Comunicación Breve

Proteolysis assessment in isolated Plasmodium falciparum living cells at the asexual blood stages

Evaluación de la proteólisis en células aisladas vivas de *Plasmodium falciparum* aisladas durante los estádios asexuales sanguíneos

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ABSTRACT

It has been demonstrated that proteases play crucial roles in Plasmodium falciparum infection and therefore have been considered as targets for the development of new therapeutic drugs. The aim of this study was to describe the specific proteolytic activity profile in all blood stages of *P. falciparum* isolated parasites in order to explore new antimalarial options. For this purpose, we used the fluorogenic substrate Z-Phe-Arg-MCA (Z: carbobenzoxy, MCA: 7-amino-4-methyl coumarine) and classic inhibitors for the different classes of proteolytic enzymes, such as phenylmethylsulfonyl fluoride (PMSF), 1.10-phenantroline, pepstatin A and E64 to study the inhibition profiles. As expected, due to the high metabolic activity in mature stages, the substrate was mostly degraded in the

trophozoite and schizont, with specific activities ~ 20 times higher than in early stages (merozoite/rings). The major actors in substrate hydrolysis were cysteine proteases, as confirmed by the complete hydrolysis inhibition with E64 addition. Proteolytic activity was also inhibited in the presence of PMSF in all but the schizont stage. However, PMSF inhibition was the result of unspecific interaction with cysteine proteases as demonstrated by reversion of inhibition by dithiotreitol (DTT), indicating that serine protease activity is very low or null. To our knowledge, this is the first report aiming to describe the proteolytic profile of *P. falciparum* isolated parasites at all the erythrocytic cycle stages. The results and protocol described herein can be useful in the elucidation of stage specific action of proteolysis-inhibiting drugs and aid in the development of antimalarial compounds with protease inhibitory activity.

Keywords: P. falciparum, proteolysis; living cells; cysteine proteases; antimalarial drugs.

RESUMEN

Se ha demostrado que las proteasas desempeñan funciones vitales en la infección por *Plasmodium falciparum*, y por lo tanto se consideran dianas en la elaboración de nuevos medicamentos terapéuticos. El objetivo del estudio era describir el perfil de actividad proteolítica específica de todas las etapas sanguíneas de parásitos aislados de *P. falciparum* con vistas a explorar nuevas opciones antimaláricas. Con ese propósito, utilizamos el sustrato fluorogénico Z-Phe-Arg-AMC (Z: carbobenzoxi, AMC: 7-amino-4-metilcumarina) e inhibidores clásicos para las diferentes clases de enzimas proteolíticas, tales como el fluoruro de fenilmetilsulfonilo (PMSF), 1,10-fenantrolina, pepstatina A y E64 para estudiar los perfiles de inhibición. Como se esperaba, debido a la elevada actividad metabólica de las etapas de madurez, el sustrato fue degradado mayormente en el trofozoíto y el esquizonte, con actividad específica ~ 20 veces superior a la de las etapas tempranas (merozoíto/ anillos). Los principales actores en la hidrólisis del sustrato fueron las cisteínas proteasas, lo que fue confirmado por la inhibición completa de la hidrólisis con la adición de E64. La actividad proteolítica también fue inhibida en presencia de PMSF en todas las etapas excepto el esquizonte. Sin embargo, la inhibición del PMSF fue resultado de una interacción inespecífica con las cisteínas proteasas, según lo demuestra la reversión de la inhibición con el ditiotreitol (DTT), lo que indica que la actividad de la serina proteasa es muy baja o inexistente. Que sepamos, este es el primer informe dirigido a describir el perfil proteolítico de parásitos aislados de *P. falciparum* en todas las etapas del ciclo eritrocítico. Los

resultados y el protocolo que aquí se describen pueden ser útiles para dilucidar la acción específica de los medicamentos inhibidores de proteólisis en cada etapa, así como contribuir al desarrollo de compuestos antimaláricos con actividad inhibidora de la proteasa.

