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Substrate specificity studies of the cysteine peptidases falcipain-2 and falcipain-3 from *Plasmodium falciparum* and demonstration of their kininogenase activity

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ABSTRACT

We studied the substrate specificity requirements of recombinant cysteine peptidases from *Plasmodium falciparum*, falcipain-2 (FP-2) and falcipain-3 (FP-3), using fluorescence resonance energy transfer (FRET) peptides as substrates. Systematic modifications were introduced in the lead sequence Abz-KLRSSKQ-EDDnp (Abz = *ortho*-aminobenzoic acid; EDDnp = *N*-[2,4-dinitrophenyl]ethylenediamine) resulting in five series assayed to map $S_3 - S'_2$ subsite specificity. Despite high sequence identity and structural similarity between FP-2 and FP-3, noteworthy differences in substrate specificity were observed. The S_1 subsite of FP-2 preferentially accommodates peptides containing the positively charged residue Arg in P_1, while FP-3 has a clear preference for the hydrophobic residue Leu in this position. The S_2 subsite of FP-2 and FP-3 presents a strict specificity for hydrophobic residues, with Leu being the residue preferred by both enzymes. FP-2 did not show preference for the residues present at P_3, while FP-3 hydrolysed the peptide Abz-ALRSSRQ-EDDnp, containing Ala at P_3, with the highest catalytic efficiency of all series studied. FP-2 has high susceptibility for substrates containing hydrophobic residues in P'_1, while FP-3 accommodates well peptides containing Arg in this position. The S'_2 subsite of both enzymes demonstrated broad specificity. In addition, radioimmunoassay experiments indicated that kinins can be generated by FP-2 and FP-3 proteolysis of high molecular weight kininogen (HK). Both enzymes excised Met-Lys-bradykinin, Lys-bradykinin and bradykinin from the fluorogenic peptide Abz-MISLMKRPPGFSPFRSSRI-NH_2, which corresponds to the Met^{375} to Ile^{393} sequence of HK. The capability of FP-2 and FP-3 to release kinins suggests the involvement of these enzymes in the modulation of malaria pathophysiology.

1. Introduction

Malaria is a disease caused by *Plasmodium* parasites and remains one of the most prevalent and persistent diseases, affecting hundreds of millions of people and causing approximately 1–2 million deaths each year [1,2]. Several proteolytic enzymes have been identified in *Plasmodium* species and appear to be involved in important aspects of the parasite biology. Among them, the papain-like cysteine peptidases derived from *Plasmodium falciparum*, the most prolific human malaria parasite, have a crucial role in the physiology of malaria parasites [3,4]. These enzymes are termed falcipains (FP) and comprise four peptidases, namely falcipain-1 [5], falcipain-2 [6], falcipain-2′ [7] and falcipain-3 [3]. Compared to papain, the prototypical cysteine protease of the family that encompasses these enzymes, the falcipains display some unique motifs. These sequences, found within the N-terminal part of the prodomain, are crucial for protein targeting to the food vacuole [4]. Also in the prodomain, it was found an inhibitory C-terminal portion containing two motifs (ERFNIN and GNFD) that are conserved in the cathepsin L [8]. The C-terminal part of the prodomain is related to inhibition, and the N-terminal part of the mature domain is required for the proper refolding of the protease. These extensions are not found in other proteases of the papain family [9]. In addition, falcipains possess a unique motif that mediates haemoglobin binding [8].

Falcipain-1 (FP-1) shares 38–40% sequence identity with falcipain-2 (FP-2) and falcipain-3 (FP-3) and has been implicated in erythrocyte invasion by merozoites [10]. Gene disruption studies

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suggest that FP-1 is not essential to the erythrocytic stage of *P. falciparum* [5,11]. Gene silencing studies have alluded to a functional biological role for FP-1 [10,12]. FP-2, FP-2′ and FP-3 are food vacuole hemoglobinases, and their joint expression, in concert with aspartic peptidases, seems to be essential for efficient haemoglobin digestion [3,7,6]. Although the parasites can survive when FP-2 expression is knocked down, the silencing of FP-3 is lethal to *P. falciparum* [13], demonstrating the importance of these proteases in the parasite's life cycle. In the late stages of its intraerythrocytic development, the parasite expresses an endogenous cysteine protease inhibitor, falstatin [14].

