RESEARCH ARTICLE

Isolation, purification and characterization of *Plasmodium yoelii* adenosine deaminase for clinical use

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ABSTRACT

Plasmodium yoelii like many other parasitic protozoans is deficient of having the advantage of the de novo pathway for purine biosynthesis and starkly acts upon the salvage pathway. Adenosine deaminase (Also known as adenosine aminohydrolase or ADA; EC 3.5.4.4), the first enzyme of the pathway was purified from *Plasmodium yoelii*, a rodent malarial species by using ammonium sulphate precipitation cut column chromatography by DEAE-Sephadex ion exchange column and Sephadex G-100 gel filtration column in successive steps and the activity of ADA was estimated afterwards. The purity of ADA was checked on 10% SDS–PAGE followed by the conduction of Western Blot to analyze the molecular weight of obtained ADA. After carrying out the western blot technique, the electrophoresis data interpreted that the purified enzyme is monomeric having the molecular weight of 41 KD. The specific activity of ADA was increased in each purification step indicating that protein was purified in each step. The enzyme was found to be pure to ~10 folds. By thwarting the requirement as well as the effect of ADA, potential anti-malarial drugs can be designed against the rage of *Plasmodium yoelii* and other parasitic protozoans and this may serve the purpose to a great extent.

Keywords: Adenosine deaminase, *Plasmodium yoelii,* Purine biosynthesis.

INTRODUCTION

The causative agents of Malaria, a mosquito-borne infectious disease of humans and other animals are the parasitic protozoans (a type of unicellular microorganism) belonging to the genus *Plasmodium*. According to the estimation carried out by WHO in 2010, 219 million cases of malaria were recorded resulting 6, 60,000 deaths [1,2]. The number of occurrence estimated by other agencies was between 350 and 550 million for *Plasmodium falciparum* malaria [3] and deaths in 2010 at 1.24 million [4] up from 1.0 million deaths in 1990 [5]. The majority of cases (65%) occurred in children under 15 years old [4].

As far as the mechanism of combatting the emergent drug-resistant strains of the genus *Plasmodium* is concerned, it has become really cumbersome to deal with the exixting anti-malerial drugs thereby compelling us to ponder over something more substantial [6,7]. *Plasmodium yoelii* like many other parasitic protozoans is deficient of having the advantage of the de novo pathway for purine biosynthesis. Conspicuosly, they do feel the urge of requirement of purine nucleotides for DNA and RNA synthesis which can be fulfilled through the salvage of preformed purine bases from host [8,9]. Therefore, the enzymes associated with this pathway can be taken into the consideration for potential targets to devise efficacious drugs of anti-malarial activities [10]. Adenosine deaminase (Also known as adenosine aminohydrolase or ADA; EC 3.5.4.4) is considered to be one of the key enzymes involved in purine metabolism [11]. It is required for the purpose of breakdown of adenosine from food and for the turnover of nucleic acid in tissues. The higher degree of the conservation of amino acid sequence of ADA is a testament of being quintessential in the purine salvage pathway where it catalyzes the conversion of adenosine into inosine. Subsequently, inosine undergoes conversion and produces hypoxanthine which is thought to be the major precursor for purine salvage pathway [12]. This present study focuses on the increment of purification level of isolated ADA from a

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protozoan source – *Plasmodium yoelii* of infected swiss mice.

MATERIAL AND METHODS

Chemicals required

Adenosine, DEPC, NEM, pCMB, acrylamide, potassium iodide (KI), urea, GdmCl, EDTA, Freund's adjuvants were obtained from Sigma (St.Louis, USA). Pre-stained SDS–PAGE protein markers were obtained from MBI, Fermentas. DEAE-Sephadex, Sephadex G-100, protein markers were obtained from Amersham Biosciences.

Infection maintenance

Swiss mice (20–25 g) were intra-peritoneally injected with *P. yoelii* parasitized RBCs in 3% tri-sodium citrate (pH 7.4) containing 0.85% NaCl.

Estimation of parasitaemia [13]

The smear was examined under oil immersion microscope. The percentage parasitmia was determined by counting the number of parasitized RBCs out of 100 erythrocytes.

