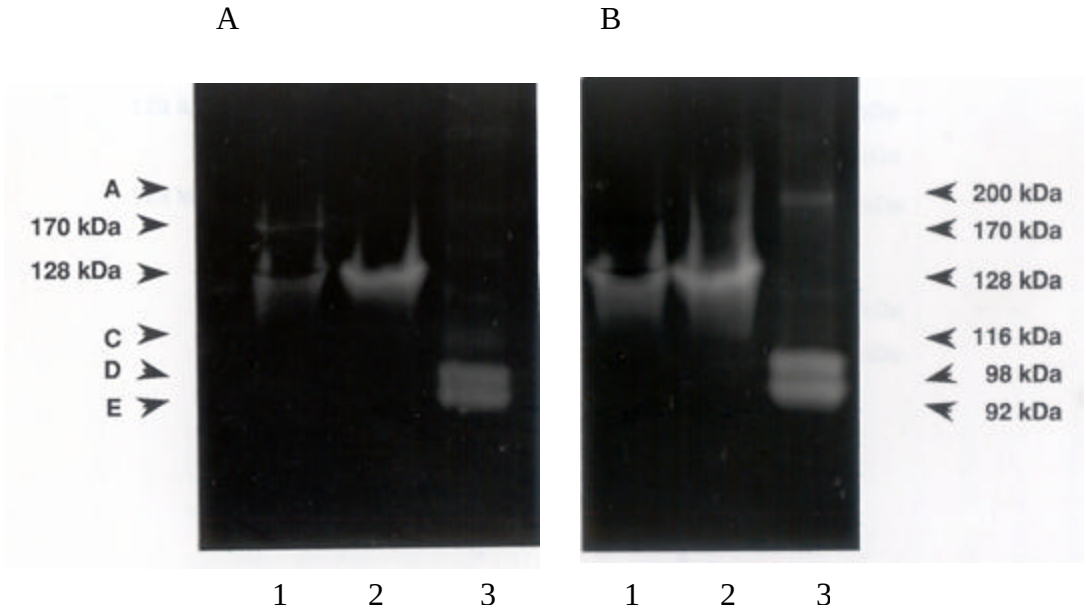

Figure 1. Proteinases in pathogenic *N.fowleri*.(strain NF3). Both supernatant and pellet samples of freeze-thawed cells were run on the gelatin gels. The cell lysate of non-pathogenic *N.gruberi* CCAP strain 1518/1A were used to compare the position of the proteinase bands in *N.fowleri*. Explanation for panels: A. the gel was incubated in 0.1M sodium phosphate buffer, pH5.5 containing 1mM DTT, B. the gel was in cubated in sodium phosphate buffer pH 8.0, without containing DTT. Both gels were incubated at 37°C. Explanation for lanes: 1;supernatant of freeze-thawed sample, 2;pellet of freeze-thawed sample and resuspended in 0.25% (v/v) Triton X-100 in PBS, 3; cell lysate of *N. gruberi*

The lysate of *N. fowleri* has been observed to cause tissue destruction *in vitro* (Fulford et al., 1985; Marciano-Cabral and Fulford, 1986). Proteinases in *N. fowleri* observed in this study could also be involved in tissue destruction and in its pathogenesis since this enzymes present in the cell lysates. The involvement of proteinases in the pathogenecity of some parasitic protozoa have been proven by Robertson and Coombs (1992) and Keene et al., (1990).

Proteinases in *N. folweri* observed in this study appear to be high molecular weight enzymes whereas proteinases reported from other protozoa generally have their apparent molecular weight in the range of 20 kDa to 96 kDa (Keene *et al.*, 1990; North *et al.*, 1990; Robertson and Coombs, 1992). Despite being high in apparent molecular weight, the proteinases in this amoeba are of the cysteine proteinase type. It is interesting to speculate why there are not many forms of proteinases present in pathogenic *N. fowleri* compared with other parasitic protozoa such as *Trichomonas* spp (Lockwood *et al.*, 1987) and *Leishmania mexicana mexicana* (Robertson and Coombs, 1992). Are the multiple forms of proteinases in these protozoa simply due to the presence of multiple genes or are they the result of posttranslational modifications so they are not related to pathogenicity? Details on the functional aspects of proteinases in *N. fowleri* however, need to be carried-out to further verify if these enzymes are related to the amoeba's pathogenesis as have been observed in *Entamoeba histolytica*.