In the present work, we performed a comparative analysis of the $S_3 - S_2'$ subsite (according to the Schechter and Berger nomenclature [15]) specificity of recombinant FP-2 and FP-3 using fluorescence resonance energy transfer (FRET) peptides derived from the lead sequence Abz-KLRSSKQ-EDDnp (Abz = *ortho*-aminobenzoic acid; EDDnp = N-[2,4-dinitrophenyl] ethylenediamine). This peptide was designed based in the results of a solid-phase substrate library for cysteine proteases [16,17] and was cleaved only at the Arg-Ser bond by FP-2 and FP-3. Five series of peptides were generated and tested: Abz-XLRSSKQ-EDDnp, Abz-KXRSSKQ-EDDnp, Abz-KLXSSKQ-EDDnp, Abz-KLRXSKQ-EDDnp and Abz-KLRSXKQ-EDDnp (X = different amino acids). Marked differences were observed in all positions analysed, suggesting a very distinct specificity between the two enzymes.

The recent description that plasmatic kininogen can be hydrolysed by *P. falciparum* intracellularly generating active peptides [18] led us to investigate whether kinins could be generated by falcipains, suggesting a real participation of these enzymes in malaria infection. Therefore, we explored the capability of recombinant FP-2 and FP-3 to release vasoactive peptides (kinins) from a synthesised fluorogenic fragment spanning the sequence from Met^{375} to Ile^{393} of human kininogen, Abz-MISLMKRPPGFSPFRSSRI-NH_2. This peptide containing the bradykinin sequence (RPPGFSPFR) extended at its C- and N-terminal sides, allowed us to evaluate the kinins that the recombinant enzymes could release. In addition, we show by radioimmunoassay that kinins can be generated by recombinant FP-2- and FP-3-mediated proteolysis of high molecular weight kininogen (HK).

2. Materials and methods

2.1. Enzymes

The enzymes falcipain-2 (FP-2) and falcipain-3 (FP-3) were expressed in *Escherichia coli* following a previously described protocol [3,19]. The recombinant enzymes were stored in 50% glycerol at −80 °C. The molar concentrations of the enzymes were determined by active site titration with E-64 according to Barrett et al. [20].

2.2. Peptides

FRET peptides containing the fluorescent group *ortho*-aminobenzoic acid (Abz) and the fluorescence acceptor N-[2,4-dinitrophenyl]ethylenediamine (EDDnp) attached to a glutamine residue were synthesised by the solid-phase synthesis method as described previously [21]. The fluorogenic kininogenase fragment Abz-MISLMKRPPGFSPFRSSRI-NH_2, which is devoid of the quenching effect, was synthesised following the same methodology. An automated bench-top simultaneous multiple solid-phase peptide synthesiser (PSSM 8 system, Shimadzu, Japan) was used to synthesise the peptides using the Fmoc-procedure. All peptides were purified by semi-preparative HPLC on an econosil C-18 column. The molecular mass and purity of synthesised peptides were checked by amino acid analysis and MALDI-TOF mass spectrometry using a TofSpec-E from Micromass (Manchester, UK). Stock solutions of the peptides were prepared in DMSO, and the concentrations were measured spectrophotometrically using the molar extinction coefficient of the EDDnp group, 17,300 M^{-1} cm^{-1} at 365 nm.

2.3. Kinetic measurements

The hydrolysis of the FRET peptides was monitored in a Hitachi F 2000 spectrofluorometer by continuously measuring the fluorescence at $\lambda_{ex} = 320 \pm 10$ nm and $\lambda_{em} = 420 \pm 20$ nm, and photomultiplier voltage set to 700 V. The assays were performed at 37 °C in 100 mM sodium acetate buffer, pH 5.5, containing 2.5 mM dithioerythritol (DTE). The 10-mm path length quartz cuvette containing the buffer was placed in the thermostated cell compartment (37 °C) of the fluorimeter until temperature equilibrium was attained. Then, FP-2 or FP-3 were added to the cuvette and left for 5 min to pre-activate the enzymes. The assays were initiated by the addition of the substrate in concentrations bellow to 1 μM, and the increase in the fluorescence with the time was continuously recorded in a final volume of 1 ml. The substrate hydrolyzed per minute was converted into micromoles based on a calibration curve obtained from the complete hydrolysis of each peptide. The experiments were carried out at low substrate concentration where the reactions followed first-order kinetics with the rate constant k_{obs} determined by the non-linear regression data analysis Grafit Version 5 program (Erithacus Software Ltd., Horley, UK). The apparent second-order rate constant k_{cat}/K_m was calculated by dividing k_{obs} by the enzyme concentration.