Isolation of parasites from infected blood

Blood collected from infected mice at 40–50% parasitaemia by cardiac puncture was passed through the CF-11 column to remove leukocytes [14]. The resultant infected erythrocytes were collected by centrifugation at 800g for 5 min and parasites were liberated by adding an equal volume of 0.15% (w/v) saponin in 1 _ phosphate-buffered saline (PBS, pH 7.4). Parasites were sedimented by centrifugation at 1300g for 5 min. The parasite pellet was washed 5 times with ice cold PBS and stored at -80 °C until used.

Purification of enzyme adenosine deaminase

The parasite lysate prepared by sonication was given 0-35% ammonium sulpahte saturation, stirred and kept at 4°C for precipitation for 2 hours. Then it was centrifuged at 12000 rpm at 4°C for 40 min. The pellet was suspended in the assay buffer and its activity was checked. The supernatant was given 35-70% saturation, stirred and kept overnight for precipitation. The precipitation protein was collected by centrifuging at 12000 rpm at 4°C for 40 mins. The pellet was resuspended in the assay buffer. Activity of ADA was checked.The fraction (35-70% containing ADA activity) was dialyzed overnight at 4°C against 0.05 M potassium phosphate buffer (pH-7.4) with 1 mM BME (β-mercaptoethanol) [15].

The 3 gm powder of DEAE sephadex was swelled in TDW for overnight. The soaked matrix was washed 3 times with TDW and then with 0.05 M potassium phosphate buffer (pH 7.4) containing 1mM BME. Then packed on the column (18 X 0.5 cm) & equilibrated with the same buffer.The active and dialyzed fraction (35-70 % cut) was loaded on prepared column. Unbound protein was eluted with the buffer. The bound protein was eluted using NaCl gradient (0.1M, 0.2M, 0.3M NaCl in same buffer). Protein profile of all the fractions was then at 280nm. Appropriate fractions were tested for enzyme activity. Fractions (20, 21, 22, 23, 24) having high activity of ADA were pooled & concentrated using sucrose. The sample was again purified using gel exclusion chromatography [16].

For the preparation of column, 3 gm of sephadex G-100 powder was weighed & soaked overnight in TDW, then washed 3 times with TDW & 3 times with 0.05 M Potassium phosphate buffer (pH-7.4) and packed into column & equilibrated with the same buffer. The concentrated protein (after anion exchange column) was loaded on the column & eluted with potassium phosphate buffer (pH-7.4) with 1mM BME and 3 ml fraction were collected. Protein was estimated in all collected fraction at 280 nm. Appropriate fractions were tested for enzyme activity. Fractions (5, 6, 7, 8) having high activity were pooled & concentrated using sucrose and considered as purified enzyme [17].

Measurement of the enzyme activity

In order to estimate the activity of the enzyme the reaction mixture was prepared. 0.05 M Potassium phosphate buffer (pH-7.4) 0.900 ml,1.35 mM Adenosine 0.025ml, Enzyme 0.025ml, TDW 2.050ml is

mixed thoroughly to prepare that reaction mixture. After preparation of reaction mixture, disappearance of adenosine was measured at 265nm. The enzyme activity is defined in terms of "units" where 1 unit is defined as "the amount of enzyme needed to convert 1 µmole of adenosine to inosine per minute at 25°C".

Estimaton and determination of molecular weight of the purified enzyme

Protein was estimated in purified fractions collected in each purification step using BSA as standard by method of Bradford [18].The purity of ADA was checked on 10% SDS–PAGE according to Laemmli (1970). Western Blot [19] was then performed to analyze the molecular weight of obtained ADA.

RESULT AND DISCUSSION

From the ADA purification and activity table (Table:1), it is conspicuous that the crude enzyme has highest activity.With the increase of the ammonium sulphate level, the enzyme activity also increases which means at this point maximum substrate can be used to convert into products. The ADA activity in supernatant also shows the same activity as in 0-35% ammonium sulphate cut enzyme shows. From the purification table, it was observed that the fold purification of the enzyme had increased after each step of the purification process. After the ammonium sulphate cut, its fold purification was found to be 1.1. However, after passing the ion exchange column, the purification fold increased to 6.6 and after the final passage through the gel permeation column, the purification fold was 10. This showed that the enzyme was fairly purified. However, after each step of the purification process, the yield of enzyme was visibly reduced such that at the end of the purification process, the percentage yield of the enzyme was less than half. The SDS-PAGE analysis of 35-70% ammonium sulphate fraction and Sephadex G-100 purified fractions shows clear bands of enzyme. The electrophoresis data can be easily interpreted after the Western Blot. It clearly shows that the enzyme is monomeric and the molecular weight is 41 Kda (Fig: 1 & 2).

