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A novel protease from *Entamoeba histolytica* homologous to members of the family S28 of serine proteases

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Abstract

Serine proteases are one of the biologically most important and widely distributed enzyme families. A protease capable of degrading the substrate Suc-AAF-AMC was isolated from axenically grown trophozoites of *Entamoeba histolytica*. The enzyme was purified by ion-exchange chromatography and electroelution, and appeared on 2D-PAGE as a spot of 60 kDa and p*I* of 4.65. Data obtained from zymogram suggest the active protease is present either as homodimer (130 kDa) or homotetramer (250 kDa). The optimal temperature of the enzyme was 37 °C, and it exhibited activity over a broad pH range. The protease was strongly inhibited by TPCK and chelating agents. The enzymatic activity was restored upon addition of calcium. BLAST analysis with the sequence of internal peptides of the protein revealed two open reading frames within the genome of *E. histolytica*, homologous to members of the family S28, clan SC of serine proteases.

Index Descriptors and Abbreviations: DPP, dipeptidyl peptidase; PRCP, prolylcarboxypeptidase; Suc-AAF-AMC, *N*-succinyl-L-Ala-L-Ala-L-Phe-7-amido-4-methylcoumarin; DFP, di-isopropyl fluorophosphate; E-64, 1-*trans*-epoxysuccinyl-L-leucilamide-(4-guanidine)-butane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; TLCK, *N*-tosyl-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis

Keywords: *Entamoeba histolytica*; Serine protease; Family S28

1. Introduction

Serine proteases are one of the biologically most important enzyme families. They are found in all organisms studied and carry out a diverse array of physiological functions, such as digestion, defense, and production of active peptides (Yousef et al., 2003). In parasites, a cytosolic serine oligopeptidase B generates the factor that mediates mammalian cell invasion by *Trypanosoma cruzi* and the deletion of this gene renders the parasite incapable of establishing an infection (Burleigh et al., 1997; Caler et al., 1998). In *Plasmodium falciparum*, a serine protease is required to process the merozoite surface protein-1 (Blackman and Holder, 1992) and another is responsible for degradation of membranal proteins of red blood cells affording invasion and infection by this parasite (Roggwiller et al., 1996).

Entamoeba histolytica, the causative agent of entamoebiasis, is a public health problem in developing countries. Trophozoites live in the human bowel and occasionally degrade the intestinal barrier and

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disseminate to other organs, mainly the liver. The majority of the proteinases detected in *E. histolytica* are cysteine proteases. Among other proteolytic activities, a collagenase (Muñoz et al., 1982), a proteasome and a high molecular weight proteinase (Scholze et al., 1996), and a serine-metallo protease (Padilla-Vaca et al., 2000) have been reported in this parasite.

The aims of this study were to purify and characterize a protease that degrades the chymotryptic substrate Suc-AAF-AMC as a step for understanding its function in *E. histolytica*. Our results demonstrate that the pathogenic amoeba has a protease homologous to members of the family S28 of serine proteases.

2. Materials and methods

2.1. Cell culture and fractionation

Entamoeba histolytica, strain HM1:IMSS, was cultured at 37 °C in TYI-S-33 medium (Diamond et al., 1978). After 2 days, trophozoites were harvested and resuspended (10^7 cells/ml) in lysis buffer (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 5 mM $CaCl_2$, and 0.05 mM E-64) and disrupted with a hand homogenizer, followed by three freeze–thawing cycles. The homogenate was centrifuged at 100,000*g* for 1 h at 4 °C. The pellet was solubilized with lysis buffer containing 1% NP-40 at 4 °C. The supernatant was obtained after centrifugation at 100,000*g* for 1 h at 4 °C and used for further experiments.