2.4. Hydrolysis of the synthetic kininogen-related fluorogenic peptide

The hydrolysis of the fluorogenic kininogen peptide Abz-MISLMKRPPGFSPFRSSRI-NH_2 by FP-2 and FP-3 was analysed by liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS) using a Shimadzu 2010 apparatus (Shimadzu Corporation, Tokyo, Japan) with an SPD-20A UV/vis detector and an RF-10AXL fluorescence detector coupled with an Ultrasphere C-18 column (5 μm, 4.6 mm × 250 mm). FP-2 (200 nM) and FP-3 (200 nM) were incubated in 0.1 M sodium acetate, pH 5.5, for 5 h at 37 °C, allowing the complete hydrolysis of the substrate (30 μM). The enzymes were pre-activated with 2.5 mM DTE for 5 min at 37 °C, before the addition of the substrate. A sample of the reaction mixture was applied in the Ultrasphere C-18 column and eluted with two solvent systems: (A) trifluoroacetic acid/water (1:1000, v/v) and (B) trifluoroacetic acid/acetonitrile/water (1:900:100, v/v) at a flow rate of 1.7 ml/min with a 10–80% gradient over 20 min after 8 min of isocratic flow. The percentage of the formation of each fragment was calculated by estimating the peak area of the generated fragment, taking the totally hydrolysed substrate to be 100%.

2.5. Radioimmunoassay determination of kininogenase activity

The ability of recombinant FP-2 (200 nM) and FP-3 (200 nM) to generate kinins from 0.52 μg of HK (Enzyme Research Co., USA) was evaluated incubating the enzymes in 0.1 M sodium acetate, pH 5.5, at 37 °C for 1 h in a final volume of 0.08 ml. The enzymes were pre-activated with 0.5 mM DTE for 5 min, at 37 °C, before the addition of the HK. The assays in the presence of 5.0 μM of E-64 were performed under the same conditions, after 30 min pre-incubation with the inhibitor. Ethanol (3:1, v/v) was added, and the mixture was centrifuged at 1000 × *g* for 15 min. The kinin content in the

supernatant was measured by radioimmunoassay as previously described [22].

3. Results

3.1. Substrate specificity studies

The FRET peptides derived from Abz-KLRSSKQ-EDDnp were used to perform a comparative analysis of the $S_3 - S_2'$ subsite specificity of FP-2 and FP-3. Five series of peptides with general sequences Abz-XLRSSKQ-EDDnp, Abz-KXRSSKQ-EDDnp, Abz-KLXSSKQ-EDDnp, Abz-KLRXSKQ-EDDnp and Abz-KLRSXKQ-EDDnp (X = different amino acids) were tested, and the relative value of the k_{cat}/K_m parameters are presented in Fig. 1. Due to the low catalytic efficiency presented by FP-3 towards some sequences, the studies with this enzyme were performed with fewer peptides than that the number used in FP-2 characterisation. Thus, for FP-3, analogues containing representative residues of each class of amino acid (hydrophobic, positively and negatively charged and non-polar) were selected. The substrates assayed were cleaved by FP-2 and FP-3 at the Arg-Ser bond or at the X-Ser and Arg-X when the modifications were introduced at $P_1 - P_1'$ positions, respectively. The only exception was the peptide Abz-KLRPSKQ-EDDnp, which was cleaved at the Leu-Arg bond by both falcipains.

The kinetic data show that FP-2 preferentially accommodates at the S_1 subsite the positively charged residue Arg ($k_{cat}/K_m = 1850\ mM^{-1}\ s^{-1}$) followed by the non-polar residue Gln ($k_{cat}/K_m = 1042\ mM^{-1}\ s^{-1}$) and all other peptides were poorly hydrolysed. In contrast, the S_1 subsite of FP-3 accommodates very well the hydrophobic aliphatic residue Leu, with the substrate Abz-KLLSSKQ-EDDnp being hydrolysed with the highest catalytic efficiency in the series ($k_{cat}/K_m = 660\ mM^{-1}\ s^{-1}$), while the lead peptide-Abz-KLRSSKQ-EDDnp was hydrolysed by FP-3 with the lowest k_{cat}/K_m value ($55\ mM^{-1}\ s^{-1}$).

