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# MÉMOIRES ORIGINAUX

# Aminopeptidase of *Eimeria nieschulzi* physico-chemical properties and action of antimalarial drugs

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SUMMARY. An aminopeptidase capable of degrading synthetic substrates and short peptides has been demonstrated in *Eimeria nieschulzi* sporulated oocysts.
Physico-chemical properties and the classic activator and inhibitory effects have been determined.

The inhibitory activity of classic antimalarial drugs has been demonstrated and the study of the inhibitory mechanism by Chloroquine has been further studied.
Lastly, the comparison between the aminopeptidase of *Eimeria* genus parasites and that of *Plasmodium* has been discussed.

Abbreviations : LNA : L-leucine-p-nitroanilide ; ANA : L. Ala-p-nitroanilide ; EDTA : Ethylenediamine tetracetic acid ; PCMB : p-chloro-mercuribenzoate ; DIFP : diiso-propyl-fluorophosphate.

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***Aminopeptidase de* Eimeria nieschulzi, *propriétés physico-chimiques et action des Antimalariques.***

*RESUME. Nous avons mis en évidence dans les oocystes sporulés d'*Eimeria nieschulzi *la présence d'une aminopeptidase capable de dégrader des substrats de synthèse et de courts peptides.*
*Les propriétés physico-chimiques ainsi que l'action d'activateurs et d'inhibiteurs classiques ont été déterminés.*
*L'activité inhibitrice des antimalariques classiques a été mise en évidence et l'étude du mécanisme d'inhibition par la Chloroquine a été abordée.*
*Enfin, la comparaison entre l'aminopeptidase des parasites du genre* Eimeria *et* Plasmodium *est discutée.*

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## Introduction

In a previous report (Charet *et al.*, 1979) we described the physico-chemical properties of an aminopeptidase from *Plasmodium* capable of playing a part in hemoglobin degradation.

The inhibitory effect of antimalarial drugs, chloroquine, primaquine and quinacrine led us to seek and study this enzyme in other sporozoans sensitive to antimalarial drugs, this enzyme being capable of playing an important role in drug action.

We naturally fixed our choice on a *Coccidium*, more particularly on a model easily used in our laboratory, *Eimeria nieschulzi.*

We were able to demonstrate the presence of aminopeptidase in these sporozoans.

While we were carrying out our study, Wang and Stotish (1978) published a paper describing some properties of an aminopeptidase from *Eimeria tenella,* but in that article no information was given concerning antimalarial drug action and some results on the role of metallic ions proved to be in contradiction to ours. These results cannot be explained simply by a difference of species and thus we tried to specify physico-chemical properties of *Eimeria nieschulzi* aminopeptidase and strictly studied the effect of the main metallic ions and antimalarial drugs.

## Material and methods

1 - *Oocyst preparation.*

The strain of *Eimeria nieschulzi* we used came from « Landers isolate » (Marquardt, 1966). 250-300 g Wistar rats were infected *per os* with 500.000 sporulated oocysts. The faeces were collected between 6,5 and 9 days after the infestation. The oocysts were purified by sucrose flotation and sporulation was carried out in a 2 % $K_2Cr_2O_7$ solution for 8 days at 20 °C. Sporulated oocysts were sterilized and purified again by flotation in a concentrated NaOCl solution (commercial hypochloride solution) at 24 French chlorometric degrees.

2 - *Preparation of enzymatic extract.*

The oocysts collected by centrifugation were suspended in 3 volumes of Trager isotonic buffer as modified by Sherman and Hull (1966) and lysed in a « French Press » with a pressure of 10,000 psi and then centrifuged (20 min., 20,000 g, 4 °C). The supernatant was collected and constituted the enzyme extract.

3 - *Determination of aminopeptidase activity and physico-chemical properties of the enzyme.*

All the methods used were described in our previous report (Charet *et al.*, 1980) : enzymatic activity on synthetic substrates, optimual pH and temperature, molecular weight, isoelectric point.

4 - *Enzymatic properties.*

Michaelis constant (Km) and inhibition constant (Ki) were determined respectively by Lineweaver and Burk plot and Dixon plot (Penasse 1974).

