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Protective Effect of Acid protease inplasmodium berghei

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Abstract

Acid protease from plasmodium berghei was purified through sephadex G-200. Five fold purification of the enzyme was achieved.The purified enzyme contained mixture of proteins ranging between Molecular weights 27K to 66K .

Acid protease induced antibody response in mice and the animals were Partially protected upon challenge . Antiprotease antibodies inhibited about 50% invasion of erythrocytes by merozoites in vitro.

Introduction

Malaria is an infectious disease caused by the *plasmodium* parasite that continues to be a health issue for Human . it is one of the most common pathogenic factors of morbidity and mortality in Saharan Africa (Legesse *et. al* ., 2004) lnvasion of erythrocytes by *plasmodium* is a complicated process involving attachment and internalization of merozoite by the host erythrocyte (Bannister *et.,al.,1975*). Since Banyal *et. al* ., (1981) reported for the first time that Invasion of erythrocyte by merozoite in *P. knowlesi* was mediated by .malarial protease, the role of parasite proteases has been established *in vitro* in hemoglobin degradation processing of merozoite surface proteins , release of invasive merozoite and their involvement in host cell invasion (Braun Breton and Pereira da Silva , 1993) . Aserine protease of *P. chabaudi* , p68, facilitates the entry of merozoite into the host cell as the invasion was prevented in the presence of inhibitors while the inhibitors were not effective when erythrocytes were pretreated with p68 (Braun Breton *et. al* .,1992) .Correct disruption of the plasmepsin 4 gene was confirmed by diagnostic PCR and southern blat analysis and the absence of PM4 expression was shown by Northern analysis and western analysis of blood. stage proteins (Kooij et.al.,2010) . Another Protease , a cytokine protease of 37k from p. falciparum was Involved in merozoite release (Deguercy et. al ., 1990)The hemozoin pigment granule produced by plasmodium has been repotedd to induse proinf lammatary immune response (Coban et. al ., 2005). ln addifion, p. falciparum mutants that lack all DV aspavtic protease activity produce less hemozoin then wild – type parasile (Bonilla et. al ., 2007) . P. berghei possesses significant activity of protease active in the acid pH range (Banyal and Makkar , 1992) . Now the question is do malarial proteases exhibit invasion blocking antimalarial role in in vivo as indicated by in vitro inhibitor studies we have purified this enzyme through sephadex G- 200 and used the purified enzyme to evaluate the protective effect of antiprotease antibodies in immunized mice and in invasion inhibition assays in vitro .

Materials And Methods

Parasite: *P berghei* NK – 65 (Zoology Deptt.Panjab University , India) was maintained in white Swiss mice (Laca strain) .Infected blood was collected in citrate saline and passed through CF-11cellulose and cell – free parasites were obtained by saponin lyses . Cell free parasites were suspended in normal saline homogenized in cold and centrifuged at 1,000g for 10 min .Clear supernatant (parasite extract) was purified through sephadex G-200 .

purification of acid protease:The acid protease was purified through sephadex G – 200 column according to the method of Andrews (1965) . The column was loaded with 2 ml of parasite extract . 45 fractions each of 5 ml were collected with flow rate 20-25 ml/h.The fractions were analyzed for protein and enzyme contents. Acid protease assay : Acid protease activity was determined by slightly modified method of Banyal et. al. (1982) . protein free supernatant was taken for color development with Folin and Ciocalteau's reagent (Lowry et. al ., 1951) .

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):The purified fractions were characterized electrophoretically by SDS-PAGE (Laemlli, 1970) using 10% separating and 3% stacking gels The gels were stained with silver nitrate (Morrissey ,1981).

lmmunization of mice : Three groups of mice were immunized with purfied acid protease antigens.0.2 ml of pooled antigen containing 50 mg of protein was emulsified with 0.2 ml Freund's complete adjuvant (Difco) and injected subcutaneously on day 0 to each mouse . Two booster doses were given intraperitoneally

two weeks apart with same amount of protein suspended in tris – KCI buffer , pH 7.5 . Control mice received only tris KCI buffer instead of protein .Seven days after the last booster dose some mice along with controls were challenged intraperitoneally with 1×10s P.berghei infected erythrocytes and the course of parasitaemia was monitored .Sera were collected on day 33 post – immunization (prechallenge) and from the mice which survived the challenge and no parasites was seen in their smears for 15 –20 days post – challenge

