2 Crawford, A R and Compston, W, *Q J Geol Soc London*, 1970, **125**, 351–372
3 Bhushan, S K, *Ind J Earth Sci*, 1985, **12**, 58–71
4 Srivastava, R K, *Mem Geol Soc India*, 1988, **7**, 77–93.
5 Bhushan, S K and Khullar, V K, Symposium on Application of Geochemistry, Shivaji University, Kolhapur, 1988 (Abstract)
6 Bhushan, S K, *Ind J Earth Sci*, 1991, **18**, 184–194
7 Yadav, Harpal Singh, PhD Thesis, University of Rajasthan, Jaipur, 1991 (unpublished)

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Immunodiagnostic potential of a filarial protease

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An antigenic fraction has been isolated from adult worms of cattle filarial parasite *Setaria digitata*. The fraction exhibited high protease activity against azocoll with optimum pH at 7.0. Elevated levels of antibodies to the protease were observed in asymptomatic microfilaraemic individuals compared to the normal people of endemic regions. Such distinction was however not observed with the whole antigenic extracts of adult worms. The potential of the protease as immunodiagnostic antigen is indicated.

PROTEOLYTIC enzymes of helminthic parasites have been attracting increasing interest recently for their diverse roles in host–parasite interactions[1]. These enzymes would have immunological, chemotherapeutic and diagnostic applications in disease control programmes. Indeed, as early as 1935, parasitic enzymes were implicated in immunological studies[2]. Biochemical characterization of antigens as functional molecules (protease/enzymes) would help in better understanding of the immunology of disease processes. While extensive information[1, 3–6] exists on the proteases of parasites such as Schistosome, Trypanosome, Leishmania, Plasmodia, Ascaris, filarial proteases other than those of *Dirofilaria immitis*[7, 8] have not been investigated. We demonstrate here that antibody response to a proteolytic fraction of *Setaria digitata* adults is highly elevated in the course of filarial infection.

S. digitata adults were obtained as described earlier[9]. Soluble extracts of adult worms (AE) were prepared as follows. The worms were crushed in a mortar and pestle for 10 min in 0.01 M phosphate pH 7.2, 0.1 M NaCl (PBS) and were further sonicated (Branson sonifier 450, 30 s each, 5 min) in ice. Soluble materials were obtained by cold-centrifugation at 15,000 rpm for 20 min (Beckmann J2-21). Proteolytic activity[6] was determined by incubating 20 µl of parasitic extracts (5 to 20 µg of protein) in 0.7 ml of 0.1 M phosphate buffer, pH 7.2, containing 50 µl of azocoll (5 mg/ml, Sigma) solution for 16 h at 37°C. The absorbance at 520 nm of the samples was measured against reagent blanks containing no parasitic extract. Protease activity was calculated by subtracting reagents blanks containing substrate alone and are expressed as change in absorbance $(O.D._{520} \times 10^3)/16$ h. The specific activity was protease activity per mg of protein. Protease activity was also checked by adding parasitic extracts in casein-agar plates (0.1% casein, fat free, 1.5% agar in 0.1 M tris buffer pH 7.5).

Serum samples from individuals of endemic area (Bajopur Khurda district) of Orissa, India were collected and categorized into different groups[10]. Microfilaraemia (MF) was detected by microscopic examination of 20 µl blood smears, obtained by finger prick between 2030 and 2330 h. Sera were classified as chronic filariasis (CP), individuals exhibiting elephantiasis and/or hydrocele; asymptomatic carriers (AS), MF carriers without any clinical symptoms, and endemic normals (EN), permanent residents of the region who are free from infection as judged by clinical and microscopic examination of the blood smear. Sera were also collected from healthy subjects of non-filarial regions (Koraput) of Orissa with similar socio-economic backgrounds, as with the filarial cases. These sera were used as the samples of non-endemic normals (control group) for serological comparisons. ELISA was performed by coating the plates with filarial antigen (2 µg/µl), essentially following the earlier procedures[9,10]. The percentage of the titre of each individual was calculated by taking a positive sample on each plate. The positive control (chronic filarial sera pooled) was assigned an arbitrary value of 100 titration units. The antibody levels of the samples were calculated as $100 \times (OD_{492}$ of sample/OD_{492} of positive control) ELISA units[11]. The positivity (> mean $A_{492} + 3SD$) was calculated with reference to a panel of non-filarial serum ($n = 20$, Koraput district of Orissa).