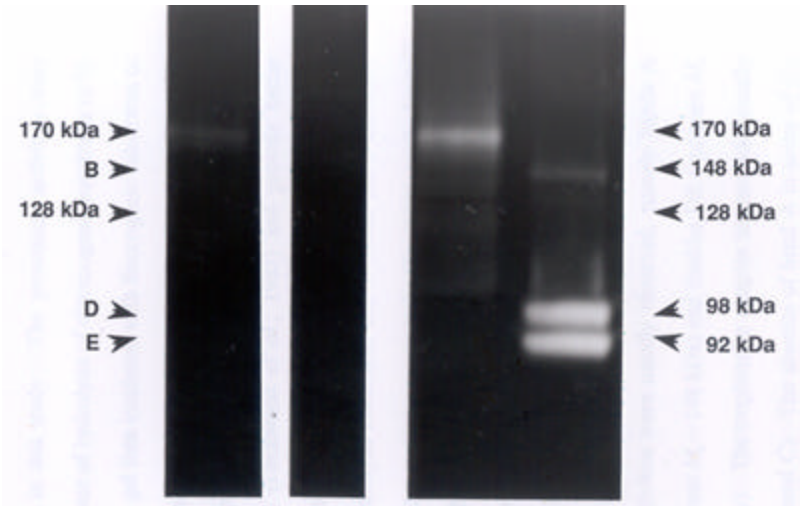

Figure 2: Effect of the inhibitors on proteinases of *Naegleria fowleri*. The gels were incubated in sodium phosphate buffer, pH 5.5 without containing DTT. Explanation for lanes: 1-inhibitors E-64, 2: antipain, 3; control, 4; cell lysates of *N. gruberi* strain 1518/A

REFERENCES

Beynon R.J. and Salvesen, G. 1989. Comercially available protease inhibitors. In *Proteolytic enzymes*: *a practical approach* (Beynon, R.J. and Bond, J.S., eds). IRL Press, Oxford University Press. pp 244-249

Chang, S.L. 1974. Etiological, pathological, epidermological, and diagnostical consideration of primary amoebic meningoencephalitis. *Critical Reviews in Microbiology* **3**: 135-159

De Jonckheere, J.F. 1977. Use of axenic medium for differentiation between pathogenic and non-pathogenic *Neagleria fowleri* isolates. *Applied and Environmental Microbiology* **33**: 751-757

Ferrante, A. and Bates, E.J. 1988. Elastase in the pathogenic free-living amoeba *Naegleria* and *Acanthamoeba sp*. *Infection and Immunity* **56**: 3320-33201

Fulford, D.E, Bradley, S.G. and Marciano-Cabral, F. 1985. Cytopathogenicity of *Naegleria fowleri* for cultured rat neuroblastoma cells. *Journal of Protozoology* **32:** 176-180

Keene, W.E., Hidalgo, M.E., Orozco, E. and McKerrow, J.H. 1990. Correlation of the cytophatic effect of virulent trophozoites of *Entamoeba histolytica* with the secretion of a cysteine proteinases. *Experimental Parasitology* **71**: 199-206

Lockwood., B.C., North, M.J., Scoot, K.I., Bremner, A.F. and Coombs, G.H. 1987. The use of a highly sensitive electrophoretic method to compare the proteinases of trichomonads. *Molecular and Biochemical Parasitology* **24**: 89-95

Marciano-Cabral, F. and Fulford, D.E. 1986. Cytopathology of pathogenic and non-pathogenic *Naegleria* species for cultured rat neuroblastoma cells. *Applied and Environmental Microbiology* **51**: 1133-1137

Martinez, A.J. 1985. Free-living amoeba: natural history, prevention, diagnosis, pathology and treatment of diseases. CRC Press, Inc. Boca Raton, Florida, USA pp:1-156

North, M.J., Robertson, C.D and Coombs, G.H. 1990. The specificity of trichomonad cycteine proteinase analysed using fluorogenic substrate and specific inhibitors. *Molecular and Biochemical Parasitology* **39**: 183-194

Robertson, C.D. and Coombs, G.H. 1992. Characterisation of three groups of cysteine proteinases in the amastigots of *Leishmania mexicana*. *Molecular and Biochemical Parasitology* **42:** 269-276