DETECTION OF ENZYMES DEHYDROGENASES AND PROTEASES IN *BRUGIA MALAYI* FILARIAL PARASITES

Y.P. Bhandary, K.N. Krithika, Sandeep Kulkarni, M.V.R. Reddy, B.C.Harinath*

Department of Biochemistry, and *Jamnalal Bajaj Tropical Disease Research Centre, Mahatma Gandhi institute of Medical Sciences, Wardha-442102, Maharashtra, India

ABSTRACT

Lymphatic filariasis caused mainly by infection from *W. bancrofti* and *B. malayi* remains a major cause of clinical morbidity in tropical and subtropical countries. Analysis of *B.malayi* mf, infective larval and adult worm lysates for the activity of enzymes led to the demonstration of activities of three key enzymes of carbohydrate metabolism viz., Malate dehydrogenase (MDH), Malic enzyme (ME) and Glucose-6-phosphate dehydrogenase (G6PDH) in all the three stages of the parasite. The specific activity of all the three dehydrogenases was significantly high in mf lysate compared to their activity in lysates of the other two stages ($P<0.001$). Analysis by native polyacrylamide gel electrophoresis (PAGE) using 7.5% non-gradient gel showed the presence of two isoforms of each of the three enzymes (MDH, ME & G6PDH) in mf lysate, while only one form of each enzyme was present in L_3 larval and adult worm lysates. Further proteolytic enzyme activity was demonstrated both in microfilarial and infective larval lysates of *B.malayi*. While both mf and L_3 larval lysates showed optimal protease activity at alkaline pH of 9.0, the mf lysate showed increased activity also at pH 3.0. The infective larval lysate was markedly inhibited by Tosylamide -L-Phenylalanine chloromethyl ketone (TPCK), a thiol protease inhibitor, while the protease activity in mf lysate was significantly inhibited by both TPCK and a serine protease inhibitor Phenyl Methyl Sulphonyl Fluoride (PMSF). In sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using gelatin copolymerized gel, the microfilarial lysate showed 3 protease molecules of 40 kDa, 180 kDa and 200 kDa and the L_3 larval lysate had 6 protease molecules of 18, 25, 37, 49, 70 and 200 kDa size.

INTRODUCTION

Lymphatic filariasis is a mosquito-borne disease of the tropics caused by infection with the nematode parasites *Wuchereria bancrofti* and *Brugia malayi*. In India, filariasis has been recognized as a disease of national importance because of continuous spread of the disease and protracted sufferings and disability caused in the affected population. India contributes to 40% cases of bancroftian filariasis in the global scenario (1). Parasite components like enzymes having certain biological function that makes them essential for parasite survival are expected to have an important role in host parasite interactions and disease process. Enzymes of filarial and other parasites have been attractive targets to be explored for the development of diagnostic assays and vaccines. The presence of high titres of antibodies against the enzymes in infections indicates that the

enzymes might be immunogenic and so need to be explored as diagnostic reagents (2). Various enzymes have been detected and some of them have been purified from whole worm homogenates or excretory secretory products of filarial and other parasites. A full complement of the important enzymes of TCA cycle were shown to be present in *Brugia pahangi* and *Dirofilaria immitis* (3). Cytosolic Malic enzyme (ME) and Malate dehydrogenase (MDH) were shown to be commonly present in helminth parasites (4, 5). Isoenzymic forms of Lactate dehydrogenase (LDH) have been demonstrated in filarial nematode (*Melanoma dessetae*) indicating that they could be valuable tools for phylogenetic studies.

Proteolytic enzymes of parasites have been given more attention than other enzymes as they are crucial for a parasite survival involved in many fundamental physiologic processes. They have been shown to mediate processes like tissue invasion, feeding, evasion of host immune response etc (6). Some of the proteolytic enzymes of filarial parasites having crucial role to play in the survival of parasite have been identified. A membrane bound enzyme of *Dirofilaria immitis* microfilariae is believed to play a major role in protecting the parasite against antibody mediated cytotoxicity via cleavage of immunoglobulins (7).