The effect of various metallic ions, chelators, blocking agents and classical antimalarial drugs was studied as follows : the enzyme extract was assayed in 0.1 M tris HCl buffer pH 7.2 containing the required concentration of additive with Alanine-4-nitro-anilide as substrate (ANA).

In some cases, we used the procedure suggested by Wang and Stotish (1978). The enzyme was incubated with inhibitor in a tris HCl buffer pH 8.5, during a variable time at 40 °C. Enzymatic activity was then determined in the same buffer with ANA as substrate.

5 - *Interaction between chloroquine and free thiol groups.*

Gerber (1964) showed an apparently substantial interaction between cysteine and chloroquine. We therefore tried to demonstrate the formation of a chloroquine-cysteine complex using the following procedure : 10 mM cysteine and chloroquine solution in 0.1 M Tris HCl buffer pH 7,2 was incubated for 18 hours at 37 °C. An aliquot was separated by descending paper chromatography (Whatman n° 3) in butanol-acetic acid-water (4/1/5 V/V/V) for 18 hours.

## Results

1 - *Enzymatic activity.*

*Table I* gave the activity of enzymatic extract on different synthetic substrates. We noted a strong activity towards ANA, whereas the activity was weaker towards L-Lys-4-nitroanilide and L-Leu-4-nitro-anilide. The extract was without activity against N-blocked substrates such as N-acetyl-L-ala-4-nitro-anilide and N-acetyl-L-Leu-4-nitro anilide. The extract released Leu from L-Leucinamide and Leu and Tryp from the short peptide Leu-Tryp-Met-Arg.

Table I. *Substrates specificities among the leucine amino-peptidases in* Eimeria nieschulzi. *Specific aminopeptidase activities* (μ *mole L-p-nitroanilide liberated/min./per mg protein*).

| Substrates | Activities |
|---|---|
| L-Ala-4 nitroanilide | 30.6 |
| L-Lys-4 nitroanilide | 12.2 |
| L-Leu-4 nitroanilide | 6.3 |

2 - *Optimal pH and optimal temperature.*

*Figure 1* shows pH and temperature enzyme activity relationships : Optimal pH 8 and optimal temperature 42 °C.

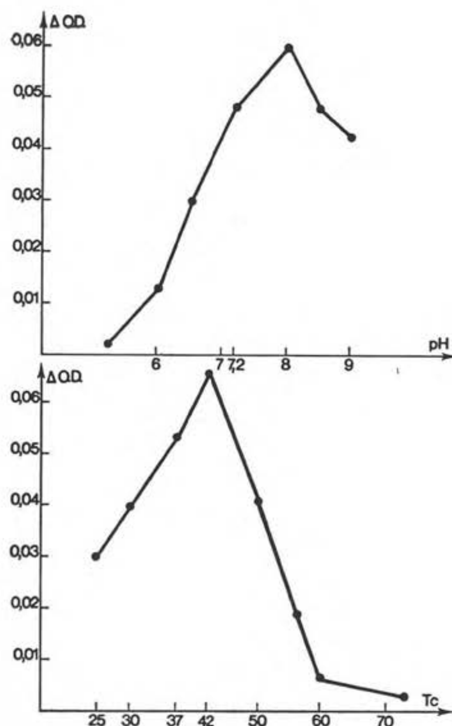


Fig. 1. pH and temperature dependence of aminopeptidase activities in *E. nieschulzi*. L. Ala-p. nitroanilide was used as substrate.

3 - *Molecular weight and isolectric point.*

Gel filtration on sephadex G 150 of 1 ml of enzymatic extracts only gave one active peak with an apparent molecular weight of 90,000. Isoelectric focusing gave only one peak of activity (*fig.* 2) pH i 5.05 for all of the above mentioned substrated.