The adult worm extract (AE) was passed through a DEAE-cellulose column (1.2 × 10 cm) pH 8.0 (0.01 M Tris-HCl) and the protease enriched, bound fractions eluted with 0.75 M NaCl, dialysed, concentrated (Centricon C-10) and further chromatographed on Sephadex G-100 column (1.0 × 45 cm). Two well-separated peaks emerged (designated as SdPI and SdPII). The first peak (SdPI) eluting after the void volume was found to have high protease activity. The

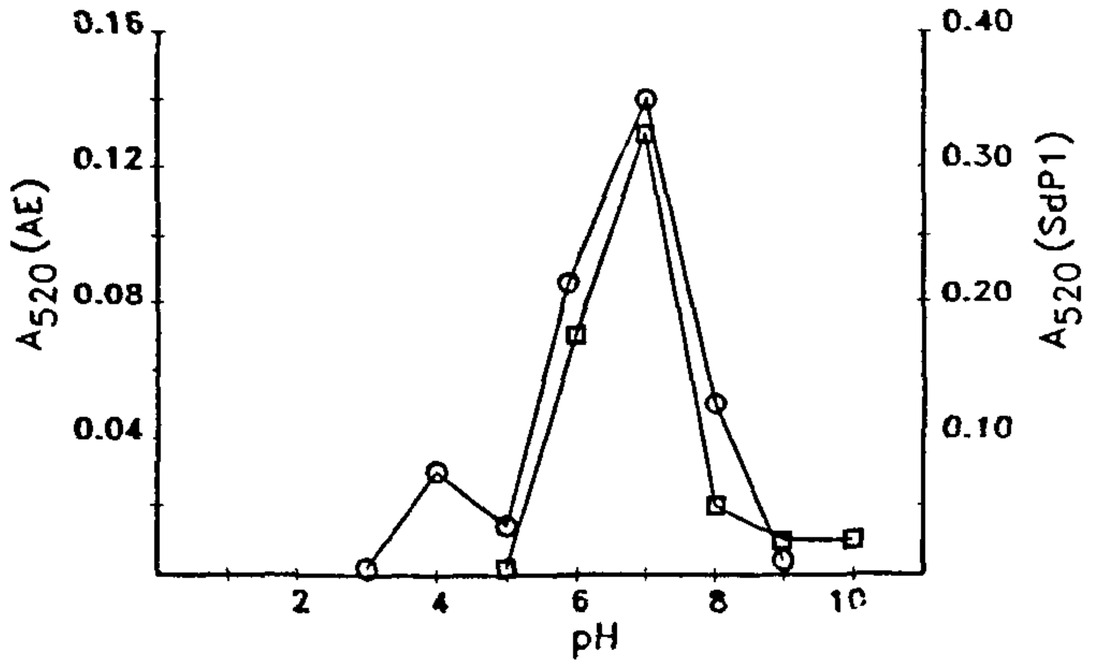

Figure 1. Effect of pH on the proteolytic activity of *S. digitata* extract, adult worms (○) and its protease SdPI (□) against azocoll.