ADA is found in bacteria, plants, invertebrates, vertebrates, and mammals, with highly conserved amino acid sequence [20]. ADA association has also been observed with epithelial cell differentiation, neurotransmission, and gestation maintenance [21]. Primarily, ADA in humans is involved in the development and maintenance of the immune system. It has also been proposed that ADA, in addition to adenosine breakdown, stimulates release of excitatory amino acids and is necessary to the coupling of A1 adenosine receptors and heterotrimeric G proteins [20]. In the present study, ADA purified from P. yoelii, showed molecular weight of 41 Kda. This result can be supported by other few recent studies [22] and it is also reported that the Km value of the enzyme for its substrate adenosine is 41µm [23] which is similar to values obtained from P. falciparum [24] but different from human erythrocyte (25 µm) and mice intestine (23 µm) and for 2 deoxy adenosine the km value is relatively lowered 25 µm For, 2 deoxy adenosine, ADA showed a relatively lower km (34 µm) indicating high specificity for the same. P. yoelii ADA contains four trytophan residues [25] and it was inhibited to different purine analogs which structurally different in nitrogenous base substitution but have common ribose sugar moiety that indicates that the inhibition is independent of sugar moiety [26]. ADA can also be used against other diseases like tuberculosis [27].

Table 1: Purification of Adenosine Deaminase of Plasmodium Yoelii

Purification Step	Activity (units/ ml)	Protein (mg/ ml)	Specific activity	Fold purification	Percentage yield
Crude	0.5246	2.92	0.1797	1	100%
35-70% cut	0.3827	1.94	0.1973	1.1	72.9%
DEAE-Sephadex	0.2901	0.244	1.1889	6.6	55.3%
Sephadex G-100	0.2469	0.140	1.7636	10.0	47.1%

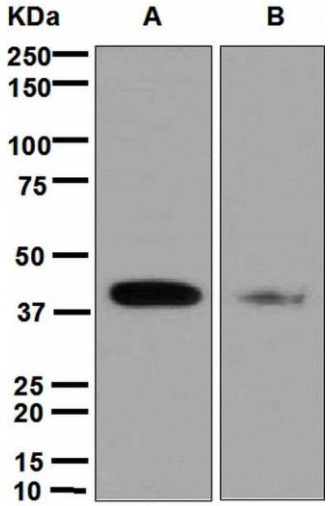

Fig:-1: Western Blot analysis of ADA
A. Pre-Stained protein ladder
B. Purified ADA

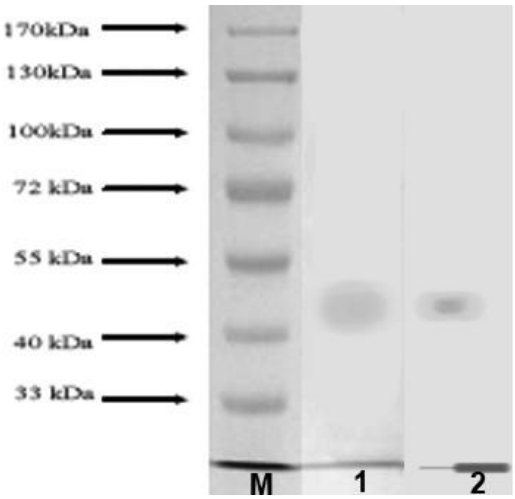

Fig: 2: SDS-PAGE analysis of ADA
M- Protein marker
1. 35-70% ammonium sulphate fraction
2. Sephadex G-100 purified fractions

CONCLUSION

The enzyme Adenosine Deaminase was isolated from *Plasmodium yoelii*. It was purified using ammonium sulphate precipitation cut column chromatography by DEAE-Sephadex ion exchange column and Sephadex G-100 gel filtration column in successive steps. The specific activity was increased in each purification steps indicating that protein was purified in each steps. The enzyme was found to be pure to ~10 folds. Pure enzyme can be obtained by the Western Blot technique. Since ADA has a highly conserved amino acid sequence, hence this enzyme can be proved to be efficacious in human for therapeutic purposes. If the utilization of the precursor molecule and the enzyme ADA required for the salvage pathway can be abrogated, the *Plasmodium yoleii* and other parasitic protozoans will no longer be capable enough to thrive and the efforts may come to fruition to a great extent.

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