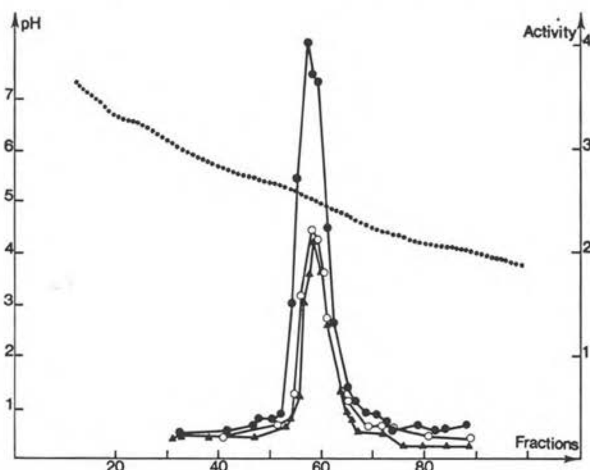


Fig. 2. Isoelectric focusing of the aminopeptidases from *E. nieschulzi.* Ala-4 nitroanilide (•———•), Lys-4 nitroanilide (○———○) and Leu-4 nitroanilide (*——— *) were used as substrates.

4 - *Michaelis constant.*

The mean apparent Km value determined from the Limeweaver-Burk plot for L-ala-4 nitroanilide was 0.7 mM $\pm$ 0.2 (fixe determinations).

5 - *Activators and inhibitors.*

The results are reported in *Table II.* Enzyme activity was strongly inhibited by $Co^{++}$ and $Zn^{++}$ and less inhibited by $Mn^{++}$ and $Mg^{++}$ at high concentration (10 mM). The $Hg^{++}$ ions and PCMB were very inhibitory. In contrast EDTA and phenanthroline were only inhibitory at high concentration as were cysteine and DTT. Hydrophobic amino-acids were strongly inhibitory.

6 - *Antimalarial drug action.*

The antimalarial drugs such as Chloroquine, Quinacrine and Primaquine had an inhibitory effect. This effect was much less marked in the case of quinine (*Table III*). Enzymatic activity of amino-peptidase inhibited by Chloroquine could be restored by removing Chloroquine on sephadex G 25 column.

Table II. *Inhibition of aminopeptidase of* Eimeria nieschulzi.

| Inhibitors | Concentration (M) | % inhibition |
|---|---|---|
| $Co^{++}$ | $10 \times 10^{-3}$ | 86 |
| | $1 \times 10^{-3}$ | 62 |
| $Zn^{++}$ | $0,1 \times 10^{-3}$ | 87 |
| $Mn^{++}$ | $10 \times 10^{-3}$ | 75 |
| | $1 \times 10^{-3}$ | 0 |
| $Hg^{++}$ | $10 \times 10^{-3}$ | 100 |
| | $1 \times 10^{-3}$ | 52 |
| $Ca^{++}$ | $1 \times 10^{-3}$ | 0 |
| $Mg^{++}$ | $1 \times 10^{-3}$ | 0 |
| PCMB | $10 \times 10^{-3}$ | 100 |
| | $1 \times 10^{-3}$ | 45 |
| DIFP | $10 \times 10^{-3}$ | 0 |
| EDTA | $10 \times 10^{-3}$ | 34 |
| | $1 \times 10^{-3}$ | 0 |
| 0-Phenantroline | $10 \times 10^{-3}$ | 88 |
| | $1 \times 10^{-3}$ | 0 |
| Leu | $1 \times 10^{-3}$ | 60 |
| Phe | $1 \times 10^{-3}$ | 0 |
| Ileu | $1 \times 10^{-3}$ | 54 |
| N-leu | $1 \times 10^{-3}$ | 50 |

Table III. *Inhibition of aminopeptidase of* Eimeria nieschulzi *by antimalarial drugs.*

| Drugs | Concentration (M) | % Inhibition |
|---|---|---|
| Chloroquine | $10 \times 10^{-3}$ | 84 |
| | $1 \times 10^{-3}$ | 54 |
| | $0,1 \times 10^{-3}$ | 10 |
| Quinacrine | $0.1 \times 10^{-3}$ | 12 |
| Primaquine | $1 \times 10^{-3}$ | 32 |
| | $0,1 \times 10^{-3}$ | 0 |
| Quinine | $1 \times 10^{-3}$ | 12 |
| | $0,1 \times 10^{-3}$ | 0 |

The study of chloroquine inhibition with classical methodology (*fig. 3 and 4*) led us to the conclusion that inhibition was apparently of competitive type (1.3 mM approximately for Ki).

7 - *Interaction between chloroquine and free thiol groups.*

Paper chromatography of chloroquine-cysteine incubates gave only 2 compounds : free cysteine and free chloroquine.

