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Journal of Pharmaceutical and Biomedical Analysis
20 (1999) 203–207

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Assay of β-hematin formation by malaria parasite

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Received 10 September 1998; received in revised form 10 November 1998; accepted 18 November 1998

Abstract

Novel leads are urgently required for designing antimalarials due to the reduced efficacy of presently available drugs. The malaria parasite has a unique reaction of heme polymerization, which has attracted much attention in the recent past as a target for the design of antimalarial drugs. The process is hampered by non-availability of a proper assay method. Currently available methods are cumbersome and require advanced instrumentation or radioactive substrates. Here, we are describing an assay for hemozoin formation that is simple and reproducible. This assay has routinely been used by us for the identification of potential compounds with antimalarial activity. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Hemozoin; Malaria; *Plasmodium*; Hemoglobin; Heme; Chloroquine

1. Introduction

Malaria parasite requires the hemoglobin of the host erythrocyte for its supply of amino acids. Hemoglobin digestion occurs inside the digestive vacuole of malarial parasite and a large amount of toxic heme is released during this process, which could cause extensive damage to membranes and inhibit a variety of enzymes resulting in the death of the parasite [1]. *Plasmodia* have a unique mechanism for detoxification of heme; they convert it into the hemozoin pigment [2–4]. Hemozoin or malaria pigment, as it is popularly known, is a polymer of heme units, linked through an iron–carboxylate bond [5]. This pigment is inert in the parasite and released into the blood of infected host after the end of the parasite life cycle [6,7]. Mechanism of hemozoin formation is not fully understood at present [1]. A histidine-rich protein (HRP II), from human malaria parasite *Plasmodium falciparum*, containing multiple repeats of a hexapeptide sequence, which could bind to heme, has been shown to initiate this polymerization [1,8–10].

Currently available assay methods for heme polymerization are cumbersome and do not provide reproducible results [11–13]. This has led to a series of contradictory reports regarding mechanism of heme polymerization. Due to the lack of a proper assay method, several reports

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PII: S0731-7085(99)00021-7

appeared in the literature describing heme polymerization as a spontaneous chemical process, which does not require presence of parasitic material [1,14]. Recently, we have shown that hemozoin formation under physiological conditions requires parasitic material [14]. These findings have lately been confirmed by other groups and a heme polymerization activity has been characterized from the rodent malaria parasite *Plasmodium berghei* [15].

Here we describe an assay for hemozoin formation, which is reproducible, sensitive and does not require radioactive substrate. This assay has been extensively used by us for screening of compounds for antimalarial activity.

2. Materials and methods

2.1. Materials

Hemin, SDS and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Parasite and experimental host

Male, Swiss albino mice, weighing 15–20 g were maintained on a commercial pellet diet and housed under the guidelines laid down by the institutional ethics committee. *Plasmodium yoelii nigeriensis*, a rodent malaria parasite, which gives high levels of parasitemia, was used for infection. Mice were infected by intraperitoneal passage of 1×10^7 infected erythrocytes, diluted in 0.5 ml of sterile acid citrate dextrose (citric acid 7.3 g, sodium citrate 22.0 g and dextrose 24.5 g, dissolved in 1000 ml of triple distilled water). Parasitemia was monitored by microscopic examination of Giemsa-stained thin blood smears [16].

2.3. Preparation of parasite lysate

Blood of infected animals, at high level of parasitemia (40–50%), was collected by cardiac puncture and centrifuged at $500 \times g$ for 10 min at 4°C to pellet the erythrocytes. Plasma and buffy coat was removed and the RBC pellet was washed twice with chilled (4°C) phosphate buffer saline (PBS) (10 mM, pH 7.4). Parasites were obtained by hypotonic lysis of RBC with chilled (4°C) Tris–HCl buffer (10 mM, pH 7.4). The RBC pellet was suspended in four volumes chilled buffer for 30 s followed by addition of one volume 5.4% NaCl. The erythrocyte lysate was centrifuged at $10\,000 \times g$ for 15 min at 4°C to pellet the parasite. The parasite pellet was again suspended in PBS containing 0.9% glucose (PBSG) and centrifuged at $5000 \times g$ for 20 min at 4°C. The parasite pellet was suspended again in four volumes PBSG. The parasite lysate was prepared by freezing the suspension in liquid nitrogen drop by drop. Frozen droplets of the lysate were stored in aliquots at −70°C until further use. When required, an aliquot of the lysate was thawed and centrifuged at $16\,000 \times g$ for 30 min at 4°C. The pellet was resuspended in sodium acetate buffer (100 mM, pH 5.0) by sonication (20 kC s^{-1} for 10 s under chilled conditions) and used for the heme polymerization assay. Parasite lysate could be stored at −70°C for 2 months, without any significant loss of activity. Inclusion of protease inhibitors was not found to have any effect on the reaction.