Palabras clave: *P. falciparum*; proteólisis; células vivas; cisteínas proteasas; medicamentos antimaláricos

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Malaria is still one of the most serious parasitic diseases affecting humankind, causing almost half million deaths annually.[1] Parasites from the genus *Plasmodium* are the causative agents of the disease, among which *P. falciparum* causes great morbidity and mortality. The *Plasmodium* life cycle is divided into two phases: sporogonic (within the mosquito host) and schizogonic (within the mammal host).[2] The erythrocytic cycle occurs during the schizogonic phase, causing the symptoms of the disease (cyclic fevers, headache and trembles), due to the infected cell rupture and the release of new parasites. During the erythrocytic cycle, the parasite develops through four stages: merozoite, ring, trophozoite and schizont.[2]

Proteases can initiate and/or regulate essential biological processes such as apoptosis, cell cycle control and cell migration. Given the significance of proteolytic events for many organisms, it is expected that the same occurs in *Toxoplasma* and *Plasmodium* apicomplexan parasites.[3] During the intraerythrocytic stages of *P. falciparum* there is a broad range of temporal expression for various classes of proteases.[4] Decades of parasite biology research has allowed defining the role of proteases in essential physiological processes such as hemoglobin degradation, erythrocyte invasion and egress.[3]

The already proved role of proteolytic events on the life cycle of the parasite led to the study of its proteases individually and the search for new inhibitors of the natural/recombinant enzymes (reviewed by.[3,5] However, considering that proteolytic events usually involve more than one enzyme, and that they are differentially expressed during the cell cycle, the study of catalytic activity as a whole arises as an alternative for a better understanding of the parasite´s biology and potential targets for compromise the parasite development.

In parasites, only a few general proteolysis studies had been conducted. As a source of proteases, cell lysates were used for the studies in *P. falciparum*[6] and *Leishmania* (*L.*) *amazonensis*.[7] However, the use of isolated living cells, which allow to measure proteolysis in physiological conditions, was mainly reported for *Plasmodium* asynchronous cultures.[8,9,10] For example, the analysis of calcium effects on Fluorescence Resonance Energy Transfer (FRET) substrates[8] and the development of a specific calpain activity assay with the substrate Z-Phe-Arg-MCA.[9] Moreover, the use of isolated parasites from asynchronous cultures was also reported in experiments to correlate inhibition of proteolysis with impairment of *P. falciparum* growth *in vitro* by potential antimalarial drugs. In this context, our group described two class of compounds: i) organotellurium compounds inhibit Z-Phe-Arg-MCA proteolysis in living cells and *P. falciparum* growth *in vitro*[10] and ii) bestatin-derived peptidomimetics inhibit *P. falciparum* growth *in vitro*, PfAM1 activity *in vitro* and also Ala-MCA proteolysis in isolated living cells.[11]

However, to better understanding the proteolytic events during parasite´s life cycle as well as to correlate inhibition of proteolysis at specific asexual blood stage with impairment of *P. falciparum* growth *in vitro* it is required to extend the hydrolytic studies in synchronized *P. falciparum* cultures at all asexual blood stages. The class of cysteine proteases is the most representative within the total protease activity of the parasite. *P. falciparum* genome analysis identified 92 possible genes for proteases, 33 of which correspond to cysteine proteases.[12] As these proteases are involved in essential processes for parasite such as the egress and invasion of parasites in erythrocytes or hepatocytes and also the acquisition of amino acids from the degradation of hemoglobin.[13] The development of inhibitors for these enzymes have a relevant antimalarial potential,[14] although is necessary a selective quantification of proteolysis during the asexual blood stages of *P. falciparum* isolated parasites and hence to develop new tools for drug discovery, which is the aim of the present work.

In order to accomplish this aim *P. falciparum* 3D7 strain[15] was cultured in RPMI 1640 (Gibco) supplemented with Albumax II (Gibco) 0.5 %, employing a method based on.[16] The parasites were synchronized with sorbitol using a procedure described elsewhere.[17] To collect the parasites, synchronized cultures were cultivated and parasite maturation was followed through Giemsa-stained smears. Cultures containing rings, trophozoites, early schizonts or late schizonts/merozoites were used for parasite collection. Parasites were isolated as previously described,[18] maintained in MOPS buffer (3-(*N*-morpholino)

propanesulfonic acid; 50 mM; NaCl 116 mM; KCl 5,4 mM; $MgSO_4 \cdot 7H_2O$ 0.8 mM; glucose 5.5 mM and $CaCl_2$ 2 mM, pH 7.2) on ice during the experiments (no more than three hours) and parasite´s integrity was verified through Giemsa-stained smears after finishing the assays. A sample of parasite´s suspension was always kept at -20 ºC and afterwards the lysates were obtained by BugBuster® Master Mix (Novagen, EUA) treatment, according to fabricant instructions. The protein concentration of parasite´s suspension was measured by Bradford's method using BSA (bovin serum albumin from Sigma) as standard.[19]