The S_2 subsite of FP-2 and FP-3 is restricted to hydrophobic residues following the typical and well-defined characteristic of the cysteine peptidases from the papain superfamily [23]. At the P_2 position, FP-2 shows a clear preference for the hydrophobic aliphatic residue Leu ($k_{cat}/K_m = 1850\ mM^{-1}\ s^{-1}$) over the aromatic residue Phe ($k_{cat}/K_m = 333\ mM^{-1}\ s^{-1}$). The S_2 subsite of FP-3 also preferred the Leu containing peptide Abz-KLRSSKQ-EDDnp, as this substrate is hydrolysed with the highest catalytic efficiency in the series ($k_{cat}/K_m = 174\ mM^{-1}\ s^{-1}$), followed by Abz-KFRSSKQ-EDDnp ($k_{cat}/K_m = 133\ mM^{-1}\ s^{-1}$) and Abz-KMRSSKQ-EDDnp ($k_{cat}/K_m = 70\ mM^{-1}\ s^{-1}$).

The subsite S_3 of FP-2 shows a broad specificity with a slight preference for the lead peptide, Abz-KLRSSKQ-EDDnp, which contains Lys in P_3 position ($k_{cat}/K_m = 1850\ mM^{-1}\ s^{-1}$). In contrast, the same peptide was hydrolysed by FP-3 with the lowest catalytic efficiency in the series ($k_{cat}/K_m = 55\ mM^{-1}\ s^{-1}$). Surprisingly, the peptide Abz-ALRSSRQ-EDDnp, containing the non-polar aliphatic residue Ala in P_3, was hydrolysed by FP-3 with the highest catalytic efficiency of all series studied ($k_{cat}/K_m = 2333\ mM^{-1}\ s^{-1}$).

The S_1' of FP-2 shows clear preference for peptides containing the aromatic hydrophobic residue Phe in P_1' with the peptide Abz-KLRFSKQ-EDDnp ($k_{cat}/K_m = 2440\ mM^{-1}\ s^{-1}$) being hydrolysed with the highest catalytic efficiency of all the series studied. Related to FP-3, the results show that the enzyme accommodates well the peptide containing the positively charged residue Arg in P_1' with the peptide Abz-KLRRSKQ-EDDnp being hydrolysed with the highest catalytic efficiency in this series ($k_{cat}/K_m = 577\ mM^{-1}\ s^{-1}$). It is of note that a Pro residue is not accepted by the S_1' of FP-1 or FP-2. Thereby, the peptide Abz-KLRPSKQ-EDDnp is cleaved at the Leu-Arg bond by both enzymes, positioning the Pro residue at P_2'. The S_2' subsite studies of FP-2 and FP-3 indicated a broad specificity, well accommodating the different class of amino acids at P_2'. However, this subsite has a marked influence on the FP-3 catalytic activity. The peptides Abz-KLRSIKQ-EDDnp ($k_{cat}/K_m = 1217\ mM^{-1}\ s^{-1}$) and Abz-KLRSVKQ-EDDnp ($k_{cat}/K_m = 1111\ mM^{-1}\ s^{-1}$), which contain the aliphatic hydrophobic residues Val and Ile, respectively, in P_2', were hydrolysed by FP-3 with approximately a 20-fold higher value of catalytic efficiency than the one presented by the lead peptide, Abz-KLRSSKQ-EDDnp ($k_{cat}/K_m = 55\ mM^{-1}\ s^{-1}$).

3.2. Hydrolysis of kininogen-related peptide and HK by FP-2 and FP-3

To verify the kinin-releasing activity of FP-2 and FP-3, a fluorogenic fragment spanning the sequence from Met^{375} to Ile^{393} of the human kininogen, Abz-MISLMKRPPGFSPFRSSRI-NH_2, was synthesised and tested with the recombinant enzymes. The hydrolysis this peptide, which is devoid of the quenching effect, was analysed by LC/ESI-MS, and the products were characterised according to their calculated molecular mass (Table 1). FP-2 and FP-3 presented a very similar pattern of cleavage, generating Met-Lys-bradykinin (MKRPPGFSPFR), Lys-bradykinin (KRPPGFSPFR) and bradykinin (RPPGFSPFR) as shown in Fig. 2. However, both enzymes