Author for Correspondence :

Dr. M.V.R. Reddy
Professor and Head,
Dept. of Biochemistry,
MGIMS, Sevagram,
Wardha-442 102

Collagenase activity was shown to be associated with ES products of *B.malayi* adult worms (8). Filarial cystatins were shown to suppress the proliferation of human peripheral blood mononuclear cells (PBMC) and murine spleen cells. This suggests that filarial cystatins down regulate hosts cellular responses as a measure of adaptation to their parasitic life style. Considering diverse roles that these parasite enzymes could play in host parasite interactions and their possible use as drug targets or as diagnostic and prophylactic tools, the present explorative study was undertaken to analyze and characterize the dehydrogenases and proteases of human lymphatic filarial parasite *B.malayi*.

MATERIALS AND METHODS

Filarial parasites

B. malayi Infective larvae (L_3) were collected from infected *Aedes aegypti* mosquitoes following Baermann's technique (9). Microfilariae were obtained by lavage of the peritoneal cavities of jirds (*Meriones unguiculatus*) with intraperitoneal *B. malayi* infection of 3 months or more duration. The mf were washed with RPMI 1640 medium, plated on sterile plastic petridishes and incubated at 37°C for 1 hr to remove jirds peritoneal exudate cells. The mf were collected from petridishes and washed with RPMI 1640 medium and adult worms were recovered from the infected amicrofilaraemic mastomys (*Mastomys natalensis*) at necropsy. The animals were sacrificed and dissected to collect adult worm from heart, lungs, kidney, testes and seminiferous tubules. The recovered worms were transferred to medium RPMI-1640.

***B. malayi* microfilarial, infective larval and adult worm lysates**

B. malayi microfilarial lysate was prepared as described by Evan's *et al.* (10). The mf were washed 3 times by suspension in ice cold Proline Balanced Salt Solution (PBSS) each time recovering them by centrifugation at 4°C (2000 x g) for 20 min. The mf were then suspended in a volume of enzyme stabilizer cocktail (2 mM Na EDTA, 2 mM E-amino caproic acid and 2 mM-dithiothreitol pH7.0) and subjected to freezing (30 sec) and thawing in liquid nitrogen. The soluble supernatant extract of lysed microfilariae was separated by high speed centrifugation at 25,000 x g for 25 min at 4°C. The protein content was estimated and the lysate was stored at -70°C until it is used.

B. malayi infective larval and adult worm lysates were prepared in a similar manner using about 2×10^3 L_3 larvae and about 500 male and female adult worm respectively.

Measurement of the activity of dehydrogenases

Enzyme malate dehydrogenase (MDH) (E.C.1.1.1.37)

was assayed as described by Yoshida *et al.* (11) using 10mM L-malate substrate with 10mM NAD in 150 mM Tris-HCl buffer, pH 8.8. Malic Enzyme (ME) (E.C.1.1.1.40) and Glucose-6-phosphate dehydrogenase (G6PDH) (E.C.1.1.1.49) were assayed as described by McManus and Smith (12) using 150 mM L-Malate with 5 mM NADP in 300 mM Tris-HCl buffer pH 6.5 and 2 mM Glucose-6-phosphate substrate with 0.05 mM NADP in 100 mM Triethanolamine/HCl buffer pH 7.4 as substrates respectively.

The assays were carried out in 3 ml volumes at 25°C. The increase/decrease in the absorbance was recorded at 10 sec intervals for 3 min at 340 nm using spectrophotometer (Gillford). Enzyme activities were calculated using mm coefficient of 6.22 for NADH/ NADPH and results were expressed in "nmoles/ min/ mg of protein".

Analysis of enzymes dehydrogenases by polyacrylamide gel electrophoresis (PAGE)

B. malayi mf, L_3 or adult worm lysates (10 g/ 50 l/ well) were applied onto 7.5% polyacrylamide Gel (7.5 cm x 8.5 cm) and electrophoresed at 4°C for 6-8 hrs (13). The gels were incubated in appropriate substrate solution at 37°C for 1 hour to visualize dark colored bands of enzymes as described by Siciliano and Shaw (14).

