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Short note

**Purification of a leucine aminopeptidase
from *Eimeria falciformis***

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Abstract – A leucine aminopeptidase was purified from the oocysts of *Eimeria falciformis* using affinity chromatography and gel filtration techniques. It had a molecular weight of 45–50 kDa. Its maximal activity against leucyl-p-nitro anilide was at pH 8.6. It is a metallo-enzyme highly inhibited by bestatin. © Inra/Elsevier, Paris

leucine aminopeptidase / purification / *Eimeria falciformis* / bestatin

Résumé – Purification d'une leucine aminopeptidase d'*Eimeria falciformis*. Une leucine aminopeptidase de la coccidie murine *Eimeria falciformis* a été purifiée par chromatographie d'affinité et filtration sur gel. L'enzyme a une masse molaire apparente de 45–50 kDa et une activité optimale vis-à-vis du leucyl-p-nitroanilide à pH 8.6. C'est une métalloenzyme qui est fortement inhibée par la bestatine. © Inra/Elsevier, Paris

leucine aminopeptidase / purification / *Eimeria falciformis* / bestatine

1. INTRODUCTION

Coccidia of the genus *Eimeria* are parasitic protozoa that infect the intestine of many animal species. They grow inside

the epithelial cells and may provoke diarrhoea and death.

Proteases catalyse a broad spectrum of biological reactions, including pro-hormone processing, blood coagulation, pro-

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tein metabolism, tissue remodelling and immune reactions [14] and they are considered virulence factors for many infectious agents. They play a critical role in host cell invasion or in the pathogenesis of parasitic diseases, for example: malaria [11], leishmaniasis [6], coccidiosis [1, 10]. Three leucine aminopeptidase (LAP) isoenzymes were found in unsporulated oocysts of *E. tenella* [20]. They undoubtedly play a role in protein synthesis during the sporulation of the oocysts. These exopeptidases not only take part in the latter stages of proteolysis initiated by endoproteases, they also play key roles in the regulatory processes of cellular metabolism [2, 4].

Following qualitative research on proteolytic enzymes in homogenates of a mouse coccidia, *E. falciformis*, we purified this enzyme (by affinity chromatography and gel filtration) in order to be able to investigate subsequently its possible implication in host-parasite relationships.

2. MATERIALS AND METHODS

2.1. Parasite and total oocyst antigen

Eimeria falciformis oocysts were obtained from experimentally infected Balb/c mice, cleaned according to the technique of Bon Temps and Yvoré [5] and allowed to sporulate in a 25 °C water bath under agitation.

Oocysts were broken with glass beads and the supernatant was sonicated at 0 °C in phosphate buffer saline (PBS) in the presence of protease inhibitors [(250 µM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 mM phenylmethyl sulphonyl fluoride (PMSF) SERVA, Heidelberg, Germany] and centrifuged at 10 000 g for 10 min.

The supernatant proteins were quantified with the Bio-Rad protein assay (Bio-Rad, Richmond, USA), stored at -80 °C and used after dilution.

2.2. Purification

2.2.1. Affinity chromatography.

The dipeptide leucine-glycine, a LAP inhibitor (Sigma, St Louis, USA) (10 mg/mL gel) was covalently linked to AH-sepharose 4B at pH 4.5 with the carbodiimide EDC (1-ethyl-3-(3-di-methyl-aminopropyl)carbodiimide), (Milwaukee, USA). The oocyst supernatant was applied to columns (8.5 × 1 cm) of the absorbent previously equilibrated with 50 mM Tris-HCl buffer pH 8.0 containing 5 mM MgCl₂. The column was washed with equilibration buffer until the unbound proteins were removed from the column (approximately 40 mL until absorbance at 280 nm of effluent was 0.05). The absorbed material was eluted at +4 °C by using 0.2 M NaCl in 50 mM Tris-HCl buffer pH 8.0. The eluted fraction was dialysed against ammonium acetate (2 g/L) buffer pH 7.1.

2.2.2. Gel filtration chromatography

Gel filtration of the active fractions from the affinity chromatography was performed on a TSK-gel 300W column (Millipore, Bedford, USA) developed with a 25 mM Bis tris-HCl pH 6.4 buffer at a rate of 0.4 mL·min⁻¹. The molecular mass for the native proteinase was estimated with calibration standards (Pharmacia, Uppsala, Sweden).

2.3. Enzyme Assay

Leucine aminopeptidase activity was measured with L-leucyl-*p*-nitroanilide as the substrate [13, 19]. 100 µL of enzyme in 50 mM Tris-HCl buffer, pH 8.0 were incubated with 10 µL of substrate (1.6 mM) at 37 °C for 1 h. The reaction was stopped with 50 µL of ZnSO₄ 5%. The release of *p*-nitroaniline was followed at 405 nm by a Titer-Tek Multiskan, ELISA reader (Flow Labs, Helsinki, Finland). Several inhibitors were added to the reaction mixture.