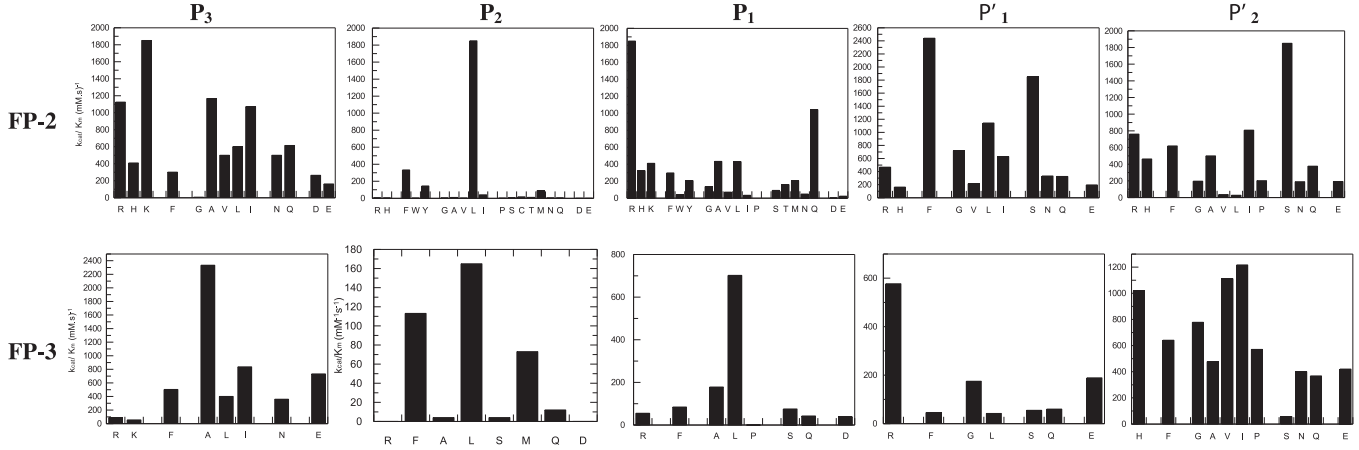

Fig. 1. Analysis of S3–S2′ subsite specificity of recombinant FP-2 and FP-3 using fluorescence resonance energy transfer (FRET) peptides derived from the sequence Abz-KLRSSKQ-EDDnp. The enzymes were incubated as described in Section 2. The assays were performed in low substrate concentration where the reactions followed first-order conditions. The *x* axis shows the different amino acid residues at P3–P2′ and the *y* axis shows the catalytic efficiencies (k_{cat}/K_m) values.

Table 1
Hydrolysis of the fluorescent-labelled kininogen related peptide Abz-MISLMKRPPGFSPFRSSRI-NH_2 by FP-2 and FP-3 and identification of the generated fragments.

Enzyme	Abz-MISL↓**M**↓**K**↓**RPPGFSPFR**↓SSRI-NH_2	Molecular weight	
		Calculated	Observed ion (*m*/*z*)
FP-2	MKRPPGFSPFR (7%)	1319.59	1319.31
	KRPPGFSPFR (63%)	1188.39	1188.29
	RPPGFSPFR (30%)	1060.22	1060.39
FP-3	MKRPPGFSPFR (20%)	1319.59	1319.22
	KRPPGFSPFR (68%)	1188.39	1188.29
	RPPGFSPFR (12%)	1060.22	1060.49

Hydrolysis conditions: The assays were performed at 37 °C in 0.1 M sodium acetate buffer, pH 5.5. The enzymes were preactivated with 2.5 mM DTE for 5 min before the addition of the substrate. Hydrolysis was followed as described in Section 2 and the generated fragments were identified by LC/ESI-MS mass spectrometry. ↓ cleavage sites.

preferentially released Lys-bradykinin, as indicated by the relative percentage of the generated kinins (Table 1).

The capability of FP-2 and FP-3 to release kinins from HK was also tested and quantified by radioimmunoassay (Table 2). We could detect that FP-3 presents a 8-fold higher catalytic activity on HK compared to FP-2. The irreversible cysteine proteinase inhibitor E-64 blocked the activity of both enzymes, demonstrating the specificity of the assay. Control of kininogenase activity with human plasma kallikrein was performed in the same experimental conditions, except for the buffer (0.05 M Tris–HCl, pH 8.0). The amount of released kinins by FP-2 and FP-3 represented 5% and 40% of the kallikrein activity, respectively (data not shown).