Measurement of protease activity by azocoll assay

Proteolytic activity of mf and L_3 larval lysates was measured spectrophotometrically as described by Knox and Kennedy (15) with few modifications using chromogenic substrate azocoll. Eppendroff vials each containing 5mg azocoll in 0.5 ml of 0.05M PBS / Glycine-NAOH buffer (pH 9.0) were added with 25 l of different concentrations of mf or L_3 lysates and incubated at 37°C for 12 h. The undigested protein was removed by centrifugation and the absorbance of supernatant was measured at 520 nm as a measure of enzyme protease activity.

Effect of pH on protease activity

The effect of pH on protease activity in parasite against azocoll was tested over the range of pH 3-11 using 0.1M Citrate buffers (pH 3-5), 0.1M Citrate-phosphate buffers (pH 5-6.5), 0.1M phosphate buffers (pH 6.5-7.5), 0.1M Tris buffers (pH 7.5-9) and 0.1M Glycine- NaOH buffers (pH 9-11).

Effect of inhibitors on protease activity

The mf/ L_3 larval lysates (25 l) were incubated separately with 25 l of different inhibitors (1 mM EDTA / 0.1 mM PMSF / 0.2 mM TLCK/ TPCK) in 0.5 ml of PBS / Glycine- NaoH buffer (pH 9.0) containing 5 mg azocoll as substrate. After 12 hrs of incubation,

the reaction mixtures were centrifuged and the absorbance of the supernatants was measured at 540 nm.

SDS-PAGE on Gelatin Co-polymerized gel for analysis of Proteases

SDS-PAGE was done according to Maizels *et al.* (16). Mf or L₃ lysates (25 µg in 25 µl) were applied on a gradient gel of 5-15% incorporated with 0.1% gelatin and electrophoresed at 25 mA for 2 hrs. The gel was washed with 2.5% triton-x-100, incubated overnight at 37°C in 0.1 M PBS and stained with Coomassie blue stain. Protein bands with enzyme activity were detected as zones of enhanced transmission relative to regions where protein substrate remains unhydrolysed.

Statistical Analysis

The results of enzyme assays were analyzed by Student's 't' test.

RESULTS AND DISCUSSION

In parasitic infections enzymes are known to have important role as the effectors of biologically important phenomena (17). Analysis of *B. malayi* mf, infective larval and adult worm lysates led to the demonstration of activities of the three key enzymes of carbohydrate metabolism viz., Malate dehydrogenase (MDH), Malic enzyme (ME) and Glucose-6-Phosphate dehydrogenase (G6PDH) in all the three stages of the parasite. The specific activities of these enzymes (Table 1) were significantly high in mf lysate compared to their activity in infective larval or adult worm lysates (P<0.001). Among the three dehydrogenases, MDH activity was significantly high compared to the other two enzymes in all three stages of the parasites (P<0.001). The presence of full complement of TCA cycle enzymes in *B. pahangi* and *Dirofilaria immitis* filarial parasites was earlier reported by Barret (3). The presence of cytosolic malic enzyme and MDH was

shown to be a common feature among helminth parasites (4, 5).

In the present study PAGE analysis showed each of these three dehydrogenases (MDH, ME and G6PDH) to be in two isoforms of each enzyme in mf lysate and in only one form in L₃ larval and adult worm lysates (Fig. 1, 2 & 3) suggesting their potential use in strain identification of filarial parasites. Marchat *et al.* (18) have separated LDH isoenzymes from filarial nematode (*Melanoma dosssetae*) and reported that these enzymes could be valuable tools for phylogenetic studies. Isoenzyme analysis has proved to be quite useful in strain differentiation in other parasites like Trypanosoma (19) Leishmaniasis (20), Entamoeba (21). Malate dehydrogenase & Glucose-6-Phosphate dehydrogenase have also been shown to be key markers for studying the genetic diversity of *Actinobacillus actinomycetemcomitans* (22).