2.4. Heme polymerization assay

The heme polymerization assay mixture contained 50 μl of the parasite extract, 100 μM hemin as the substrate and sodium acetate buffer (100 mM pH 5.0) in a total volume of 1.0 ml. Polymerization of heme could also be started by PfHRP II. In one of the experiments, recombinant PfHRP II, expressed and purified as described earlier [8], was used for initiating heme polymerization. Two controls were always run simultaneously, one without the substrate and another without the parasite extract (containing only 100 μM heme in acetate buffer). Each assay was set up in triplicate and incubated at 37°C for 6 h on a rotary shaker. The reaction was stopped by centrifugation at $16\,000 \times g$ for 5 min at 25°C and pellets were resuspended in Tris–HCl (100 mM, pH 7.4) containing 2.5% SDS and incubated at 37°C for 1 h, followed by centrifugation at $16\,000 \times g$ for 15 min at 25°C. The pellets were

suspended in Tris–HCl buffer by sonication at 30 kC s^{-1} for 10 s and kept at 37°C for 30 min. The pellet was collected again by centrifugation as above and resuspended in sodium bicarbonate buffer (100 mM, pH 9.0) and incubated at 45°C for 1 h. The pellet was again collected by centrifugation and washed once more with bicarbonate buffer. This step dissolves most of the free heme attached to the hemozoin and other non-hemozoin adducts. This is necessary for the removal of hemozoin-like adducts which form spontaneously during the assay as described by us previously [1,10,14]. Final washing was done with 95% ethanol. The pellet thus obtained was of polymerized heme (hemozoin).

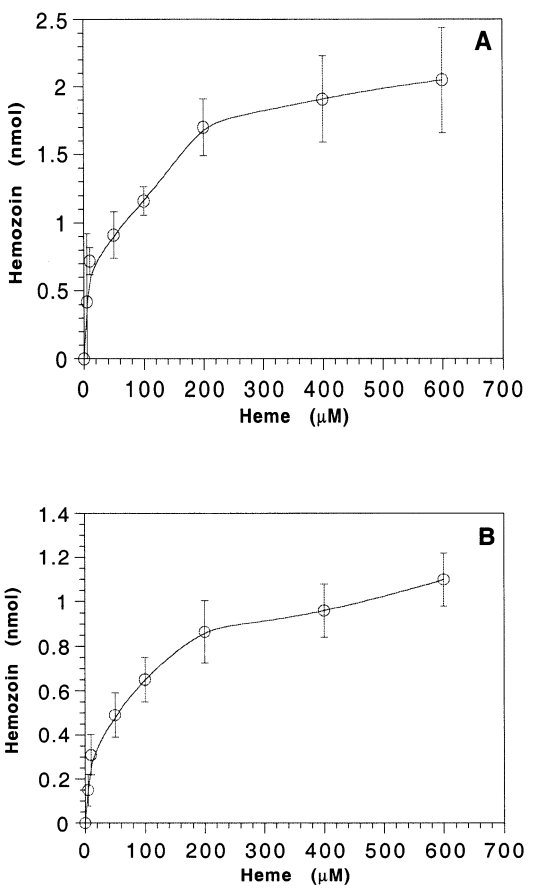

Fig. 1. Effect of increasing substrate concentration on heme polymerization activity of *Plasmodium yoelii* lysate (A) and PfHRP II mediated heme polymerization (B). Each data point represents mean ± SD of triplicate observations.

2.5. Estimation of heme

For quantification of hemozoin, the pellets were solubilized in 2 N NaOH (50 μl) and kept at 37°C for 1 h to convert the polymerized heme (hemozoin/β-hematin) to hematin. SDS (2.5%, 950 μl) was added to each tube and absorbance was measured at 400 nm. A millimolar extinction coefficient of 91 mM^{-1} cm^{-1} at 400 nm was used for quantification of heme as described previously [9,17].