Indirect fluorescent antibody (IFA) test : Sera were analyzed by IFA (Collins and Skinner,1972) and The slides Counter stained with Evane 's blue

ln vitro invasion inhibition assay : The short – term culture of P. berghei was maintained in vitro (Trager and Jensen 1976) . Differential count in Giemsa stained Smears was made and percent inhibition of merozoite invasion was calculated as :

$$\% \text{ inhibition} = 100 - \frac{\text{No . of ring in Exp . culture}}{\text{No . of ring in Control culture}} \times 100$$

Results

Maximum activity of *P. berghei* acid protease was eluted In fraction 12 along with maximum parasite protein content Significant activity of the enzyme was also observed in fractions 20 to 30. 495 fold recovery of the enzyme and 5-8 fold purification were achieved in major fractions (Table l) . protease appears to be a mixture of proteins of different molecular weights . Fractions 11 and 12 Contained proteins of 66, 40 ,36 ,32 and 28 KDa. Fractions 23, 24, 25, 26, 27 and 28 contained protein of 66 , 49 , 41 ,32 , 28 And 27 KDa .Asingle band protein of molecular weight 27k was eluted in fraction 13.(Fig . 1)

lmmuization with acid protease: Acid protease antigens used in immunization were designated as API , APII and APIII . API was pooled fractions 11 and 12 , APII contained fractions 23,24 and 25 and APIII had fractions 26 , 27 and 28. During immunization 5 mice from APl were found dead on day 15 , i.e., next day of first booster dose . On day 35 ,2 mice From API , 4 from APII, 4 from APII I and the placebo controls Were challenged .Control mice showed gradual increase in Parasitaemia and all of them died by day 4 (Fig. 2A). 0f the Mice immunized with API, APII and APIII , only 2 survived, One each from API and APII (Figs . 2B .2C. 2D). In others prepatent, and patent period were though prolonged mice died due to infection .

Indirect immunofluorescence antibody assay : with sera Of API, the prechallenge titre was 1: 1024 while in APII the titre in pre – and post challenge sera was 1:2048 . In APIII , the prechallenge titre was Iow (1:32) . Fluorescence was seen on whole of the parasite and not restricted to ang particular .

ln vitro invasion inhibition : 2.5 to 5.0% immune sera from prechallenge / postchallenge mice added to the culture showed inhibition of newly formed rings by 50% 56% and 39% with prechallenge API, APII and APIII sera respectively (Table 2) . Free merozoites were seen in cultures with Prechallenge immune sera as compared to the normal controls. Merozoites were slso found adhered to the erythroeytes