In the present study, significant protease activity has been demonstrated both in microfilarial and infective larval lysates of *B. malayi* (Fig. 4). While both mf and L₃ larval lysates showed optimal protease activity at alkaline pH of 9.0, the mf lysate showed additional increased activity also at pH 3.0 suggesting the differences in the set of proteases associated with each stage (Fig. 5 and 6). This is also in agreement with variations in the manner in which protease activity in the mf and L₃ larval lysates is affected by different inhibitors. While the azocolytic activity of infective larval lysate was markedly inhibited by TPCK, a thiol protease inhibitor, the protease activity in mf lysate is significantly inhibited by both TPCK and a serine protease inhibitor PMSF (Fig. 7 and 8).

The proteases of both mf and L₃ larval lysates were analyzed by SDS-PAGE using gelatin copolymerized gels. The gels stained for protease activity showed complex array of proteases with varying molecular weights. While microfilarial lysate showed 3 protease molecules of 40kDa, 180 kDa & 200kDa, the L₃ larval

Table 1. Activity of enzymes dehydrogenases in *Brugia malayi* microfilarial, infective larval (L₃) and adult worm lysates

Enzyme	Substrate	Enzyme activity (n moles /min/ mg protein)* in		
		L ₃ lysate	Mf lysate	Adult worm Lysate
Malic Enzyme	L-Malate	15.86±0.74	57.01±22.93	17.92±0.82
Malate Dehydrogenase	L-Malate	46.08±1.8	88.6±39.19	38.15±2.6
Glucose-6-Phosphate Dehydrogenase	Glucose-6-phosphate	8.57±1.8	19.82±10.0	13.52±0.17

*Mean±SD of three Experiments.

Fig. 1. Isoenzymes of Malate dehydrogenase (MDH) of *B. malayi* separated by polyacrylamide gel electrophoresis and stained for enzyme activity. (L_3 = infective larval lysate; Mf = microfilarial lysate; Ad = adult worm lysate)

Fig. 2. Isoenzymes of Malic Enzyme (ME) of *B. malayi* separated by polyacrylamide Gel electrophoresis and stained for enzyme activity. (L_3 = infective larval lysate; Mf = microfilarial lysate; Ad = adult worm lysate)

lysate had 6 protease molecules of 18, 25, 37, 49, 70 & 200 kDa size (Fig-9). This heterogeneity in the composition of proteases in mf and L_3 larval stages might be appropriate considering the different host environments to which they are exposed.

We have no indications on the exact function of proteases *in vivo*, although the collagenolytic and elastinolytic activity would be consistent with their tissue migratory function. In parasitic infections, diverse roles have been attributed to these enzymes, such as facilitation of invasion of host tissues (23),

Fig. 3. Isoenzymes of Glucose-6-Phosphate dehydrogenase (G6PDH) of *B. malayi* separated by polyacrylamide Gel electrophoresis and stained for enzyme activity. (L_3 = infective larval lysate; Mf = microfilarial lysate; Ad = adult worm lysate)

Fig. 4. Enzyme protease activity in *B. malayi* microfilarial (mf) and Infective larval (L_3) lysates using azocol (5 mg/ ml in 0.05 M phosphate buffer saline at 37°C for 12 hrs) as substrate

digestion of host proteins (24), evasion of the host's immune response (7, 25), retardation of blood coagulation (26) and nutritive role (27). The other important role of proteases could be to help in moulting and development of parasite. In Several nematode infections, parasite proteases have been demonstrated to induce protection. Proteases secreted by liver flukes (*Fasciola hepatica*) showed immunoprotection when used as vaccine candidates in cattle (28). An anticoagulant metalloprotease produced by *Ascaris caninum* (29) has been shown

Fig. 5. The effect of pH on protease activity of *B. malayi* microfilarial lysate using Azocoll as a substrate

Fig. 6. The effect of pH on protease activity of *B. malayi* Infective larval lysate using Azocoll as a substrate

Fig. 7. Effect of various inhibitors on protease activity of *B. malayi* microfilarial lysate. The concentrations of inhibitors used were Phenyl Methyl Sulphonyl Fluoride (PMSF-1 mM), N-a-p-Tosyl-L-Lysine chloromethyl Ketone (TLCK-0.2 mM), N-Tosylamide-L-Phenylalanine Chloromethyl Ketone (TPCK-0.2 mM), Ethylene Diamine tetraacetic acid (EDTA- 1 mM)

