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Secretome Analysis of *Plasmodium Falciparum* Intraerythrocytic Asexual Stage Using SDS-PAGE Electrophoresis

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Abstract: ***Plasmodium falciparum causes fatal cerebral malaria and more knowledge is required on its proteome. Proteomic studies require the adaptation of P. falciparum field isolates to continuous in vitro cultivation. The objective of this study is to identify and characterize some of the proteins secreted by P. falciparum trophozoites in the host cell using in vitro conditions. For this purpose, propagation protocols of asexual erythrocytic stages of P. falciparum have been established. The parasites were grown in standard culture medium without human serum but supplemented by AlbuMax 1. The secreted proteins were characterized by gel electrophoresis. Two staining methods were used to detect the separated proteins. The secretome profile for P. falciparum was characterized that revealed over 24 excreted proteins. The protocols described here will be useful in functional analysis by proteomics of different pathogenic patient isolates***

Keywords: Plasmodium falciparum – serum free media – secretome – trophozoites – SDS-PAGE

1. Introduction

Malaria remains one of the world's most devastating infectious diseases. *Plasmodium falciparum* causes the most severe form of Malaria in humans, with several hundred million infections each year and 1-2 million deaths. It is therefore, not surprising that the majority of the research efforts are going worldwide on molecular biology, chemotherapy and vaccine development in different laboratories have focused on *P. falciparum*, aided by *in vitro* cultivation of the parasite that previously described [1].

The stages in the life cycle of *P. falciparum* in humans are well defined, beginning with the sporozoites that initiate infection in the liver, merozoites that infect erythrocytes where the parasite goes through asexual multiplication (schizogony) via the ring, trophozoite and schizont stages, as well as sexual maturation from ring to I–V gametocyte stages [2]. Unlike *P. vivax*, *P. malariae* and *P. ovale* in which all the intraerythrocytic stages in their life cycles are detectable in human peripheral blood, *P. falciparum* circulates mainly at the ring and mature gametocyte stages, except in relatively rare infections where all the stages could also be detected. A clear understanding of the different stages in the life of the blood forms of *P. falciparum* and the changes they induce in host erythrocytes has been tremendously facilitated by *in vitro* cultivation of the parasites. Efforts and time have been invested in trying to improve the *in vitro* growth of the asexual stages of the *Plasmodium* life cycle, as these stages give rise to the most common pathological conditions of malaria and are thus a main target for research into antimalarial drugs [3]. AlbuMaxI (lipid rich bovine serum albumin) has been reported to improve growth, yielding more than twofold parasites at any stage of the growth cycle, and is suitable for *in vitro* antimalarial screening as *in vitro* drug susceptibility assays have shown the similar sensitivity to antimalarials and natural product extracts of parasites grown in this medium and human serum–supplemented media [4].

The most virulent Malaria parasite has asexual stages those change the host cell through Export remodelling & virulence proteins and there is a specific NH2-terminal signal [conserved] which plays central role in this export. iRBCs become rigid & develop ability to cytoadhere to a number of cells e.g. vascular endoethial cells, a mechanism that prevents parasitized host cells from passing through to spleen where they would be cleared from the blood stream. These cellular modifications of iRBCs are the result of a dramatic remodelling process induced by the parasite that ultimately serves to induce cytoadherence by exposing several ligands on the iRBCs surface for host cell receptors and facilitate nutrients import into the infecting parasite [5]. Some work has identified a host-targeting (HT) signal on malaria proteins. This signal predicts a secretome of 300–400 effectors for the human malaria parasite *P. falciparum*, vastly expanding the number of potential vaccine and drug targets [6]. Although transcriptome analysis provides useful understanding at the level of gene expression, they do not reflect the active protein component of a cell [7]. Further, parasites such as *Plasmodium*, interact with and respond to host environmental cues which can often be revealed by proteomic analyses of the parasite as it is present within the host [8]. Proteins secreted from the malaria parasite *P. falciparum* to the host erythrocyte have been elusive but of great interest because they remodel the host cell for parasite survival and underlie crucial disease pathologies [6]. Several years ago, a motif within the exported proteins was discovered that allowed them to be exported, which was used to predict the total set of proteins exported to the host cell (the secretome) [9].

P. falciparum causes malaria by replicating inside red blood cells of infected individuals. By exporting many different proteins into the host cell, the parasite changes many of its properties. Knowledge of the identity and function of all the exported proteins will both increase our understanding of the modifications required for parasite survival and provide us with targets that can be inhibited to block the growth of the

parasites. Therefore, the aim of this research is to make use of *in vitro* growth conditions for analysis and identification of *P. falciparum* secretome of the asexual stage (trophozoite) by means of SDS-PAGE electrophoresis.

