Purification of a leucine aminopeptidase from Eimeria falciformis

M.M. Kaga, Fabrice Laurent, A. Doh, G. Luffau, P. Yvore, P. Pery

▶ **To cite this version:**

M.M. Kaga, Fabrice Laurent, A. Doh, G. Luffau, P. Yvore, et al.. Purification of a leucine aminopeptidase from Eimeria falciformis. Veterinary Research, 1998, 29 (1), pp.107-111. hal-02691885

HAL Id: hal-02691885
https://hal.inrae.fr/hal-02691885

Submitted on 1 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Vet Res (1998) 29, 107–111
© Elsevier/Inra

Short note

Purification of a leucine aminopeptidase from *Eimeria falciformis*

Madjanakona M. Kaga[a, c], Fabrice Laurent[a], Ananivi Doh[c], Gérard Luffau[a], Pierre Yvoré[b], Pierre Péry[a*]

[a]Unité de virologie et immunologie moléculaires, Inra, 78352 Jouy-en-Josas, France
[b]Unité de pathologie aviaire et parasitologie, Inra, 37380 Tours-Nouzilly, France
[c]Laboratoire de biochimie et de nutrition appliquées, Université du Bénin, B.P. 1515, Lomé, Togo

(Received 20 June 1997; accepted 26 September 1997)

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-

* Correspondence and reprints
Tel: (33) 1 34 65 26 25; fax: (33) 1 34 65 26 21

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 endoproteinases, 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 experimentaly infected Balb/c mice, cleaned according to the technique of Bontemps 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 $MgCl_2$. The column was washed with equilibration buffer until the unbound proteins were removed from the column (approximatively 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 $ZnSO_4$ 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 $\mu M \cdot min \cdot mg^{-1}$ (*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 mM and V_{max} = 0.77 $\mu M \cdot min^{-1}$).

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 Ki = 210 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 slighly 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 ($\mu M \cdot min^{-1} \cdot mg^{-1}$)	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 ($\mu M \cdot min^{-1}$)
None	–	50
$MgCl_2$	5	76
$ZnSO_4$	25	1.50
$MgCl_2 + ZnSO_4$	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 eluciding 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-naphtylamides [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 dipyridyl 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 metalloenzyme as are other LAPs [17] and that bestatin is a specific inhibitor with Ki = 210 mM.

The immobilised Leu-gly affinity chromatography and gel filtration methods described in this study are reliable and gave a reproductible 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 antobodies to determine the location of LAP in the parasites and to elucidate its possible involvement in host–parasite relationships.

REFERENCES

[1] Adams J.H., Bushell G.R., The effect of protease inhibitors on *Eimeria vermiformis* invasion in cultured cells, Intern. J. Parasitol. 18 (1988) 683–685.

[2] Amoscato A.A., Balasubramaniam A., Alexander J.W., Babcock G.F., Degradation of thymopectin by human lymphocytes: Evidence for aminopeptidase activity, Biochim Biophys Acta 955 (1988) 164–174.

[3] Auriault C., Pierce R., Cesari I.M., Capron A., Neutral protease activities at different developmental stages *of Schistosoma mansoni* in mammalian hosts, Comp. Biochem. Physiol. 72B (1982) 377–384.

[4] Bachmair A., Finley D., Varshavsy A., In vivo half-life of a protein is a function of its amino-terminal residue, Science 234 (1986) 179–186.

[5] Bontemps M., Yvoré P., Technique de purification des suspensions de sporozoïtes d'*Eimeria* sur colonne de fibres synthétiques, Ann. Rech. Vet. 5. (1974) 109–113.

[6] Bordier C., The promastigote surface protease of *Leishamania*, Parasitol. Today 5 (1987) 151–153.

[7] Coombs G.H., Mottram J.C., Parasite proteinases and amino acid metabolism: possibilities for chemotherapeutic exploitation, Parasitology 114 (1997) S61–S80.

[8] Farooqui A.A., Lujuan R., Hanson W.L., Acid hydrolases of the coccidian *Eimeria tenella*, Experientia 39 (1983) 1368–1370.

[9] Forney J.R., Yang S., Du C., Healey M.C., Efficacy of serine protease inhibitors against *Cryptosporidium parvum* infection in a bovine fallopian tube epithelial cell culture system, J. Parasitol. 82 (1996) 638–640.

[10] Fuller A.L., Mc.Dougald L.R., Reduction in cell entry of *Eimeria tenella* (coccidia) sporozoites by protease inhibitions, and partial

characterization of proteolytic activity associated with intact sporozoites and merozoites, J. Parasitol. 76 (1990) 464–467.

[11] Hadley T., Aikawa M., Miller L.H., *Plasmodium knowlesi*: studies on invasion of *Rhesus* erythrocytes by merozoites in the presence of protease inhibitors, Exp. Parasitol. 55 (1983) 306–311.

[12] Laemmli J.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[13] Lin W.Y., Lin S.H., Van Wart H.E., Steady-state kinetics of hydrolysis of dansyl-peptide substrates by leucine amino-peptidase, Biochemistry 27 (1988) 5062–5068.

[14] McKerrow J.H., Parasite proteases, Exp. Parasitol. 68 (1989) 111–115.

[15] Moon R.P., Tyas L., Certa U., Rupp K., Bur D., Jacquet C., Matile H., Loetscher H., Grueninger-Leitch F., Kay J., Dunn B.M., Berry C., Ridley R.G., Expression and characterization of plasmepsin I from *Plasmodium falciparum*, Eur. J. Biochem. 244 (1997) 552–560.

[16] North M.J., Motram J.C., Coombs G.H., Cysteine proteinases protozoa, Parasitol. Today 6 (1990) 270–275.

[17] Taylor A., Aminopeptidases: towards a mechanism of action, Trends Biochem. Sci. 18 (1993) 167–172.

[18] Troeberg L., Pike R.N., Morty R.E., Berry R.K., Coetzer T.H., Lonsdale-Eccles J.D., Proteases from *Trypanosoma brucei brucei*. Purification, characterisation and interactions with host regulatory molecules, Eur. J. Biochem. 238 (1996) 728–736.

[19] Tuppy H., Wiesbauer U., Wintersberger E., Aminosaure-*p*-nitroanilide als substrate fur Aminopeptidasen und andere proteolytische, Fermente. Z. Physiol. Chem. 329 (1962) 278–288.

[20] Wang C.C., Stotish R.L., Multiple leucine amino-peptidases in the oocysts of *Eimeria tenella* and their changes during sporulation, Comp. Biochem. Physiol. 618 (1978) 307–313.