Fig. 8. Effect of various inhibitors on protease activity of *B. malayi* Infective larval lysate. The concentrations of Inhibitors used were Phenyl Methyl Sulphonyl Fluoride (PMSF- 1mM), N-a-p-Tosyl-L-Lysine Chloromethyl Ketone (TLCK-0.2mM), N-Tosylamide-L-Phenylalanine Chloromethyl Ketone (TPCK-0.2 mM), and Ethylene Diamine tetraacetic acid (EDTA-1 mM)

Fig. 9. Enzymes proteases in *B. malayi* infective larval (L_3) and microfilarial (mf) lysates separated by SDS-polyacrylamide gel electrophoresis on gels Co-polymerised with Gelatin

to be an important target for vaccine development. The *invitro* released proteases of the tissue stage larvae of nematode *Ascaris suum* have been to be highly antigenic and the antibody obtained from infected animals can inhibit parasite proteases (30). The role of proteases in immunoprotection has also been shown in Leishmania infection by Mc Kerrow (31).

The present study did help in demonstrating the different isoforms of selected dehydrogenases and multiple forms of proteases in *B. malayi* filarial parasite. Further studies as their multiform of enzymes in filarial parasite populations collected from different endemic areas and their role in host parasite interactions will help to explore their possible use for epidemiological typing or as diagnostic and prophylactic tools.

ACKNOWLEDGEMENTS

This work was supported by the Dept of Biotechnology and the JBTDRC Research scheme of Mahatma Gandhi Institute of Medical Sciences, Sevagram. Our thanks to Shri. Dhirubhai Mehta, President, KHS and Dr. (Mrs). P. Narang, DEAN, Mahatma Gandhi Institute of Medical Sciences for their keen interest and encouragement in this work.

REFERENCES

- Ramaiah, K.D., Das, P.K., Michael, E. and Guyatt, H. (2000). The economic burden of Lymphatic filariasis in India. *Parasitol.Today*. 16, 251-253.
- Leid, R.W and Sequet, C.M. (1986) A superoxide dismutase of metacestodes of *Taenia taeniaeformis* *Mol.Biochem.Parasitol*. 18, 301-311.
- Barrett, J. (1983). Biochemistry of filarial worms. *Helminthological Abstracts* 52, 1-18.
- Barrett, J. (1981). Biochemistry of parasitic helminths. *Macmillan Press, London*. p. 308.
- Saz, H.J. (1981). Biochemistry of parasites: helminths. In *Medical Microbiology and infectious diseases* (Edited by Braude A.T). W.B. Saunders, Philadelphia, U.S.A.
- Tort, J., Brindely, P.J., Knox, D., Wolfe, K.H. and Dalton, J.P. (1999). Proteinases and associated genes of parasitic helminths. *Adv.Parasitol*. 43,161-266.
- Tamashiro, W.K., Rao, M. and Scott, A.L. (1987). Proteolytic cleavage of IgG and other protein substrates by *D.immitis* microfilarial enzymes. *J. of Parasitol*. 73,149-154.
- Petralanda, I., Yarzabal, L. and Piessens, W.F. (1986). Studies on a filarial antigen with collagenase activity. *Mol. Biochem. Parasitol*. 19, 51-59.
- Suzuki, T. and Soregey, I.G. (1979). Mass dissection technique for determining infectivity rate of filariasis vector. *Jpn. J. Exp. Med*. 49, 117-121.
- Evan, D.A. (1989). Handbook on isolation, characterization and cryopreservation of Leishmania. Geneva: World Health Organization, 15.
- Yoshida, A. (1969). L-malate dehydrogenase from *B. subtilis* in *Methods in Enzymology*. X111 Academy press, New York, 141-145.
- McManus, D.P. and Smith. J.D. (1982). Intermediary carbohydrate metabolism in protoscoleces of *Echinococcus granulosa* (horse & sheep strains) and *E. multilocularis*. *Parasitology* 84, 351-366.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- Siciliano, M.J. and Shaw, C.R. (1976). Separation and visualization of Enzymes on gels in Chromatographic and Electrophoretic technique Vol. 2 (I. Smith ed) 185-209. William Heinmann Medical Book Ltd. London.
- Knox, D.P. and Kennedy, M.W. (1988). Proteinases released by the parasitic larval stages of *Ascaris suum* and their inhibition by antibody. *Molecular and Biochemical Parasitology* 28, 207-216.
- Maizels, R. M. and Robertson, B.D.(1988). *Molecular Parasitology Lab Manual*.
- Ashall, H.F. and Maizels, R.M. (1991). Characterization of proteolytic enzymes from larval and adult *Nippostrongylus brasiliensis*. *Immunology* 103, 305-314.
- Marchat, L., Loiseau, P.M., Pous, C. and Petek, F. (1994). Isoenzymatic diagnosis of filariae: a method for separation of lactate dehydrogenase isoenzymes from *Molinema desetae*. *Biochem. Mol. Biol*. 109, 451-457.
- Enrique, E., Montamat, G.M., Luca, Doro., Rafael, H., Raul, Sosa and Antonio, B. (1996). Characterization of *Trypanosoma cruzi* populations by zymodemes: Correlation with clinical picture. *Am. J. Trop. Med. Hyg*. 55 (6), 625-628.
- Chatterjee, M., Madhumita, M., Bhaduri, A.N. and Sarkar, D. (1995). Recent kala azar cases in India: Isoenzyme profiles of *Leishmania* parasites. *Ind. J. Med. Res*. 102, 165-172.
- Sargeaunt, P.G. and Williams, J.E. (1979). Electrophoretic isoenzyme patterns of the pathogenic and non pathogenic intestinal amoebae of man. *Trans. R. Soc. Trop. Med. Hyg*.

