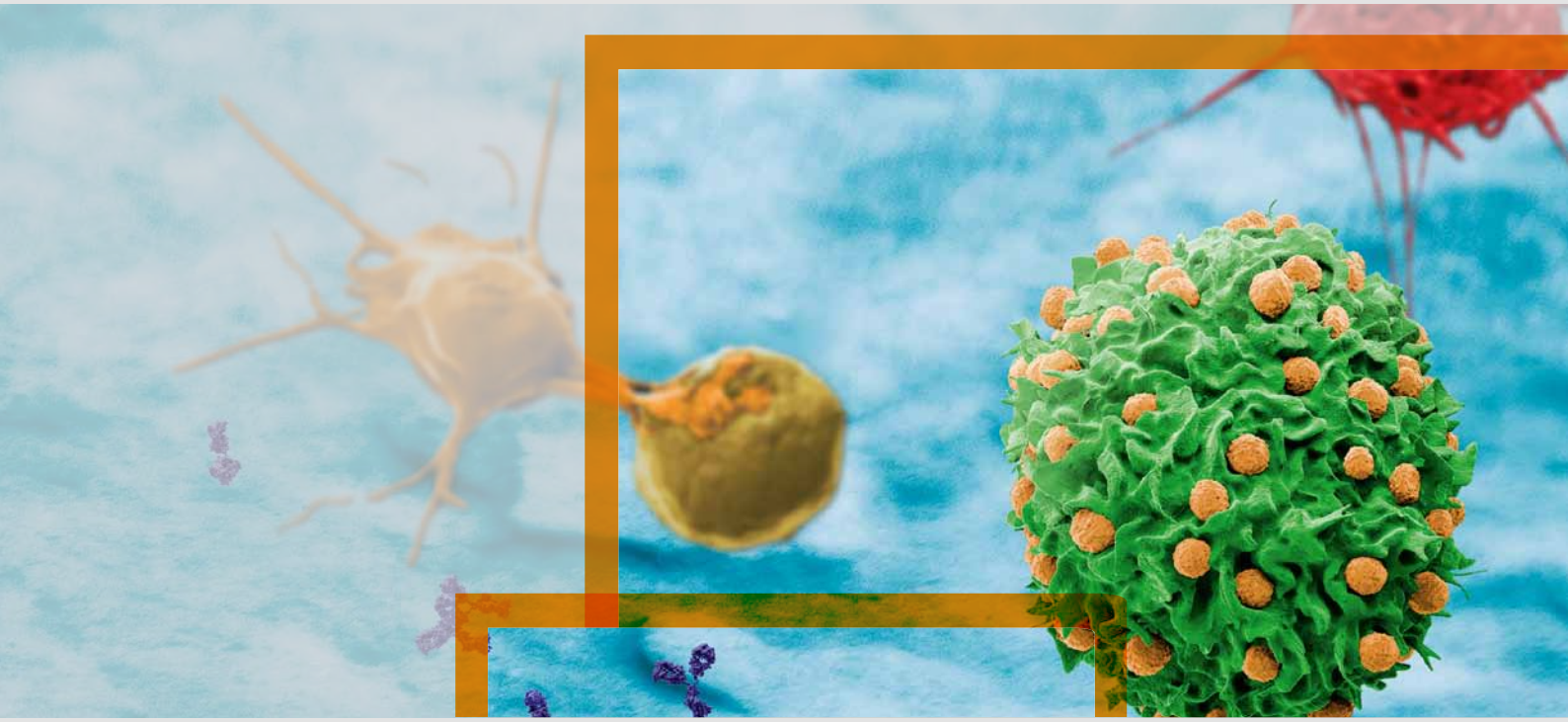
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Measuring prolyl aminopeptidase activity in extracts prepared from magnetically purified malaria parasites





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Introduction

Despite extensive eradication campaigns malaria remains a major cause of morbidity and mortality worldwide with an estimated 1–3 million deaths each year[1]. The prevention and treatment of this disease is becoming increasingly difficult due to the spread of drug-resistant parasites and, therefore, there is an urgent need to discover novel targets for new drug development.

The aminopeptidases are a group of enzymes identified within the genome of the most lethal malaria parasite, *Plasmodium falciparum*, and which may have the potential to become new targets for antimalarial drug development. These enzymes catalyze the cleavage of the N-terminal amino acids from proteins and peptides and one such enzyme, the prolyl aminopeptidase (PfS33PAP) is currently being investigated in our laboratory. As a first step in characterizing the PfS33PAP, it was necessary to identify the enzyme activity in extracts of malaria parasites. However, for biochemical studies it is often crucial to obtain pure preparations of parasite-infected red blood cells (RBCs) that are relatively free of uninfected cells.