2. Materials and Methods

2.1 Culture Maintenance

3D7 cell lines of *P. falciparum* parasites were maintained in human O+ erythrocytes isolated from O+ human blood collected from healthy volunteers at 5% hematocrit in RPMI 1640 (Sigma) buffered with HEPES containing 10% AlbuMaxI (GIBCO) and supplemented with 5% sodium bicarbonate. The culture was grown at 37^0 C incubator in a candle jar. The culture media was changed with fresh one every 24 hr. Parasite growth was assessed by monitoring the parasitemia every 48 hr through microscopic examination of Giemsa-stained smears. The parasitemia was calculated from the percentage (%) of parasite – infected RBCs over uninfected RBCs. The culture was safely continued for 6-7 days without need for addition of fresh erythrocytes (subculture) provided the parasitemia not exceed 4%. Beyond this level, the glucose consumption will interfere with the buffering capacity of the medium. As a result the culture may gradually die out.

2.2 Percoll Treatment

Percoll solutions (90% and 70%) were prepared by appropriate dilution with PBS buffer and were filter sterilized with 0.45 µM filter and 12.5 ml were poured on an autoclaved 50 ml falcon tube using Pasteur' pipette and was kept in a vertical position without shaking. A volume of 40 ml culture was washed with 1x PBS, resuspended in an appropriate volume of 1x PBS and was layered on the top of the Percoll solution and the tube was spun at 2000 rpm for 20 min at RT. The supernatant was removed till the interphase and the hemozain band was removed carefully. The mature stages (trophozoites and schizonts) were collected in to a clean sterile 15 ml falcon tube (~3.5 ml) and was washed twice with 1x PBS.

2.3 Saponin release of parasite from infected-RBCs

Two volume of saponin solution (0.15% in 1x PBS) were added to the pellet of the infected – RBCs (washed in 1x PBS) into 15 ml falcon tube. They resuspended gently and were then incubated for 3 mins. To quench the saponin reaction, the volume was completed to 12 ml with 1x PBS. The tubes were then centrifuged for 45 min at 3450 rpm. The resultant pellet was washed with 1x PBS solution.

2.4 Parasite Growth in SFM

The resultant culture pellet (free parasites) were then resuspended in 1 ml of Serum Free Medium (SFM) which contains 20 µl of glucose (500 mM) and 3.6 µl of hypoxanthine (100mM). A volume of 200 µl was aliquot on each well of 96-well-flat bottom plate and the plate was incubated at 37^0 C. The spent media was collected every 8 hrs for 24 hr. A volume of 50 µl of protease inhibitors cocktail (Sigma) were added to the collected supernatants and stored at – 80^0 C freezer until to be used. RBCs only, grown in SFM were used as controls.

2.5 Analysis of supernatants on SDS-PAGE

The appropriate amount of supernatant preparation were mixed with 1/5 volume of SDS sample buffer that contains β-mercaptoethanol which was added to ensure complete breakage of disulfide bonds. The mixture was then boiled at 95^0C for 5 min, briefly spun, before loading on 12% SDS-PAGE denaturing gel. The run was carried out at 75 V initially, and then the voltage increased to 110 V.

2.6 Coomassie staining

The gel was then stained by agitation for 1 hr in Coomassie Brilliant Blue R-250 (0.25% CBB, 10% glacial acetic acid, 40% methanol). Finally, the gel was washed in destaining solution (10% glacial acetic acid, 40% methanol).

2.7 Silver Staining

The gel was fixed in fixing solution (50% ethanol – 12% Glacial acetic acid – 0.05% formalin) for 2 hrs. The fixing solution was discarded and the gel was rinsed in Milli-Q water and then washed 3 times with washing solution (20% ethanol) 1 minute each. The washing solution was discarded and the gel was incubated for 2 mins in the sensitizing solution (0.02% Sodium thiosulfate). After decanting the sensitizing solution, the gel was washed twice with Milli-Q water 1 min each. The gel was incubated for 20 min in cold silver staining solution (0.2% silver nitrate – 0.076% formalin). After decanting the staining solution, the gel was washed twice with Milli-Q water 1 min each. The gel was then rinsed shortly in developing solution (6% Sodium carbonate – 0.0004% Sodium thiosulfate – 0.05% formalin). After discarding the developing solution, the reaction was stopped by adding the terminating solution (12% Glacial acetic acid) and agitation for 10 mins. Finally the gel was placed for 2 hrs in the drying solution (20% ethanol).