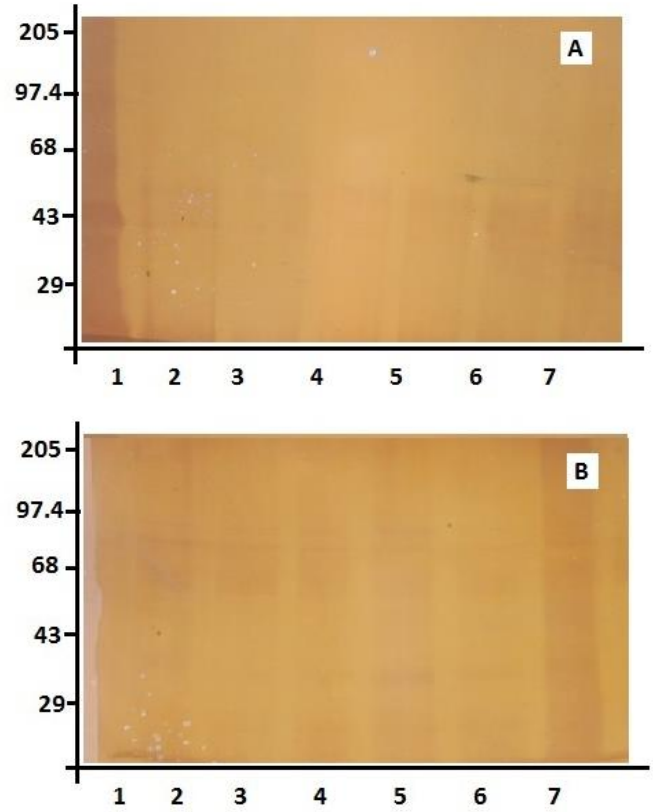


Fig. 1(A , B) : SDS – PAGE of fraction purified through sephadex G-200 column , A Lane 1- Protein standard (PS) , Lane 2 to 6 - Fractions 11, 12 , 13, 23 and 24 respectiively , B Lane 1 – Protein standard (PS) , Lanes 2 to 6 – Fractions 25, 26, 27, 28 and 32 respectively

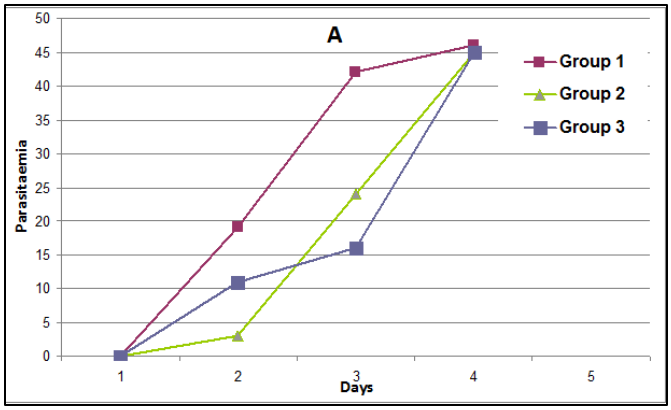
Table 1 Purification of acid protease from p . berghei by sephadex G-200

Fraction	Volume (ml)	protein (mg / ml)	Acid protease Unit/ml	Acid protase Units / mg	Yield (%)	purification (fold)
Acid protease						
Crade	2,0	8.14	1.9574	0.240	-	-
11,12	10.0	0.44	0.5555	1.154	141.9	4.81
23,24,25	15.0	0.24	0.4633	1.966	177.5	8.19
26,27,28	15.0	0.23	0.4585	2.0	175.6	8.30

Table 2 Merozoite invasion inhibition in the presence of pre-and postchallenge sera of mice immunize with purified acid protease antigens

Sera	Cone. Of Serum (%)	No of parasitized RBCs /5000 RBCs Parasitaemia (%)	R	T	S	% invasion inhibition
0hr	-	0.20	0	3	7	-
After 20h Normal	5.0	0.70	18	1	16	-
Immune	5.0	0.28	7	0	7	61
API pre-challenge	2.5	0.54	9	0	18	50
API post-challenge	2.5	0.28	5	0	9	67
APII pre-challenge	5.0	0.28	8	0	6	56
APII post-challenge	2.5	0.42	7	2	12	61
APIII. pre-challenge	5.0	0.38	11	0	8	39

R = ring T – trophozoite S - schizont



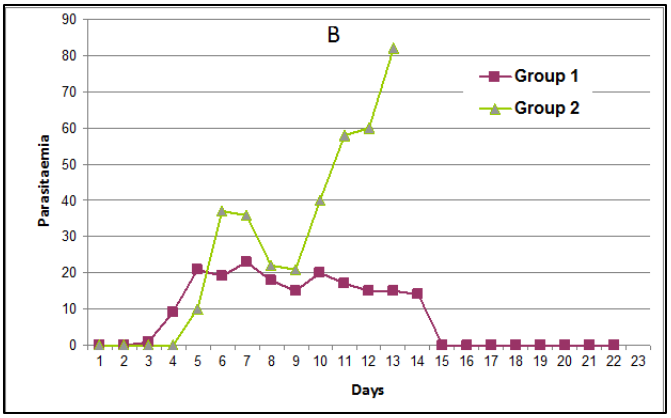
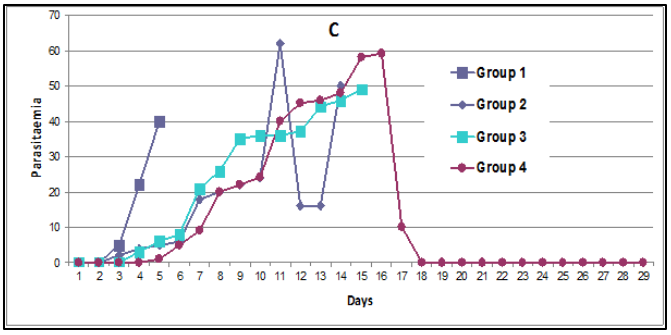


