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Summary

Title

Isolation, Purification and Characterization of Clinically important proteinases of filarial parasites

Research Institute

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Scientific Background and scope/objectives

Lymphatic filariasis is one of the public health problem in tropical countries and affecting more than a billion people at a risk of infection in the world(Williams et. al , 2000). Available anti filarial drugs, diethylcarbamazine, ivermectin and others are microfilaricidal and not effect to the adult parasite. The World Health Organization has aimed to eliminate the disease globally by the year 2020. Therefore development of adulticide is necessary for complete evacuation of the diseases.

Proteolysis play a major role in cellular catabolic and anabolic processes and essential for survival. Parasites use proteases in a number of roles crucial for the parasitic life cycle and survival, including host invasion/release, nutrient digestion and uptake, evasion of host immune response. Thus some of the proteases will be a excellent targets for the design of novel anti-parasitic drugs. However no studies reported on proteinase from filarial parasite *Brugia malyi.* or *Wuchereria*

bancrofti. Collection of human filarial parasite in gram quantities for protein isolation is extremely difficult. Therefore we have started to characterize type of proteinases present in adult filarial parasite *Seteria digitata*. Human filarial parasite proteinases are isolated by gene cloning and expressing identified cDNA in *E .coli* to produce recombinant human filarial parasite proteinase for their characterization. If specific proteinases are identified as key therapeutic target point, inhibitors of the proteinases might offer a novel therapeutic approach to control filariasis in Sri Lanka and world over.

Experimental methods

Adult female parasite, *Seteria digitata* were dissected into three parts as digestive system(esophagus and intestine), reproductive system and body wall under the microscope. Crude extract of parasite *Seteria digitata* was prepared by homogenizing the parasite(20g) or dissected tissues(5g) in 200 ml or 50 ml of 0.2M phosphate buffer, pH 7.5. Acid proteinase and neutral proteinase activities were analysed by using denatured heamoglobin at pH 2.0 and casein at pH 8.0 as the substrates.

Purification and characterization of acid proteinases.

Acid proteinases were purified by successive chromatographies on DEAE Cellulose 52, Sephacryl S-200 gel filtration, pepstatin Sepharose affinity chromatography and FPLC over mono Q column. Purity and approximate molecular weights of of the purified enzymes were analysed by SDS-PAGE. Enzymatic properties of purified proteinases were analysed to identify the type of proteinases.

Cloning of aspartic proteinase genes of human filarial parasite *Brugia malyi*

PCR primers were designed based on homologous amino acid sequences of active site residues and other regions of reported aspartic proteinase in the data bases. mRNA was isolated from *Brugia malyi* and cDNA library was constructed. Three cDNA clones of aspartic proteinases were isolated by RT-PCR cloning and colony hybridization.

Expression of recombinant enzyme

Two cDNA was expreesed in *E.Coli* by using the plasmid vector T-7(PAR 2016) and BL21-codon plusTM(DE3)-RIL *E. Coli* strains. Recombinant protein was appeared as inclusion body. Inclusion body was purified by sequential washing and recombinant protein was purified by preparative electrophoresis. Polyclonal antibodies were produced by immunizing the rabbits with recombinant proteinases.

Immunohistochemical analysis

Polyclonal antibodies were produced by immunizing the rabbits with recombinant proteinases. Tissue localization of the two proteinases were identified by using immuno-histochemical analysis of permeabilized whole mount adult parasite of *Brugia malyi.*

Results and Discussion

Higher proteolytic activity was observed in the acidic pH and very low activity observed at the neutral pH. This high acid proteinase activity of crude extracts suggesting its functional importance in filarial parasite biology. Therefore this study was mainly focused on acid proteinases of filarial parasite.

Three activity zones were observed with crude extract of reproductive system, where as

only two activity zones were observed for crude extracts of intestine and body wall. Proteolytic activity was detected in fractions of three peaks in DEAE cellulose chromatography as sample injection, eluted at 0.2M NaCl and eluted at 0.8M NaCl, respectively for crude extract of whole parasite and reproductive system. However only two peaks, eluted at 0.2M NaCl and 0.8M NaCl were observed for crude extracts of intestine and body wall, respectively. Further observed proteolytic activity of crude extract was completely inhibited by pepstatin suggest that acid proteinases of filarial parasite belongs to family of aspartic proteinases. These results suggest the presence of three aspartic proteinases in filarial parasite and their charge at pH 7.5 are differ. Further their tissue localization also differ and specific. Relatively higher activity was observed in crude extract of reproductive system.