P. falciparum passes through a series of morphologically distinguishable stages as they mature within RBCs, developing from ring stages into trophozoites and then into schizont-stage parasites. Each parasite contains up to 16 daughter merozoites, which are released into the blood stream to invade new RBCs. During its 48-hour development cycle the parasite digests 65–70% of the host cell hemoglobin producing insoluble hemozoin as a by-product. The magnetic properties of hemozoin allow the isolation of parasitized erythrocytes. Techniques for the *in vitro* cultivation of *P. falciparum* are well established and relatively high parasitemia can be achieved, though this is dependent on the particular parasite line and skill of operator. In this report we present a protocol for the rapid magnetic purification of *P. falciparum*–infected RBCs from *in vitro* cultures using the autoMACS® Pro Separator. Extracts prepared from purified parasites exhibited prolyl aminopeptidase activity.

Materials and methods

Preparation of parasite cultures

P. falciparum parasites were cultured and synchronized *in vitro* using established methods[2,3]. An *in vitro* culture of trophozoite-stage parasites at 3–5% parasitemia was used for further processing. Ten mL of the parasite culture (equivalent to 500 µL packed cell volume) were centrifuged at 400×*g* for 5 minutes, and the supernatant was discarded. Cells were washed twice with pre-warmed (37 °C) RPMI. Subsequently, the 500-µL cell sediment was resuspended in 50 mL RPMI (equivalent to a 1% hematocrit), and incubated in a water bath at 37 °C for 30 minutes.

Purification of *P. falciparum*–infected erythrocytes using the autoMACS® Pro Separator

Following the 30-minute incubation in RPMI, the cells were gently resuspended. The sample was split into five 10-mL aliquots in 15-mL tubes and kept at 37 °C prior to separation. The first sample was placed in position A1 of a Chill 10 Rack at room temperature. Empty 15-mL tubes for the collection of the negative and positive fractions were place in position B1 and C1, respectively. The rack was placed onto the MACS® MiniSampler of the autoMACS® Pro Separator. Cell enrichment was performed using program PMalaria. The remaining samples were consecutively processed in the same way. Prior to separation each sample was gently mixed to resuspend cells.

Assessment of yield

All positive fractions were combined and centrifuged at 400×*g* for 5 minutes. The supernatant was discarded. Approximately 3 µL of the cell pellet was smeared onto a microscope slide, fixed with methanol and stained with Giemsa solution. Parasitemia was determined microscopically.

Prolyl aminopeptidase activity assay

Purified parasites were hypotonically lysed with distilled water to obtain active malarial extract. Enzymatic activity of the prolyl aminopeptidase, PfS33PAP, in malaria

parasites was determined by addition of extract aliquots to the fluorogenic substrate H-Pro-NHMec. Reactions were carried out in 96-well microtiter plates (100 µL total volume, 60 min incubation at 37 °C) using a spectrofluorimeter with excitation at 370 nm and emission at 460 nm. Initial rates were obtained over a range of substrate concentrations in 50 mM Tris-HCl, pH 7.5. To ensure the prolyl aminopeptidase activity was not due to malarial or human metalloproteases, aliquots of malarial extract were pre-incubated (10 min, 37 °C) with 2 mM of the metal chelator *o*-phenanthroline in 50 mM Tris-HCl and then assayed for activity against H-Pro-NHMec, H-Ala-NHMec, H-Leu-NHMec, and H-Glu-NHMec.

To distinguish the PfS33PAP activity from a second putative *P. falciparum* prolyl aminopeptidase, extracts from a PfS33PAP knockout parasite line were tested for H-Pro-NHMec activity and compared to 3D7 wild type parasites. Assays were performed in the presence of 2 mM phenathroline to inhibit metalloprotease activity.

Results and discussion

Enrichment of *P. falciparum*–infected RBCs

Cultures of trophozoite stage *P. falciparum*–infected RBCs at between 3 and 5% parasitemia were enriched to give a parasitemia of 80–85% following one round of enrichment using the autoMACS Pro Separator (fig. 1).

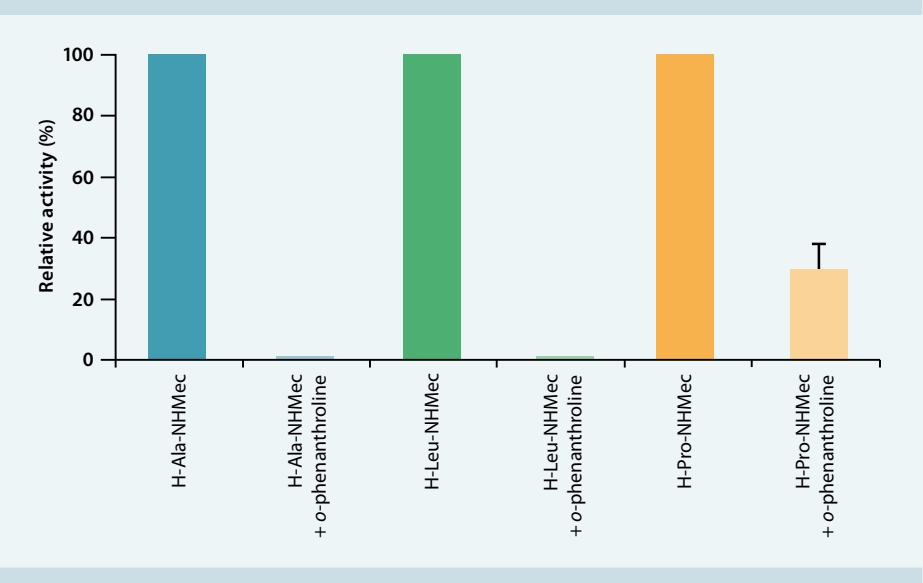


Figure 2 H-Pro-NHMec hydrolysis in *P. falciparum* extracts. Hydrolysis was measured in the presence or absence of the metal chelator *o*-phenanthroline (2 mM) as described in the materials and methods section. Data reflect the mean relative hydrolysis activity (n=2) of 3D7 extract with 200 µM H-Pro-NHMec, 10 µM H-Ala-NHMec, and 5 µM H-Leu-NHMec as substrates.