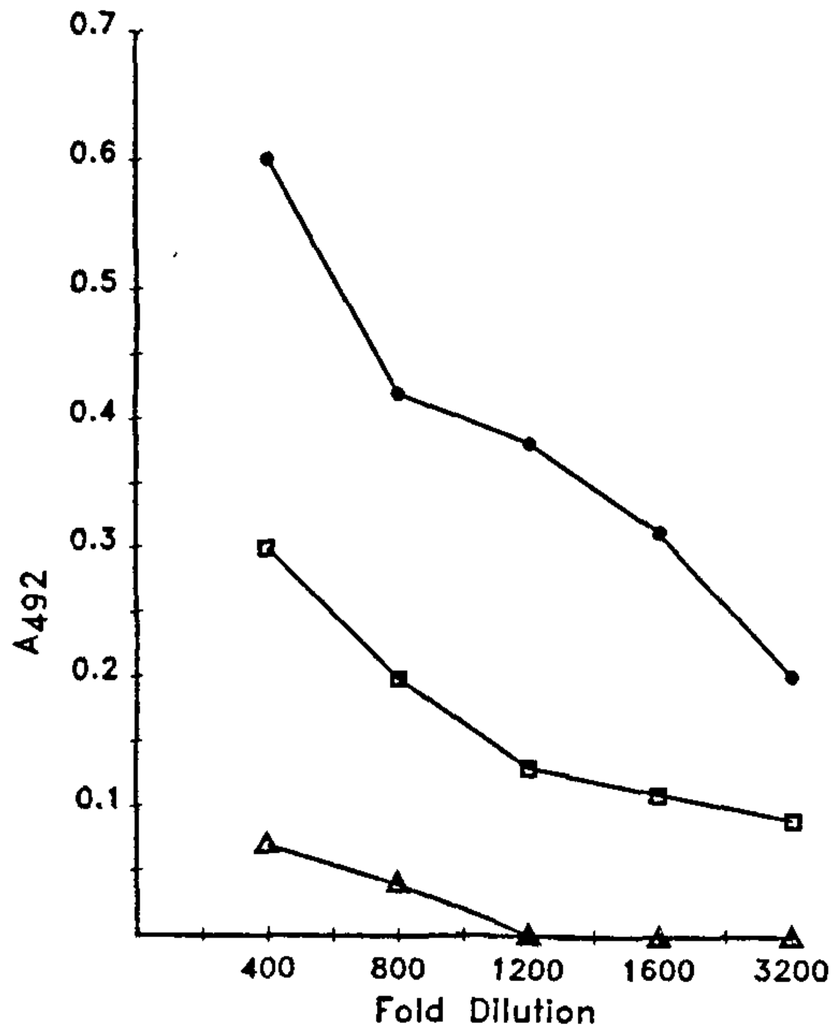

Figure 2. IgG antibody levels to SdPI in pooled filarial sera: chronic filariasis (●), Asymptomatic microfilaraemic carriers (□) and endemic normals (△).

specific proteolytic activities of AE, SdPI and SdPII against azocoll as protein substrate are 103.2 ± 32.15, 1332.5 ± 162.1 and 870 ± 183.8 respectively. SdPI constitutes 3.5% of total soluble proteins of *S. digitata*. Proteolytic activity of SdPI was measured over a wide pH range (Figure 1). AE of *S. digitata* was also studied for comparison. No activity below pH 3 could be demonstrated. A sharp pH optimum at 7.0 was determined for SdPI. However, there are two pH optima at 4.0 and 7.0 in AE, indicating the presence of multiple protease activities.

IgG responses to SdPI in different groups of pooled filarial sera are shown in Figure 2. The elevated antibody level in chronic and asymptomatic MF carriers compared to that in endemic normals at different dilution of sera is quite striking. This is further

Table 1. IgG levels to *S. digitata* protease (SdP1) in different groups of filariasis. ELISA unit was determined at 1:500 fold dilution of the serum

Groups	Number tested	IgG unit	Positives (%)
Endemic normals (EN) Asymptomatic	45	10.95 ± 9.73 (0–35)	7 (15.5)
MF-carriers (AS)	71	61.55 ± 45.85* (4–282)	62 (87.3)
Chronic filariasis (CP) (Elephantiasis = 30, Hydrocele = 14)	44	78.88 ± 38.36* (0–165)	42 (95.4)

*$P < 0.01$, compared to endemic normals.

confirmed by testing individual samples belonging to filarial groups for the evaluation of IgG seropositivity (Table 1). IgG level in AS (or in CP) is significantly ($P < 0.01$) higher than that in endemic normals. The degree of seropositivity in healthy subjects of non-filarial (Koraput) and filarial regions are 0% and 15% whereas in AS and CP groups the values are 87% and 95% respectively. A sharp increase in seropositivity is noticed in filariae-infected people. At the individual level, 42 out of 44 in CP group, 62 out of 71 in AS, 7 out of 45 in EN and none out of 20 in control group are seropositive. It may be mentioned that EN and AS individuals could not however be differentiated using whole extract of adult worms as antigen. A similar extent of IgG seropositivity (70%) was noticed in both cases (data not shown).

The absence of antibody response in control group indicates that IgG response to SdPI is specific for filariasis. This is corroborated by the presence of markedly reduced antibody levels in the normal population of endemic regions compared to the levels in the infected cases.

That a significantly higher percentage of infected individuals both asymptomatic and symptomatic is reactive to SdPI compared to normal people suggests that the IgG antibody levels to SdPI could be an immunological marker for filariasis in humans. The two individuals who are sero-negative in CP group are found to be suffering from hydrocele. This is not surprising since hydrocele due to non-filarial origin cannot be ruled out even in highly endemic regions[12]. In summary, the results presented here describe the isolation of a somatic proteolytic fraction of *S. digitata* and indicate its potential application as an immuno-diagnostic antigen in human filariasis.

1. Mckerrow, J. H., Sun, E., Rosenthal, P. J. and Bouvier, J., *Annu Rev Microbiol*, 1993, **47**, 821–853
2. Chandler, A. C., *Am J. Hygiene*, 1935, **22**, 157–168
3. Mckerrow, J. H. and Doenhoff, M. J., *Parasitol Today*, 1988, **4**, 334–340
4. Ltges, R., Bouvier, J. and Bordier, C., *J Biol Chem*, 1986, **261**, 9098–9101