Optimum temperature and pH for acid proteinase activity were observed at 48 ^{0}C and pH 2.5 , respectively. Three acid proteinases were purified by using successive chromatographies on DEAE-cellulose-52, Sephacryl S-200, Pepstatin Sepharose and Mono Q. Molecular weights of purified proteinases were determined as 42kDa and 40 kDa by SDS-PAGE and gel filtration, respectively. Protolytic activity of purified proteinase was inhibited completely with 0.01M pepstatin, but not inhibited by soyabean trypsin inhibitor, E-64, Phenol methane sulphonyl fluoride(PMSF) and EDTA. This suggest that three acid proteinases of filarial parasite belong to family of aspartic proteinases.

cDNA library was constructed and the titre of amplified library was 1.5 x 108 pfu and it was suitable for RT-PCR cloning of filarial proteins.

Three cDNA clones BmAsp-1, BmAsp-2 and BmAsp-3 were isolated. Complete full

length cDNA sequence of BmAsp-1 and Bm Asp-2 and partial sequence of BmAsp-3 were determined. Protein sequences of BmAsp-1 and BmAsp-3 were deduced and resulting sequences were compared with sequences of other aspartic proteinases. Result suggest that BmAsp-1 is belongs to the family of pepsinogen like aspartic proteinase and Bm Asp-2 is belongs to cathepsin D/E like aspartic proteinase. Results of phylogenic analysis of BmAsp-1 and BmAsp-2 sequences with other aspartic proteinases, suggest that these two sequences belongs to novel two classes of aspartic proteinases of parasites. These two enzymes shows higher homology to aspartic proteinase from parasites and less homology to mammalian aspartic proteinase. This explore the higher possibility of using these two proteinase as the possible theraputic intervention point.

Recombinant proteins of BmAsp-1 and Bm Asp-2 genes was produced in *E. coli* successfully with molecular weight of 40,000kd and 42,000 kd respectively . Antibody against recombinant proteins were produced in rabbit and resulting anti serums showed higher titer. Histo-chemical analysis suggest that BmAsp-1 protein was expressed in the GI tract(intestine and esophagus) and Bm Asp-2 protein was expressed in intestine, body wall and reproductive system.

Further BmAsp-1 clone was not identified in libraries of *Brugia malyi* microfilaria and L3 larva by PCR amplification or colony hybridization. This suggest that BMAsp-1 gene is specifically transcribed in adult stage of parasite and thus exploring the possibility if its specific function in adult parasite. However BmAsp-2 clone was identified from libraries of above three stages of life cycle of *Brugia malyi*.

BmAsp-1 gene was specifically expressed in intestine, suggest that it might involve in

nutrient digestion and uptake. BmAsp-2 gene was expressed in most of the body tissues and suggest that it might involve in intracellular protein catabolism or precursor protein processing.

Further characterization of these proteinase are necessary to identify their exact biological function and to clarify possibility of using them as a possible therapeutic intervention points. Recombinat protein production of *Brugi malyi* active aspartic proteinases are in progress to clarify the above.

Conclusions and recommendations

1 Three different aspartic proteinases and two neutral proteinases were isolated from adult filarial parasite. Tissue localization of three aspartic proteinases were differ.

2. Full length cDNA of two of the aspartic proteinases were isolated and their DNA sequences were determined. Deduced protein sequence of BmAsp-1 amd BmAsp-2 gene suggest that these two proteinases represents the novel two classes of parasitic aspartic proteinases. Homology of two proteinases with human aspatic proteinases were very low and exploring the possibility of targeting these two steps as the therapeutic intervention points.

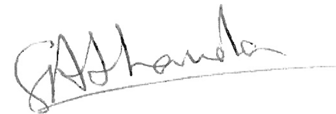
3. Isolated two cDNA were expressed in *E. Coli* to produce recombinant proteinase for further characterization. Yield of recombinant expression was high and will be useful for further characterization of proteinases.