Fig.2 (A &B) : Course of parasitaemia in mice immunized with acid protease antigens , API – APIII , and subsequently challenged with 1 *10 berghel – parasitized erythrocytes . Signs denote the data of single mouse A-Plcaebo controls B – APII



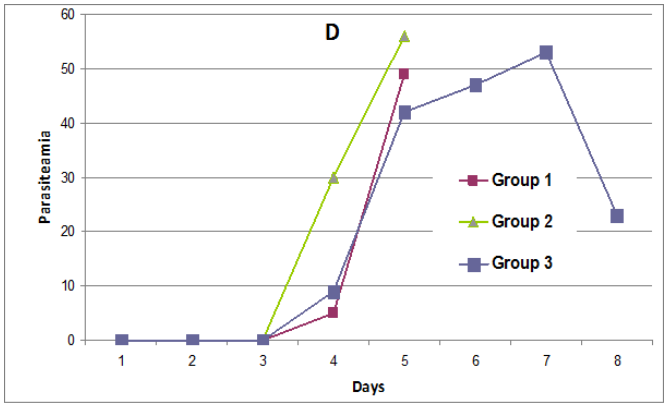


Fig.2(C & D) : Course of parasitaemia in mice immunized with acid protease antigens , API – APIII , and subsequently challenged with 1 *10 *berghel* – parasitized erythrocytes . Signs denote the data of single mouse C- APII , D - APIII

Discussion

P berghei possesses significant activity of acid protease ln our repeated experiments we found that P. berghei acid protease is a mixture of proteins of molecular weights ranging between 27Kand 66like P. falciparum protease (Bailly et al., 1991) . Further , a68K neutral endopeptidase of P. berghei has been associated with the apical complex of the merozoite (Bernard and Schrevel , 1987) . In the present study , p. berghei Acid protease exhibited partial ln vitro invasion – inhibition and in vivo protection. There seems a relationship between the antibody level and inhibition /protection achieved in this study . Since APIII generated low antibody response , in vivo inhibition was only 39% and mice did not survive the challenge as compared to the other two antigens. Effect of antiprotease antibodies on parasite s invasion may be analogous to protease inhibitor s effect on the same process (Banyal et al., 1981), (Kooij et. al ., 2010) found that all mice infected with 10^4 to 10^5 parasites were protected which inoclatcd from a Δ pm4 . In the smear s of cultures with prechallenge sera we found that merozoites could attach to the erythrocyte s surface but were unable to penetrate the host cell . It is quite likely that the antiprotease antibodies present in the culture did not inhibit the attachment of the merozoite to the erythrocyte but further entry of the parasite into the erythrocyte was prevented . However, the antibodies elicited partial inhibitory /protective effect as compared to the significant inhibition by serine-protease inhibitors (Braun Breton et al ., 1988) . This may be because : either the concentration of antigen inoculated was not enough to generate sufficient amout of antibodies to completely eliminate the parasite or the invasion process is not exclusively mediated by proteases thereby antiprotease antibodies unable to check the invasion process or delay interaction between antibody and parasite- erythrocyte contact/penetration as it is a quick process . To answer the last two questions antibody labeling studies may provide further informations . Our results, however , clearly indicate that antiprotease antibodies significantly interfere in merozoite invasion process in vitro and in vivo.

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