2.4. Polyacrylamide gel electrophoresis

Total oocyst antigen and purified leucine aminopeptidase were analysed by SDS poly-

acrylamide gel electrophoresis (PAGE) under reducing conditions [12] using a 12.5 % running gel.

3. RESULTS

Affinity chromatography resulted in a 52 % yield, whereas gel filtration gave an almost complete recovery. Leucine aminopeptidase was purified 207-fold with a specific activity of $236 \mu\text{M}\cdot\text{min}\cdot\text{mg}^{-1}$ (table I).

The molecular mass of the purified enzyme was estimated to be 45–50 kDa by gel filtration and 50 kDa by SDS-PAGE. The active form of the enzyme is thus a monomer.

The enzyme showed a high affinity for the leucine-*p*-nitroanilide, which is an excellent substrate ($K_M = 1.32 \text{ mM}$ and $V_{\text{max}} = 0.77 \mu\text{M}\cdot\text{min}^{-1}$).

Optimum pH for the enzyme activity was found to be pH 8.6. The isoelectric point of the purified enzyme was estimated to be 5.0 by capillary electrophoresis.

Bestatin (calculated $K_i = 210 \text{ mM}$) and 1-1-dipyridyl strongly inhibited the activity (respectively 90 and 60 %) but DTT and iodoacetamide did not. EDTA and the dipeptide leu-glycine slightly decreased the activity. The divalent cations Mg^{++} increased the proteinase activity (table II).

4. DISCUSSION

During the host-parasite relationship proteases may be used by the parasite to penetrate the host cells during infection [8, 9] or to grow inside the parasitophorous vacuoles [15] and may also directly interfere with the immune system of the host as shown in studies on schistosomiasis [3], on

Table I. Purification of leucine aminopeptidase from *E. falciformis*.

Purification steps	Total protein (mg)	Specific activity ($\mu\text{M}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	Purification (fold)	Yield (%)
Sporulated occysts	38	1.14	1	...
Affinity chromatography	3.75	6.11	5.5	52
Column gel filtration	0.18	236	207	99

Table II. Effects of chemical reagents on LAP from *E. falciformis*.

Reagents	Concentration (mM)	Activity ($\mu\text{M}\cdot\text{min}^{-1}$)
None	–	50
MgCl_2	5	76
ZnSO_4	25	1.50
$\text{MgCl}_2 + \text{ZnSO}_4$	5 + 25	0.8
Bestatin	1	0.5
1-1 dipyridyl	1	22
DTT	1	50
Iodoacetamide	1	48
EDTA	4	27
Leucine-glycine	2	39

leishmaniasis [6] or trypanosomiasis [18]. Proteases could thus possibly represent interesting antigens for vaccination, diagnosis or be targets for chemotherapy [7].

The mechanism of host cell invasion by *Eimeria* spp. sporozoites is still not fully understood. In recent years, there has been increasing interest in elucidating the role of various proteases during this process [16]. LAP isozymes have been identified in *E. tenella* unsporulated oocysts [20]. In this paper we presented how we purified a LAP from *E. falciformis* sporulated oocysts using affinity chromatography and gel filtration chromatography. Affinity chromatography increased the specific enzyme activity 5.5-fold (table I). A further purification by gel filtration chromatography yielded a 35-fold increase in specific activity. The purified *E. falciformis* LAP had an apparent molecular mass of 45–50 kDa in gel filtration and 50 kDa by SDS-PAGE. This enzyme would appear to be a monomer, the difference in molecular weight between the native and denatured protein may be due to glycosylation.

E. falciformis LAP shares several other properties with *E. tenella* LAP, such as the optimal pH and metal ion requirement during preincubation and assaying. It also hydrolyses several aminoacyl β -naphthylamides [20]. Its activity was enhanced by 5 mM $MgCl_2$ (table II), and its optimal pH was 8.6. The purified enzyme showed a high affinity for leucine-*p*-nitroanilide ($K_M = 1.32$ mM and $V_{max} = 0.77$ mM \cdot min $^{-1}$). Bestatin, and 1-1 dipyriddylyl strongly decreased the activity of the purified enzyme (respectively by 90 and 60 %), whereas DTT and iodoacetamide had no effect. These results suggest that the *E. falciformis* LAP is a metallo-enzyme as are other LAPs [17] and that bestatin is a specific inhibitor with $K_i = 210$ mM.

The immobilised Leu-gly affinity chromatography and gel filtration methods

described in this study are reliable and gave a reproducible purification of LAP. Only one isozyme was observed here and further studies are needed to determine if this fact reveals differences between Eimerian species or if other *E. falciformis* isozymes can be obtained by other methods. Further studies will also be undertaken with monoclonal antibodies to determine the location of LAP in the parasites and to elucidate its possible involvement in host–parasite relationships.

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