3. Results and discussion

We have found that when free heme is incubated with particulate fractions of the *P. yoelii* lysate or PfHRP II, under acidic conditions, a fraction of heme is polymerized that could be identified as a product insoluble in SDS (2.5% w/v) and alkaline bicarbonate solution. However, no such product was formed when heme alone (without any parasite extract) was processed in a similar manner. We found that the formation of hemozoin by *P. yoelii* extract is dependent on amount of heme (Fig. 1A) as well as protein concentration of the parasite extract and incubation time (Fig. 2). Similar patterns have been observed earlier by other workers for heme polymerization by *P. falciparum* and *P. berghei* [3,11]. Assay is linear till 6 h of reaction and is dependent upon the amount of protein present in the system. Recently, a histidine-rich protein (PfHRP II) has been shown to catalyse polymerization of heme [8]. We used PfHRP II as a source of heme polymerization activity in one of the experiments on hemozoin formation in vitro (Fig. 1B). Use of PfHRP II to promote heme polymerization has the advantage of providing zero basal levels of hemozoin. When parasite lysate is used, hemozoin already present in the lysate has to be deducted from the total pigment at the end of the reaction.

The inhibition pattern of heme polymerization by chloroquine and quinine (Fig. 3) was similar to that reported for *P. falciparum* or *P. berghei* as reported earlier [3,11]. The assay was used to study the effects of potential antimalarial compounds on heme polymerization. Since, all the

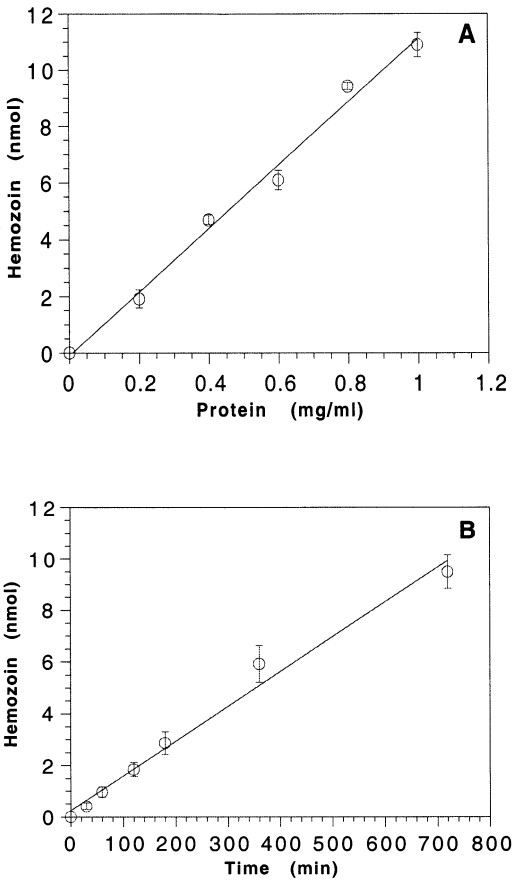

Fig. 2. Effect of protein concentration (A) and time variation (B) on heme polymerization *by Plasmodium yoelii*. Each data point represents mean ± SD of triplicate observations.

blood schizontocidal antimalarial drugs reported so far, bind to heme and inhibit hemozoin formation, this assay could be utilized as a primary screen for potential candidates for antimalarial development.

Washing steps described by us in this assay could differentiate between hemozoin and free heme with reproducible results. This takes care of any ambiguity in the results due to mixing up of free heme with the hemozoin pigment. Since large amount of heme is present in the reaction mixture, which binds to proteins of the parasite lysate, it is necessary to remove the non polymerised heme completely before dissolving the hemozoin for detection. Raising the temperature to 45°C during bicarbonate washing step was found to give better reproducibility as compared to room temperature incubations. As we have shown earlier [1,9,14], solubility properties of native hemozoin, as well as synthetic β-hematin are different from free heme. Moreover, although heme could form an iron–carboxylate bond with the organic acids present in the reaction mixture that has similar infra red absorption patterns, the heme–acid complex could be easily distinguished from hemozoin on the basis of solubility properties.