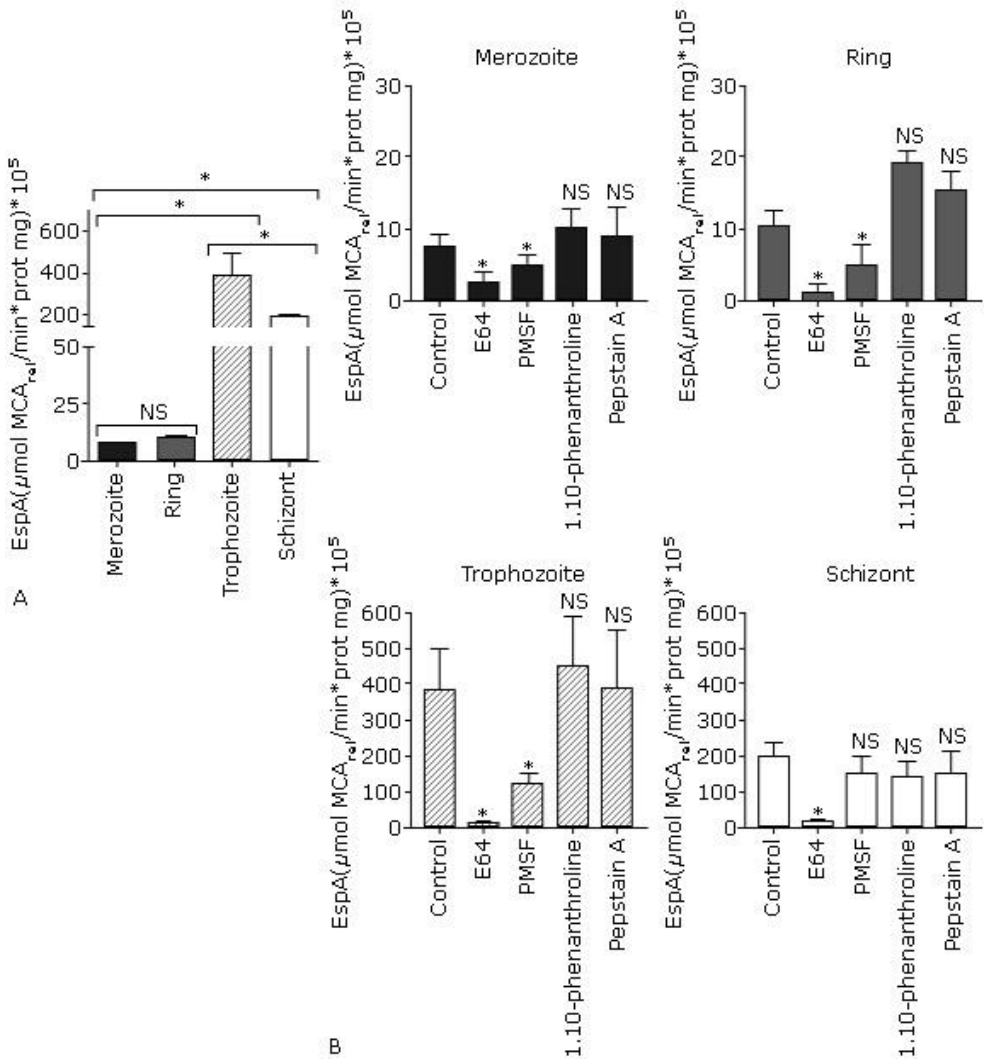
The fluorogenic substrate Z-Phe-Arg-MCA was chosen in our studies because it is a broad specificity substrate (serine and cysteine proteases) and has been used previously in parasite living cells proteolysis assays.[9,10]