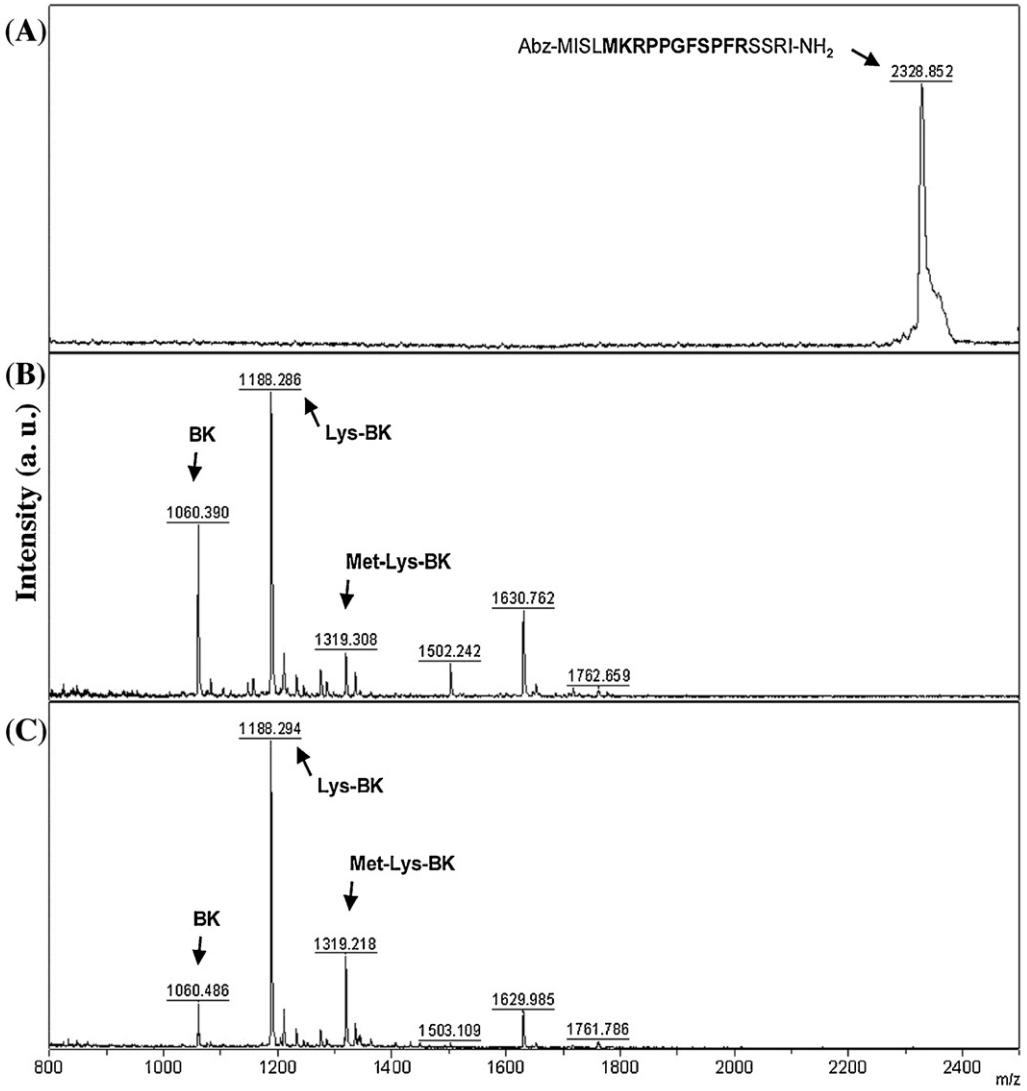

Fig. 2. Mass spectrometry analyses of Abz-MISLMKRPPGFSPFRSSRI-NH_2 (A) hydrolysis by FP-2 (B) and FP-3 (C) and the identification of the generated kinins. Assay conditions are described in Section 2.

Table 2
FP-2 and FP-3 kininogenase activity on HK measured by radioimmunoassay.