Use of recombinant PfHRP II could increase the reproducibility of assay as the error due to the basal hemozoin pigment level in the samples is removed. Washing of free heme from PfHRP II-induced reaction is easier as the protein–heme complex is easy to remove, resulting in pure preparations of the newly formed pigment. The method described here by us enables quick and reproducible measurement of hemozoin formation and could be adopted for screening of drug candidates. This method could easily be adapted to work in a 96-well plate format, that would enable

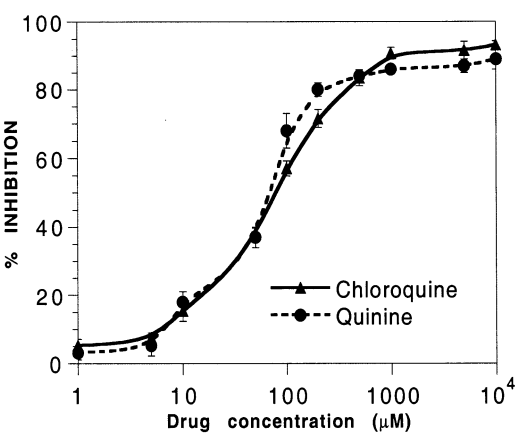

Fig. 3. Inhibition of *Plasmodium yoelii* mediated heme polymerization by chloroquine and quinine. Experimental conditions were as described Section 2. Chloroquine was dissolved in water and quinine was dissolved in 70% ethanol, 50 μl of the appropriate dilution was added in the reaction mixture to reach the desired concentration. A control with equal amounts of ethanol was found to be similar as the one without ethanol. Parasite lysate (200 μg of protein) was added in the tubes to start the reaction and incubation was continued for 6 h at 37°C with constant stirring. Each data point represents mean ± SD of triplicate observations.

processing of a large number of samples with minimal reagents. Using the automation devices available for microwell plate assay systems, heme polymerization could be performed in high throughput assay format for screening large molecular libraries.

Acknowledgements

The authors thank Dr V.C. Pandey for helpful suggestions and discussions. Help of Mr G.P. Dixit and V.K. Nigam in maintaining *P. yoelii* in mice is gratefully acknowledged. NS is recipient of a Senior Research Fellowship from the Council of Scientific and Industrial Research (CSIR), New Delhi, India.

References

[1] A.V. Pandey, V.S. Chauhan, Curr. Sci. 75 (1998) 911–918.
[2] A.F.G. Slater, Pharmacol. Ther. 57 (1993) 203–235.
[3] A.F.G. Slater, A. Cerami, Nature 355 (1992) 167–169.
[4] A.V. Pandey, B.L. Tekwani, FEBS Lett. 402 (1997) 236–240.
[5] A.F.G. Slater, W.J. Swiggard, B.R. Orton, et al., Proc. Natl. Acad. Sci. USA 88 (1990) 325–329.
[6] A.V. Pandey, B.L. Tekwani, V.C. Pandey, Biomed. Res. 16 (1995) 115–120.
[7] A.V. Pandey, B.L. Tekwani, Parasitol. Today 11 (1996) 370.
[8] D.J. Sullivan, I.Y. Gluzman, D.E. Goldberg, Science 271 (1996) 219–221.
[9] A.V. Pandey, S.K. Joshi, B.L. Tekwani, V.S. Chauhan, Anal. Biochem. 268 (1999) 159–161.
[10] A.V. Pandey, R.M. Joshi, B.L. Tekwani, R.L. Singh, V.S. Chauhan, Mol. Biochem. Parasitol. 90 (1997) 281–287.
[11] A.C. Chou, C.D. Fitch, Biochem. Biophys. Res. Commun. 195 (1993) 422–427.
[12] A. Dorn, R. Stoffel, H. Matile, A. Bubendorf, R.G. Ridley, Nature 374 (1995) 269–271.
[13] K. Bendrat, B.J. Berger, A. Cerami, Nature 378 (1995) 138.
[14] A.V. Pandey, B.L. Tekwani, FEBS Lett. 393 (1996) 189–192.
[15] C.D. Fitch, A.C. Chou, Mol. Biochem. Parasitol. 82 (1996) 261–264.
[16] N. Singh, S.K. Puri, J. Parasit. Dis. 20 (1996) 45–48.
[17] T. Asakura, K. Minakata, K. Adachi, M.D. Russel, E. Schwartz, J Clin. Invest. 59 (1977) 633–640.