The hydrolysis of Z-Phe-Arg-MCA (λ_{ex}= 380 nm and λ_{em}= 460 nm, slit parameter of 10/10 nm) was monitored for 10 min in a spectrofluorometer Synergy (BioTek Instruments Inc., USA), at 37 °C. Isolated parasites were transferred to a 96-well plate (enough quantity to reach a slope of the curve Fluorescence *vs.* time of at least[20] containing MOPS buffer (final assay volume 200 µL). The assay was initiated by addition of 10 µM of the substrate. To enable the quantification of released MCA a calibration curve was developed (Fluorescence *vs.* degraded substrate) incubating increasing quantities of Z-Phe-Arg-MCA with isolated parasites as source of enzymes. One unit of enzymatic activity was defined as the amount of enzyme able to release 1 µmol of MCA per min under specified conditions ($cot_{cal\ curve}$= 0.000126658 $\mu mol \cdot L^{-1}/F$). The specific activity was expressed as enzymatic activity units related to protein concentration in the assay.

In inhibition assays, the parasites were pre-incubated 30 min at 37 ºC with inhibitors for the different class of proteases: serine proteases (phenylmethylsulfonyl fluoride (PMSF) (0.5 mM); aspartyl proteases, pepstatin A (1 µM); metalloproteases, 1,10-phenanthroline (5 mM), and cysteine proteases, trans-epoxysuccinyl-L-leucylamido-(4-guanidine) butane (E64) (5 µM). After incubation, the assays were carried out in the conditions described above. As control, parasites were incubated in the same conditions without the inhibitors. Reducing agents promote a proteolysis increase in non-covalent inhibited cysteine proteases, which reveals remaining active proteases. Thus, to determine inhibition reversibility by reducing agents as dithiotreitol (DTT) each experiment was performed with a further addition of DTT 5 mM for 10 min measurement. All results are expressed as mean ± SD of three individual experiments (with three replicates each one). ANOVA was used for comparisons of more than three groups, followed by Tukey or Dunnet post-tests. A p value

less than 0,05 was considered indicative of a statistically significant difference. GraphPad Prism software (San Diego, CA, USA) was used for all statistical tests.

As shown in figure 1, A, a higher specific activity was observed in trophozoites and early schizonts. This result is similar to that obtained for parasite´s lysate proteolytic activity using FRET substrates.[6] The substrate Z-Phe-Arg-MCA cannot be degraded by exopeptidases because it has both its C- and N-terminal portions blocked so that only endopeptidases can hydrolyze it. In *P. falciparum*, this substrate can be degraded by cysteine proteases such as falcipains (FP-1, FP-2, FP-2´ and FP-3)[20] and calpain.[9]



A: Comparison of Z-Phe-Arg-MCA proteolysis in isolated *P. falciparum* parasites at the four erythrocytic stages. The data were compared with one way ANOVA followed by Tukey *pos-test.* B: Effect of typical protease inhibitors on Z-Phe-Arg-MCA proteolysis at *P. falciparum* asexual blood stages.

The data were compared with one way ANOVA followed by Dunnett *post test*. In all instances, asterisks indicate significant differences with $p< 0.05$ for the comparison between groups (A) or the comparison with the control (B) (n= 3, three independent experiments).

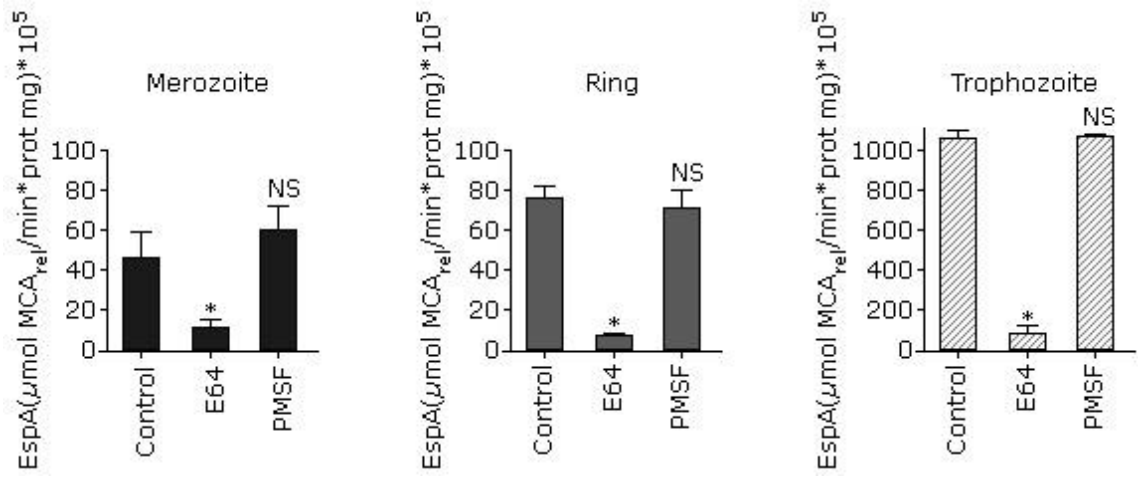
Fig. 1 - Profile of Z-Phe-Arg-MCA hydrolysis by isolated *P. falciparum* parasites at asexual blood stages.