- 73 (2), 225-227.
22. Shah, H.N. and Andrews, D.M. (1994). Malate dehydrogenase and Glucose-6-phosphate dehydrogenase, key markers for studying the genetic diversity of studying the *Actinobacillus actinomycetemcomitans*. Microbiol lett. 122, 69-73.
23. McKerrow, J.H., Brindley, P., Brown, M., Gam, A.A., Staunton, C. and Neva, F.A. (1990). *Strongyloides stercoralis*: Identification of a protease that facilitates penetration of skin by infective larvae. Experimental Parasitology 70, 134-143.
24. Keene, W.C., Jeong, K.H., Mckerrow, J.H. and Werb, Z. (1983). Degradation of extracellular matrix by larvae of *S.mansoni*.II. Degradation by newly transformed and developing schistosomula. Laboratory Investigation 49, 201-207.
25. Verwaerde, C., Auriault, C., Neyrinck, J.L. and Capler, A. (1988). Properties of serine proteases of schistosomula involved in regulation of IgE synthesis. Scandinavian J. of Immunology 27,17-24.
26. Hotez, P.J. and Cerami, A. (1983). Secretion of a proteolytic anticoagulant by *Ancylostoma* hookworms. J. of Exp. Med. 157, 1594-1603.
27. Maki, J.,Furuhashi, A and Yanagisawa, T. (1982). The activity of acid proteases hydrolysing haemoglobin in parasitic helminths with special reference to interspecific and intraspecific distribution. Parasitology 84,137-147.
28. Dalton, J.P., McGonigli, S., Rolph, T.P. and Andrews, S.J. (1996). Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with haemoglobin. Infect. Immun. 64, 5066-5074.
29. Hotez, P.J., Letang, N. and Mc Kerrow, J.H. Ceramia (1985). Isolation and characterization of proteolytic enzyme from the adult hook worm *ancylostoma caninum*. J. Biological Chemistry 260, 7343-7348.
30. Knox, D.P. and Kennedy, M.W. (1988). Proteinases released by the parasitic larval stages of *Ascaris suum* and their inhibition by antibody. Molecular and Biochemical Parasitology 28, 207-216.
31. Mckerrow, J.H. (1989). Parasite proteases. Experimental Parasitology 68, 111-115.