2.2. Assay of protease activity

The activity was routinely determined using the fluorogenic substrate Suc-AAF-AMC (Bachem). Fifteen microliters of sample was added to 185 μl of reaction buffer (50 mM Tris–HCl, pH 8.0, 10 mM NaCl, and 50 mM $CaCl_2$) with 0.05 mM substrate and incubated for 15 min at 37 °C. To test the effect of temperature on the activity, a range of 20–60 °C was investigated. The reactions were stopped by adding 2.8 ml of 0.1 M glycine, pH 10.0, and fluorescence was read at 380 nm excitation and 460 nm emission in a Perkin-Elmer LS-5B luminescence spectrometer. To determine the inhibition profile of the enzyme, different concentrations of either allicin, antipain, aprotinin, benzamidine, DFP, E-64, EDTA, leupeptin, pepstatin A, PMSF, SBTI, TLCK, TPCK, and 1,10-phenanthroline were incubated with the protein at room temperature for 15 min prior to adding it to the reaction buffer. All the inhibitors were purchased from Sigma, except allicin which was purified from garlic as described (Ankri et al., 1997). To test the effect of calcium, the buffer used was 50 mM Tris–HCl, pH 8.0, 10 mM NaCl, 2 mM EGTA plus the indicated concentrations of $CaCl_2$. To determine the effect of pH, reactions were performed in 100 mM sodium acetate (pH 4.0–7.0) and 100 mM Tris–HCl (pH 7.0–10.0). For direct visualization of protease activity, SDS–PAGE was carried out under semi-denaturing conditions. Samples were loaded in 8% SDS–polyacrylamide gel and run at 4 °C. After electrophoresis, the gel was washed with 2.5% Triton X-100 for 1 h at 4 °C and incubated in 50 mM Tris–HCl, pH 8.0, 50 mM $CaCl_2$, and 0.15 mM Suc-AAF-AMC for 10 min at 37 °C. Fluorescence was detected with a UV transilluminator.

2.3. Purification of the serine protease

The solubilized fraction obtained from 1×10^9 trophozoites containing E-64 was equilibrated to 20 mM NaCl and 20 mM ammonium acetate, pH 5.0, and applied to an equilibrated High S column (Bio-Rad). The serine protease activity was found in the non-bound material, which was dialyzed against 150 mM NaCl and 20 mM Tris–HCl, pH 8.5, and applied to an equilibrated High Q column (Bio-Rad). After extensive washing of the column, bound proteins were eluted with a linear gradient of NaCl (150–500 mM). The serine protease eluted as a single peak at 230 mM NaCl. The sample was dialyzed, lyophilized, and subjected to semi-denaturing SDS–PAGE to visualize the active protease. A fluorescent band corresponding to the activity was carefully excised from the gel, electroeluted at 100 V for 3 h, dialyzed, lyophilized, and subjected to 2D-PAGE. Protein concentration was determined using the DC Protein Assay (Bio-Rad).

2.4. 2D-PAGE

Lyophilized sample (20 μg) was resuspended in 125 μl of rehydration buffer (8 M urea, 2% Chaps, 0.5% IPG buffer, 0.2% DTT, and 0.002% bromophenol blue) and used to rehydrate an immobilized pH gradient strip (7 cm, pH 4–7) (Amersham) overnight at room temperature. The strip was subjected to isoelectric focusing for 8000 V h in a Ettan IPGphor II system (Amersham) and equilibrated in 50 mM Tris–HCl, pH 8.3, 6 M urea, 2% SDS, and 0.01% bromophenol blue plus 0.5% DTT. A second equilibration was performed using 1.2% iodoacetamide. The strip was then run on a 10% SDS–polyacrylamide gel. Molecular markers were from Bio-Rad.

2.5. Tryptic peptide sequencing

Following 2D-PAGE, the gel was stained with 0.25% Coomassie brilliant blue in 50% methanol, 2% acetic acid for 1 h, followed by destaining (50% methanol, 2% acetic acid), and extensive washing with double-distilled water. The spot was excised and sent for LC/MS/MS analysis at the Australian Proteome Analysis Facility.