4. Purification and folding of recombinant protein are in progress to produce active enzyme for further characterization and clarification of their specific biological role.

Future work-stemming from conclusions

Further characterization of two aspartic proteinases of *Brugia malyi* and clarification of their biological roles at t various stages of life cycle of parasite.

Purification and cloning of neutral proteinases of filarial parasites

Date 30th November , 2001 Signature of the Grantee: 

Recommendation of the Head of the Department Excellent progress. Further work needs to carried out. Recommend a grant for future work.

Signature of the Head of the Department

Prof. P. A. J. PERERA
Head Department of Biochemistry
FACULTY OF MEDICINE
UNIVERSITY OF PERADENIYA

Date 11th Jan 2002

(ix) *List of equipments purchased under NSF*:

PCR-Perkinelmer 2400. It is located in the department of Biochemistry, Faculty of Medicine, University of Peradeniya.

(3) Publications / Communications

1. Purification and characterization of a minor acid proteinase from filarial parasite *Seteria digitata*, Athauda SBP, Wijeratne Banda YM and Takahashi K(1997) Proceeding of the Sri Lanka Association for the Advancement of Science; 53: 39-40
2. Tissue localization of acid proteinase from filarial parasite *Seteria digitata*, Jayaratne HGUP, Athauda SBP and Takahashi K(1998)
   Proceeding of the Sri Lanka Association for the Advancement of Science; 54: 25-26.
3. Characterization of acid proteinase present in digestive and reproductive systems of *Seteria digitata*, Jayaratne HGUP, Athauda SBP and Takahashi K(1999)
   Proceeding of the Sri Lanka Association for the Advancement of Science; 55: 24-25.
4. Cloning of aspartic proteinase from filarial parasite *Brugiya malyi*
   Athauda SBP, Nomura H, Inoue H and Takahashi K(1999)
   Proceedings of the Japan Biochemical Society; 71: 734-735
5. Acid proteinases and their enzymatic properties of filarial parasite *Seteria digitata*, Jayaratne HGUP, Athauda SBP and Takahashi K(2000)
   Fifth International medical congress, p 64
6. Molecular cloning of aspartic proteinases from filaraial parasite *Brugia malyi*
   Athauda SBP, Noumura H, Inoue H and Takahashi K(2000)
   Proceedings of 8th International conference on aspartic proteinases, Madeira, Portugal
7. Properties of acid proteinases of filarial parasite *Seteria digitata*
   Athauda,SBP., Jayaratne , HGUP, Perera PAJ & Takahashi K (2001)
   Proceedings of Kandy Society of Medicine; 23:p44
8. RT-PCR cloning of non lysosomal aspartic proteinase of filarial parasite *Brugia malyi*,

Athauda SBP, Nomura H, Inoue H and Takahashi K (2001)

Proceedings of Kandy Society of Medicne; 23 : p43

9. Molecular cloning of lysosomal aspartic proteinase from filarial parasite *Brugia malyi*

   Athauda SBP, Nomura H, Inoue H and Takahashi K (2001)

   Proceedings of Kandy Society of Medicne; 23 : p47

10. Isolation and purification of two neutral proteinases from reproductive system of filarial parasite *Seteria digitata*

    Athauda,SBP., Wijeratne SR, Jayaratne , HGUP, Perera PAJ & Takahashi K (2001)

    Proceedings of Kandy Society of Medicine; 23:p65

11. Purification and characterization of acid proteinases of filarial parasite *Seteria digitata*, Athauda SBP, Jayaratne HGUP and Kenji Takahashi

    Biomedical Research (2002)(in preparation)

12. Molecular cloning, expression and tissue localization of aspartic proteinase from filarial parasite *Brugiya malyi*(BmAsp-1)

    Athauda SBP, Nomura H, Inoue H and Takahashi K

    Experimental parasitology(2001)(submitted)

13. RT-PCR cloning, expression and tissue localization of aspartic proteinase from filarial parasite *Brugiya malyi*(BmAsp-2)

    Athauda SBP, Nomura H, Inoue H and Takahashi K

    International Journal of parasitology(2002) (in preparation)