Next, in order to determinate the class of proteases that are acting on this substrate, classical protease inhibitors were tested (Fig. 1, B). There is no significant inhibition observed by pepstatin A or 1.10-phenanthroline, which considering that aspartic and metalloproteases, respectively, are expressed during the intraerythrocytic stages[4,21] indicates that Z-Phe-Arg-MCA is not degraded by these enzymes at any erythrocytic stage. This outcome is expected, taking into account the specificity described for *P. falciparum* metalloendopeptidases such as falcilisin,[22] metalloaminopeptidases (reviewed in[23]) and likewise for aspartic proteases such as plasmepsins.[24] Similar results were obtained in trophozoites by Gomes and coworkers using similar conditions. [9]

On the other hand, E64 inhibits the proteolytic activity in all stages tested, suggesting that cysteine protease activity is present (Fig. 1, B). These results are in accordance with those previously obtained in similar conditions for trophozoites[9] and for recombinant falcipain 2 and 3.[25]

In addition, there is a significant decrease of proteolytic activity in the presence of PMSF in merozoites, rings and trophozoites whereas there is no inhibition in schizonts (Fig 1, B). In the case of trophozoites, these results differ with that obtained by *Gomes et al*. (2014), which did not observe PMSF inhibition with this substrate. These differences could be due to the cell-inhibitor incubation time and the inhibitor concentration. In the present work, we incubated 0.5 mM of inhibitor for 30 min instead of 0.01 mM for 10 min used in that study,[9] which probably allowed a better enzyme-inhibitor interaction.

Nevertheless, taking into account that PMSF is a serine protease inhibitor but it could also inhibit cysteine proteases and that inhibition can be reversed by DTT,[26] a reversibility inhibition assay was performed. As shown in figure 2, DTT addition annulled PMSF inhibition but had no effect in cysteine protease inhibition by E64. This result indicates that the PMSF inhibition observed was the result of unspecific interaction with cysteine proteases and therefore the serine protease activity is very low or null under these conditions.



The data were compared with one way ANOVA followed by Dunnett *post test*. Asterisks indicate significant difference with $p< 0.05$ compared to control. (n= 3, three independent experiments).

Fig. 2 - Effect of PMSF reversible inhibition of Z-Phe-Arg-MCA proteolysis in isolated *P. falciparum* parasites.

The results suggested that the main enzymatic activity observed in these conditions is due to cysteine proteases in accordance with previous studies using isolated trophozoites with the same substrate[9] or in parasite lysates with broad specificity FRET peptides.[6] Considering the present work and the differential expression of proteases during *P. falciparum* erythrocytic cycle,[4,21,25,27,28] it can be postulated that the proteases responsible for the hydrolysis of Z-Phe-Arg-MCA are mostly cysteine proteases in merozoites (FP-1), rings (FP-1 and Pfcalpain), trophozoites (FP-2, 2´, 3 and Pfcalpain) and schizonts (FP-3, SERA6 and Pfcalpain). Our results shows a interestingly proteolytic profile of cysteine proteases during the asexual blood stages and the most active period observed is trophozoite stage, which is 20 fold higher than schizont and 40 fold against ring/merozoite.

Taken together, this is the first study of proteolysis in parasite living cells isolated from synchronous cultures of *P. falciparum* asexual erythrocytic stages, and it establishes a routine assay that may be useful: i) to identify new inhibitors effect on endogenous stage specific proteolysis and indication of suitable therapeutic window and ii) to determine the mechanism of action for molecules with antimalarial activity *in vitro*.

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Conflict of interests

The authors do not declare a conflict of interest.