P. falciparum extracts contain prolyl aminopeptidase activity

Soluble extracts of hypotonically lysed wild type parasites exhibited prolyl aminopeptidase activity towards H-Pro-NHMec, which was only partially inhibited by the metal chelator *o*-phenanthroline (fig. 2). In contrast, aminopeptidase activities towards the substrates H-Ala-NHMec, H-Leu-NHMec were completely inhibited by the chelator. This result indicates that at least part of the prolyl aminopeptidase activity was not due to malarial or human metalloproteases. To distinguish between PfS33PAP activity and that of a second putative malarial prolyl aminopeptidase, we compared the enzymatic activity in extracts of wild type and a PfS33PAP knockout strain and found that *o*-phenanthroline-insensitive prolyl aminopeptidase activity was reduced in the knockout (fig. 3). The remaining activity in the knockout might be due to a second as yet uncharacterized prolyl aminopeptidase.

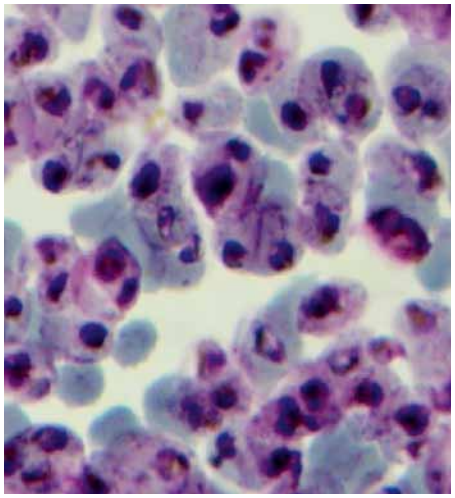


Figure 1 *P. falciparum*–infected erythrocytes isolated using the autoMACS Pro Separator. The image shows a light-microscopic view of a Giemsa-stained thin smear of enriched infected cells.

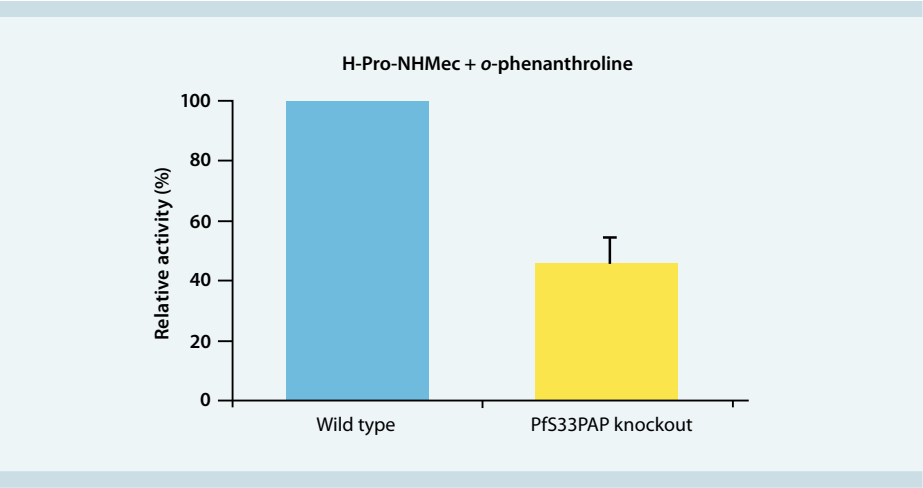


Figure 3 H-Pro-NHMec hydrolysis in extracts from 3D7 wild type and PfS33PAP knockout. Prolyl aminopeptidase activity was measured using extracts prepared from wild type and the PfS33PAP knockout strain. Tests were performed in the presence of 2 mM *o*-phenanthroline.

Conclusion

- The autoMACS® Pro Separator allows the quick and efficient isolation of *P. falciparum*–infected RBCs to high purities.
- Use of the autoMACS Pro Separator minimizes manual handling steps during separation, enabling operator-independent separation results.
- Enzyme activity can be measured in extracts prepared from *P. falciparum* malaria parasites purified with the autoMACS® Pro Separator.
- PfS33 PAP enzyme activity was detected in extracts prepared from wild type *P. falciparum* parasites, and was reduced in extracts prepared from a transgenic PfS33 PAP knockout parasite line.

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2. Trager, W. and Jensen, J.B. (1976) Science 193: 673–675.
3. Lambros, C. and Vanderberg, J.P. (1979) J. Parasitol. 65: 418–420.

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