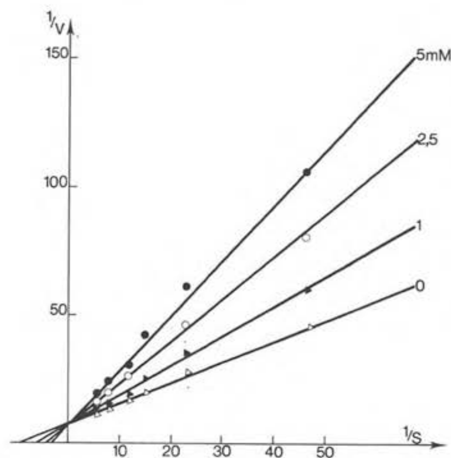


Fig. 3

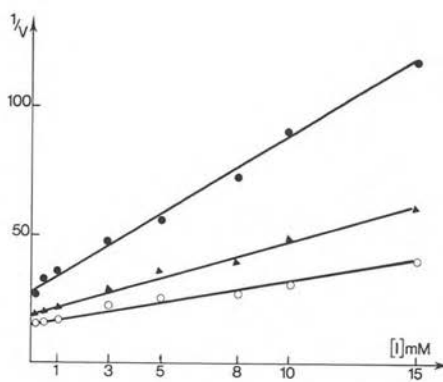


Fig. 4

Fig. 3. *ki* determination with increasing concentration of choroquine, ○ (★ ——— ★) 1 mM (* ——— *) 2.5 mM (○ ——— ○) and 5 mM (• ——— •).

Fig. 4. $\frac{1}{V}$ = F (I) with different substrate concentration : 0,43 mM (• ——— •), 0,87 mM 0.87 mM (* ——— *) and 1.29 mM (○ ——— ○).

## Discussion

The aminopeptidase in the extracts of sporulated oocysts from *Eimeria nieschulzi* had physico-chemical and enzymatic properties very similar to those found in *Plasmodium extracts* (Charet *et al.*, 1980). A similar enzyme has also been found in the *Babesia* genus (Aissi *et al.* 1980, unpublished results). On the other hand, some properties we described were contrary to the results of Wang and Stotisch (1978) with *Eimeria tenella* oocyst extract : polyacrylamid gel electrophoresis of sporulated oocyst extract revealed 2 bands of enzymatic activity whereas column iso-electric focusing gave us only one pealn of activity whatever the synthetic substrate used. Furthermore, Wang and Stotisch showed that their aminopeptidase was activated by $Mg^{++}$ ions and even more so by $Mn^{++}$ ions. In our conditions, $Mg^{++}$ ions were

without effect whereas Mn++ was a strong inhibitor. In the conditions used by Wang and Stotisch (1978) (pH 8,5 0.05 M tris HCl buffer, $MnCl_2$, 2 mM at 40 °C) we found that $Mn^{++}$ ions precipitated as unstable $Mn(OH)_2$, leading to the formation of brown $MnO_2$, which could interfere with the activity determination.

A fact which drew our attention was the inhibitory effect of antimalarial drugs. Chloroquine gave the most marked effect whereas Quinacrine, Primaquine and especially Quinine had a lesser effect. The inhibition by Chloroquine was apparently of the competitive type. This type of inhibiton by antmalarial agents has already been shown for glucose-6-phosphate dehydrogenase (Cotton and Sutorius, 1971) and alcohol dehydrogenase (Fiddick and Heath, 1967). It is interesting that we have demonstrated the same effect for a hydrolase.

It will be interesting to determine which is the target of Chloroquine on the enzyme. As early as 1964, Gerber showed a significant interaction between Chloroquine and the thiol groups of cysteine. Coccidial amino-peptidase is probably a thiol-containing enzyme since its proteolytic action was inhibited by $Hg^{++}$ and P.C.M.B. We therefore tried to show the existence of the compound Chloroquine-cysteine, unfortunately without success.

In conclusion, the action of antimalarialdrugs on aminopetidase is probably of a limited interest (inhibition obtained with high concentrations and lack of specificity). On the other hand, the remarkable analogy between the *Eimeria* genus and *Plasmodium* and also *Babesia,* leads to suggest, considering the different nutritional requirements of these 3 species, that this enzyme plays a more important role in its own basic metabolism than in host protein degradation.

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