3. Results and Discussion

Synchronized cultures of *P. falciparum* containing up to 40% parasitized cells have been obtained through the use of diluted red blood cell (RBC) suspensions and daily replacement of culture medium as previously described [10]. During this study, the synchronization and the enrichment procedures have successfully produced a pure culture of *P. falciparum* trophozoites. Percoll synchronization method was used to shorten the cycle window to 4–6 h as previously described [11]. Erythrocyte reinvasion by schizont progeny was enhanced by diluting cultures to a 1% hematocrit immediately after synchronization with Percoll as previously described [12]. Highly synchronized high parasitemia cultures are needed for comparative studies on global gene expression at specific parasite stages based on proteomic or microarray analysis [4]. Low hematocrit cultures with only small proportions of uninfected RBCs considerably reduce interference in proteomic studies [10, 13]. Also desirable is that the 23-Mb genome of *P. falciparum* with ~5,800 protein-encoding genes [14] should be amenable to accurate functional analysis by proteomics of different pathogenic

patient isolates. New insights into diseases have been recently provided by comparing genomes across different cell phenotypes, and proteomic studies on the most experimentally accessible stages of *P. falciparum* clones have made considerable progress in characterizing parasite subproteomes [13, 15, 16]. However, although these proteome-wide studies have identified approximately half the 5,800 predicted genes of the genome sequence of *P. falciparum*, we still need to identify less abundant proteins arising from different experimental conditions across the asexual cycle over a shorter window than the duration of the stage's rings, trophozoites or schizonts [17].

Proteomics is much more complicated than genomics. Most importantly, while the genome is a rather constant entity, the proteome differs from cell to cell and is constantly changing through its biochemical interactions with the genome and the environment. One organism will have radically different protein expression in different parts of its body, in different stages of its life cycle and in different environmental conditions [18]. In this study the proteome of *P. falciparum* trophozoite was investigated through SDS-PAGE electrophoresis analysis of the secretome. The separated proteins were identified according to their molecular weight; however, 2D-GE is required to identify them according to their charges. A standard curve was created for the protein maker for the migrated distance by each band of known molecular weight (MW), from which the MW of the separated secretome was determined [Data not shown].

When comparing the protein profile produced by the two experimental sets of the free parasites, it was observed that some of the secreted proteins degrade after another 8 hrs, most of them are of large size (Figure 1). Two distinct expressed protein patterns (up-or-down regulation) were separated from *P. falciparum* trophozoites in the two different incubation time (Figure 1). As shown in Table 1, a total of 24 secreted proteins were detected; 14 of them were detected after the first 8 hrs incubation, while 5 of these 14 ones were degraded after 8 hrs incubation. Marked reduction in the number of the expressed proteins was observed after 16 hrs incubation period. These 5 proteins may be down-regulated after another 8 hrs of trophozoites growth. On the other hand, 4 proteins were up-regulated after another 8 hrs of trophozoites growth. The third group of secreted proteins has shown expression on both preparations but with high intensity after 8 hrs of growth (Figure 1). Six bands > 212 kDa were observed after the first 8 hrs of growth, while only 2 bands > 212 kDa were observed after the second 8 hrs of growth. The optimal conditions to obtain this pattern of protein expression was to grow free parasites culture in SFM media containing glucose and hypoxanthine at 37^0 C incubator and analyze the spent after 8 hrs on denaturing SDS-PAGE [19] and using the silver staining for detection of the separated proteins. However, the coomassie staining was insensitive when used for the same purpose (Data not shown). As the neutral ionic species of coomassie brilliant blue (CBB) binds to proteins by a combination of hydrophobic interactions and heteropolar bonding with basic amino acids, this can provide some information on the nature of these secreted proteins. The silver staining was more sensitive than CBB staining in detecting the secreted proteins of *P. falciparum* in the SFM. Multi –bands pattern was observed on Lanes 3 and 4, while only two bands appeared on the controls (Figure 1). The gel was over-stained since it was incubated longer time in the developer solution, this why the protein marker (Broad Range, (2-212 kDa), New England – BioLabs) appeared like this.