Sample	Kinin released (pg)
HK	7
FP-2	6
FP-3	6
HK + FP-2	240
HK + FP-3	1965
HK + FP-2 + E-64	6.5
HK + FP-3 + E-64	2.7

Assay conditions: FP-2 (200 nM) and FP-3 (200 nM) were incubated with 0.52 μg of HK in 0.1 M sodium acetate, pH 5.5 for 1 h, at 37 °C, in a final volume of 0.08 ml. The assays in the presence of 5.0 μM of E-64 were performed under the same conditions, after 10 min of pre-incubation. Experimental conditions were as describe under Section 2.

4. Discussion

Despite the great efforts made by the scientific community, malaria remains one of the most prevalent and persistent diseases that affect people worldwide, with hundreds of millions of people infected [24]. The disease is caused by *Plasmodium* parasites that use several processes to complete the different stages of their life cycle, and a series of peptidases are essential to the achievement of one or more of these phases.

Proteins and peptides can reach and be processed by *P. falciparum* proteases inside erythrocytes [18,25]. Among them, the *P. falciparum* cysteine peptidases, especially falcipain-2 and falcipain-3, stand out because they are involved in the hydrolysis of haemoglobin and erythrocyte rupture [26]. In the present study, we used FRET peptides to explore the $S_3 - S'_2$ subsite specificity requirements of recombinant FP-2 and FP-3. For this purpose, we synthesised and tested analogues of the lead sequence Abz-KLRSSKQ-EDDnp. Our results show some differences from those obtained in previous studies with a library with general sequence Z-peptidyl-MCA [27], making clear that the subsites are not independent and are influenced by residues occupying "primed" subsites. This is an advantage of our methodology because when a fixed residue occupies the position P'_1, such as the MCA group in the compound Z-peptidyl-MCA, it is not possible to analyse the S′ subsites.

As expected for a cysteine peptidase [23], the highest selectivity of FP-2 and FP-3 is given by the S_2 subsite, which clearly prefers peptides containing aliphatic or aromatic hydrophobic residues at P_2. Ramjee et al. [27], using Z-peptidyl-MCA substrates, observed that the S_2 subsite of FP-2 preferentially accepts Leu and Phe residues, while FP-3 hydrolyses only peptides containing Leu or Val in position P_2. Our studies indicated that Leu is the preferred residue by the S_2 subsite of FP-2 and FP-3; however, the latter also well accepts well Phe and Met residues at the P_2 position. Indeed, the structural data showed the preference of a Leu at P_2, as this hydrophobic aliphatic residue fits comfortably into the narrow S_2 subsite of both enzymes, thus allowing it to be involved in a number of favourable interactions [28–30].

With respect to the S_1 subsite, we observed that FP-2 preferred the peptides containing Arg and Gln at the P_1 position, as Abz-KLRSSKQ-EDDnp and Abz-KLQSSKQ-EDDnp were hydrolysed with the highest values of k_{cat}/K_m of the series. In contrast, the compound Abz-KLQSSKQ-EDDnp was one of the worst substrates in the series for FP-3. This enzyme preferentially hydrolysed the peptide Abz-KLLSSKQ-EDDnp, which contains the hydrophobic residue Leu at P_1. Reinforcing our findings, the crystal structure of FP-3 complexed with vinyl sulphones showed that the nonpolar environment of the S_2 and S_1 pockets favour the interaction with the hydrophobic P_1 and P_2 residues of the inhibitor, which allow favourable contacts [30]. Ramjee et al. [27] obtained results similar to ours for FP-2 but did not detect the hydrolysis of Boc-LL-MCA by FP-3. The explanation for this discrepancy may be the fact that depending on the peptide sequence and interaction of the side chains of amino acid residues with subsites S and S′, variations can be observed in the susceptibility to hydrolysis.

In relation to the S_3 subsite, we highlight the major influence of this subsite on FP-3 activity, as the peptide Abz-ALRSSKQ-EDDnp showed the highest value of catalytic efficiency of all series studied ($k_{cat}/K_m = 2333\ mM^{-1}\ s^{-1}$). This result may be explained by the narrowing of the S_2 subsite of FP-3 observed by Kerr et al. [30]. Thus, a peptide containing a small residue at P_3, such as Ala, may be better accommodated at the active site of the enzyme than the peptides containing residues with long side chains.