5 Rosenthal, P J , Wollish, W S , Palmer, J. T. and Rasnick, D , *J Clin Invest* , 1991, **88**, 1467–1472.
6 Knox, D P and Kennedy, M W , *Mol Biochem Parasitol.*, 1988, **28**, 207–216
7 Richer, J K , Sakanari, J A , Frank, G. R and Grieve, R. B., *Exp Parasitol* , 1992, **75**, 213–222
8 Sato, K , Nagel, Y and Suzuki, M., *Mol Biochem Parasitol* , 1993, **58**, 293–300
9 Beuria, M K and Das, M K , *J Biosci* , 1992, **17**, 453–461
10 Das, M K , Beuria, M. K. and Dash, A P., *Int Arch Allergy Immunol* , 1992, **99**, 118–122.
11. Del Giudica, G , Engers, S. D , Tougene, C , Bitto, S., Weiss, N , Verdini, A. S , Pressi, A , Degremont, A , Frey Vogel, T. A , Lambert, P. H and Tanner, M , *Am. J Trop. Med Hyg* , 1987, **36**, 203–212
12 Dandapat, M C , Mohapatra, S. K. and Mohanty, S S., *Br J Surg* , 1986, **73**, 77–88.

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Glycosaminoglycan fractions in the cornea of two fishes

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Four glycosaminoglycan fractions – chondroitin 4-sulphate, chondroitin 6-sulphate, keratan and dermatan sulphates were isolated from the cornea of fishes: *Cyprinus carpio* and *Stromateus argenteus*. Glycosaminoglycans are essential for the maintenance of corneal structure and function.

GLYCOSAMINOGLYCANS (GAG) and their various fractions have been reported in the cornea of some higher vertebrates[1]. But studies on the various fractions of GAG and their significance in the cornea are yet to be reported though the corneal transparency and its consistency plays a significant role in photo-adaptation in aquatic medium. However, polysaccharide composition of corneal glycoprotein in a few fish has been reported and some interesting roles have been attributed to them[2, 3]. Here we report the occurrence of various fractions of GAG and their possible significance in the maintenance of corneal structure of common carp, *Cyprinus carpio* and pomfret, *Stromateus argenteus*. The fishes are from two habitats, the former is a freshwater fish while the latter is a marine one.

Histochemical preparations of cornea exhibited positive metachromatic reactions in both the cornea when stained with toluidine blue and paraldehyde fuchsin[4] but the reaction was a little weaker in the marine fish. The sections, when stained with PAS became purple. Alcian blue reaction indicated the presence of sulphated glycans[4]. GAG from each cornea was extracted for qualitative and quantitative estimation. Electrophoretic and chromatographic analysis of the extracted GAG confirmed the occurrence of the fractions – chondroitin 4-sulphate, chondroitin 6-sulphate, keratan sulphate and dermatan sulphate[5, 6]. Quantitative estimation indicated proportionately more GAG in pomfret cornea. Each extract was further hydrolysed and analysed chromatographically to detect various sugar components[7]. The sugars identified from their Rf values were glucose, galactose, mannose, fucose and xylose.

GAG, thus detected in the cornea may play a significant role in visual excitation since they act as selective ion barriers[8]. The transparency and elasticity of cornea is regulated by GAG[1]. Further GAG fractions may be responsible for the determination of consistency of cornea, since the cornea of pomfret is much thicker than the carp cornea, that is, active thickness control is influenced by GAG by filling the corneal interfibrillar spaces and repelling water since cornea of pomfret shows relatively weak metachromasia. This is because metachromasia is dependent on hydration and pH[9, 10]. We have also observed this phenomenon in carp cornea. Thus it is obvious that pomfret cornea is strongly hydrophobic. In this context, it is worth mentioning that hydration and dehydration are limiting factors in aquatic medium, and the various GAG fractions probably play a crucial role in maintaining corneal structure and its osmolarity in aquatic domain.

1. Maurice, D. M. and Riley, M V , in *Biochemistry of the Eye*, Academic Press, London, 1970, pp 50–70
2 Anseth, A , *Exp. Eye Res* , 1961, **1**, 106–115
3. Moczar, M and Moczar, E , *Comp Biochem Physiol.*, 1973, **45**, 213–223
4 Humason, G L , *Animal Tissue Techniques*, W H Freeman and Co , San Francisco, 1972, pp. 190, 317, 347
5. Jaques, L. B , *Methods Biochem Anal* , 1977, **24**, 206
6. Wessler, E., *Anal. Biochem* , 1968, **26**, 439–444
7. Trevelyan, W. E., Procter, D. P and Harrison, J S , *Nature*, 1950, **166**, 444
8 Jeanloz, R. W , in *The Carbohydrates*, Academic Press, 1970
9 Ashton, N., in *The Transparency of the Cornea, Symposium*, Blackwells, Oxford, 1960
10 Cejkova, J and Bolkova, A , *Albrecht von Graefes Archiv fuer Klinische Experimentelle Ophthalmologie*, 1974, **190**, 353–360

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