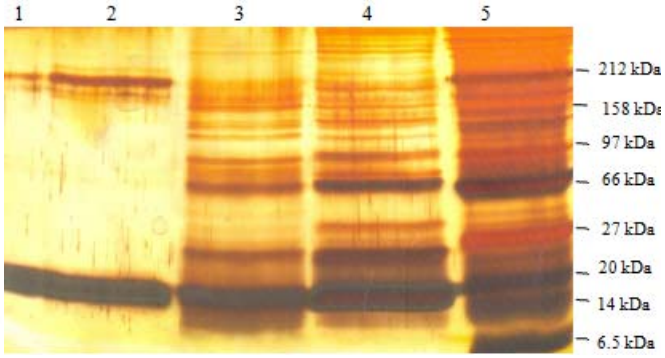


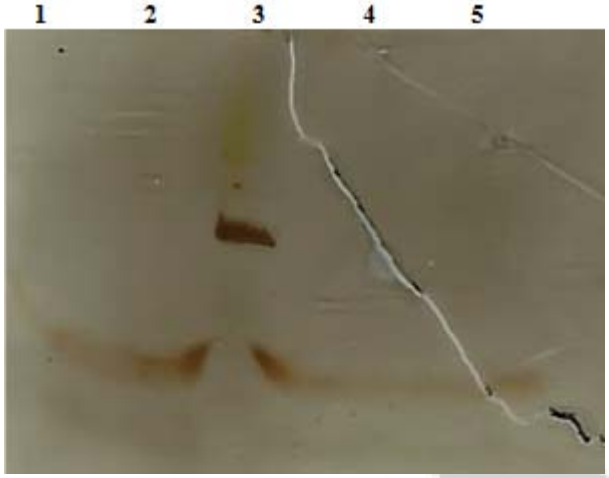
Figure 1: *P. falciparum* secretome Analysis after optimization of growth conditions

The spent supernatants were loaded (32 µl) on to 12% SDS-PAGE gel. The separated proteins were visualized by silver staining. Lanes 1 the spent supernatants of 8 hrs old culture of control un-infected RBCs & lane 2 the spent supernatants of 16 hrs old culture of control un-infected RBCs culture. Lane 3 the spent supernatants of 16 hrs old culture of *P. falciparum*. Lane 4 the spent supernatants of 8 hrs old culture of *P. falciparum*. Lane 5 the protein maker.

Table 1: MW (kDas) of protein profiles of two preparations of *P. falciparum* trophozoites secretome characterized by SDS-PAGE

Protein (kDa)			
8 hrs incubation		*16 hrs incubation*	
Control sample	*Parasite sample*	*Control sample*	*Parasite sample*
212.0	212.0	212.0	212.0
-	185.0	-	185.0
-	-	-	180.0
-	158.0	-	-
-	-	-	130.0
-	116.0	-	116.0
-	97.2	-	97.2
-	-	-	90.0
-	80.0	-	-
-	66.4	-	66.4
-	-	-	62.0
-	60.0	-	-
-	55.0	-	-
-	42.7	-	42.7
-	40.0	-	-
-	27.0	-	27.0
20.0	20.0	20.0	20.0
-	14.3	-	14.3

Figure 2 shows the analysis before optimization of growth conditions. These conditions included the concentration of hypoxanthine, Percoll treatment, incubation period and adjustment of the initial parasitemia.



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The definitive identification of a protein requires mass spectrometry, sequencing, or immunodetection. Immunodetection methods, such as western blotting, use antibodies that specifically recognize the proteins of interest. Such antibodies can provide positive identification. *P. falciparum* is predicted to export a ‘‘secretome’’ of several hundred proteins to remodel the host erythrocyte. Prediction of protein export is based on the presence of an ER-type signal sequence and a downstream Host-Targeting (HT) motif (which is similar to, but distinct from, the closely related Plasmodium Export Element [PEXEL]) [9]. Previous studies revealed a positive prediction rate of ~70%. In addition, several proteins were identified that are very likely to play an essential role in infection, with at least one involved in the formation of a structure required for nutrient import. Recently, the proteome of *P. vivax* isolated directly from patients was described [8]. They identified 153 proteins majority of which do not show homology to any previously known gene products and 29 new proteins that were specific only to *P. vivax*. Several proteins previously implicated as anti-malarial targets, were also found in *P. vivax*. Similar results could possibly be obtained if the clinical *P. falciparum* isolates from different hosts were analysed and their proteome were compared. The proteome of the drug resistance of *P. falciparum* was investigated that revealed a total of 25 protein difference between the resistant and the sensitive clone [20]. During this study, excreted proteins were characterized by SDS-PAGE electrophoresis, and ultimately should be characterized by mass spectrometry (MS). Few trials were carried out to analyze the *P. falciparum* secretome on MS (MALDI-TOF) (Data not shown) however more optimization is required to obtain reliable results.

4. Conclusion

The proteins pattern expressed in intraerythrocytic asexual stage of *P. falciparum* was confirmed by SDS-PAGE analysis and sufficient proteins were produced that amenable for proteomic studies by MS methods. Eight hours of growth is the best for secretome analysis of *P. falciparum* and some proteins degrade then after. The analysis shows the up – and down – regulation of some of the secreted proteins and that their temporal expression is confirmed.

5. Future Scope

The protocols described in this study can possibly be used to generate whole-parasite antigens of a given stage from highly synchronized cultures and soluble antigens by the lysis of parasites or culture supernatants. Moreover, these protocols may also be used in functional analysis by proteomics of different pathogenic patient isolates. Further study to identify the chemical structures of these proteins by MS is required. Spatial study of these proteins is also required.

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