Our studies indicated that the S′ subsites have a notable influence on the specificity of FP-2 and FP-3. The presence of a Phe residue in position P'_1 of the Abz-KLRFSKQ-EDDnp made the peptide very suitable to hydrolysis by FP-2, being the substrate cleaved with the highest catalytic efficiency of all the series tested ($k_{cat}/K_m = 2440\ mM^{-1}\ s^{-1}$). It is noteworthy that FP-2 hydrolyses the substrate Z-LR-MCA with k_{cat}/K_m of $103\ mM^{-1}\ s^{-1}$ [8] and FP-3 with k_{cat}/K_m value of $0.590\ mM^{-1}\ s^{-1}$ [3], which confirmed the favourable effect of the interaction of a more relaxed substrate with the enzyme S and S′ subsites occupied. Thus, our comparative analysis of the specificity FP-3 and FP-2 showed differences at all analysed positions, demonstrating that the two enzymes have very different substrate specificities. Whereas haemoglobin is the natural substrate for both, the two enzymes cleave the protein at different sites, generating fragments equally important for the parasite [4]. The results presented here may be useful for developing selective inhibitors for each of the enzymes studied and for drug development.

The capability of FP-2 and FP-3 to generate active kinins was also tested to verify the participation of these enzymes in infection caused by malaria. Kininogenase activity of some cysteine peptidases has been described for enzymes from *Porphyromonas gengivalis* [31], *Streptococcus pyogenes* [32], *Trypanosoma cruzi* [33] and *Fasciola hepatica* [34]. In the late 1960s, some studies suggested that kinins could be involved in the pathological manifestations of malaria [35]. Recently, Bagnaresi et al. described the uptake of kininogen by malarial parasites, followed by the generation of kinins, which are products of the parasite's proteolytic activity [18]. These generated peptides can elicit a calcium response in HUVEC cells in culture, suggesting that these fragments have biological activity and could be involved in the disease progression. Here, we demonstrated that FP-2 and FP-3 are able to hydrolyse the fluorogenic peptide Abz-MISLMKRRPGFSPFRSSRI-NH_2, which contains the kinins sequence in kininogen. Lys-BK was the predominant kinin generated by both enzymes, followed by BK and Met-Lys-BK. Interestingly, the profile of the fragments generated from the processing of the peptide Abz-MISLMKRRPGFSPFRSSRI-NH_2 by recombinant falcipains differs from that reported for parasites by Bagnaresi et al. [18]. These authors have shown the formation of des-Arg^9-BK, but not Met-Lys-BK, after cell parasites incubation with these kininogen-related fluorogenic peptide, demonstrating that besides FP-2 and FP-3 other proteases and cellular events are also involved in kininogen processing during malaria development, as already described for haemoglobin [37]. Enzymes such as *Plasmodium* aminopeptidases, which X-ray structure and substrate specificity was recently characterised [36–39], are probably involved in kinin formation. In addition to aminopeptidases, *P. falciparum* carboxypeptidases may be involved in the processing of the generated kinins, thereby justifying the formation of des-Arg^9-BK; however, these enzymes had not yet been described.

The hydrolysis of HK by FP-2 and FP-3 was also analysed and quantified by radioimmunoassay, and the kininogenase activity presented by both enzymes towards the protein corroborates

the results obtained with the mimetic-HK peptide. Our results demonstrated that the well-known inhibitory effects of the two cystatin-like domains of kininogen on falcipains [7] are not sufficient to block FP-2 and FP-3 activity but can be responsible for the high enzyme concentrations required to yield a sufficient amount of kinins for detection, particularly in a very sensitive method such as an radioimmunoassay. Bagnaresi et al. [18] verified that HK fragments resulting from FP-2 and FP-3 cleavage were biologically active and were able to promote the contraction of the isolated guinea pig ileum, which was prevented by the B2 receptor antagonist HOE-140. When HK was incubated with FP-2 and FP-3 in the presence of the specific cysteine protease inhibitor E-64, the contraction response was ablated. The answer obtained in this pharmacological system, together with our radioimmunoassay data demonstrate that active kinins are generated as a result of HK processing by falcipains.

Taken together, the comparative specificity studies and the description of the kininogenase activity of the recombinant enzymes from *P. falciparum* FP-2 and FP-3 may contribute to a better understanding of the role of these enzymes in parasite biology.

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