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RESEARCH ARTICLE

Expression of a *Haemonchus contortus* cysteine protease in the baculovirus system

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Abbreviations: CP: Cysteine Protease
FR-AMC: Phe-Arg-Amino Methyl Coumarin
HCP: Helminth Cysteine Protease
ORF: Open Reading Frame.
PAGE: Polyacryl Amide Gel Electrophoresis
PCR: Polymerase Chain Reaction
PMSF: Phenylmethylsulfonyl Fluoride
PVDF: Polyvinylidene Fluoride
RR-AMC: Arg-Arg-Amino Methyl Coumarin
YVAD-AMC: Tyr-Val-Ala-Asp-Amino Methyl Coumarin

A *Haemonchus contortus* recombinant Cysteine Protease (CP) was expressed in the baculovirus system. The CP gene was isolated by PCR from *H. contortus* cDNA, the PCR amplicon was cloned downstream to the polihedrin promoter within a bacterial expression vector, Sf9 insect cells were used for simultaneous co-transfection with the CP-vector and baculovirus naked DNA, which originated recombinant viruses by homologous recombination capable to express recombinant CP in an insect cell culture. A recombinant protease was identified as a fusion protein with a Ni lithium affinity 6XHis group. Recombinant CP was purified by affinity chromatography to obtain active recombinant protease identified by *H. contortus* experimentally infested ovine sera on a western blot as a 37 kDa protein, as well as by enzyme activity on PAGE-gelatin. Cysteine protease activity was assayed against synthetic substrates including the dipeptides: Phe-Arg, cathepsin B substrate: Arg-Arg, the caspase tetrapeptide substrate: Tyr-Val-Ala-Asp. Maximum CP activity was detected at pH 6.0 for all synthetic substrates and total inhibition was achieved by E-64 but not by EDTA, pepstatin or PMSF. Recombinant *H. contortus* CP can be obtained in large amounts from

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transfected insect cell culture and may be applied to control experiments of ruminant Haemonchosis.

The Cysteine Proteases from parasitic helminths, belong to a wide range of proteases family known as cathepsin B like proteases (Skuce et al. 1999), this papain related enzymes may be subdivided in over ten subfamilies based on the amino acid sequence and affinity for synthetic substrates (Falcone et al. 2000). Helminth Cysteine Proteases (HCP) enzymatic activity may play important roles on helminthic parasite activities such as host invasion, cuticle molting and blood digestion during feeding of a large variety on parasitic helminths, making HCPs a potential target for new immunotherapeutic and immunodiagnostics strategies (Jasmer and McGuire, 1991; Shompole and Jasmer, 2001). Several studies on HCP highlight the importance of these enzymes for the parasites physiology, including the nematodes *Haemonchus contortus, Ancylostoma duodenale, Toxocara canis* and *Onchocerca volvulus* (Lustigman et al. 1996; Falcone et al. 2000; Shompole and Jasmer, 2001; Vervelde et al. 2002), as well as on the trematodes *Fasciola hepatica, Schistosoma mansoni Schistosoma japonicum* and *Paragonimus westermani* (Brady et al. 1999a; Brady et al. 1999b; Yun et al. 2000; Law et al. 2003). HCPs are involved in the apolysis or detachment of the cuticle from the underlying epidermis, an essential process during the exystment of different larval stages of parasites helminths which implies the formation of a new cuticle and the shedding of the previous one or ecdisis (Lustigman et al. 1996; Hashmi et al. 2002). One possibility for the control of helminth parasites is to induce a neutralizing antibody in the host capable to inhibit the HCP activity by antigen-antibody interaction (Munn, 1997; Knox, 2000), this line of research is a favored one for ruminant Haemonchosis since an *H. contortus* intestinal proteases enriched fraction induced host protection against Haemonchosis diminishing in a 77% the parasite burden and in a 95% the egg count in feces from experimentally inoculated sheep (Knox and Smith, 2001; Vervelde et al. 2002). Another line of research on helminth parasite proteases is related to the potential use of these enzymes for immunodiagnostics purposes since the infestation of the host may induce host antibodies against these proteases with a distinctive reaction on several diagnostics formats. (Schallig et al. 1995; Poot et al. 1997). In this study a baculovirus recombinant *H. contortus* Cysteine Protease (CP) recognized by *H. contortus* infected sheep sera was obtained and its catalytic properties were tested for distinctive CP activities which may provide a reliable supply of antigen for immunodiagnostics and immunotherapy research projects as well as a guidance to investigate these enzymes in relation to normal biological functions.

MATERIALS AND METHODS

Cysteine Protease gene isolation

mRNA was isolated from 50,000 L3 H. contortus larvae according to the protocol of the commercial kits Totally RNA™ and RNAqueos® available on line (www.ambion.com), from which cDNA was synthesized following the protocol of the cDNA synthesis commercial kit RETROscript™ available on line (www.ambion.com). A PCR reaction was performed using the forward primer 5'-ATGAAATACTTGGTGCTTGC-3' and the reverse primer 5'-GTAATCCAGCTTTTATCGAA-3' designed from GenBank sequences (access number M31112 and AAA29177). 10 pMol of each primer were used in a 20 µl total reaction volume in a 50 mM KCl, 10 mM Tris-HCl,

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AAA29177    M-VLVFTLCA VLCAASGASI NAAQEIPLEA QTLTGEPLVA VLRKNQNLFE VNSEPTPNFE QKIMDIKFKN QKLNF    74
M31112      MKVLVLALCT VLCSQTGADE NAAQGIPLEA QRLTGEPLVA VLRRSQNLFE VNSAPTPNFE QKIMDIKVKH QKLNL    75
EF371879    MKVLVLALCT VLCSRTGADE NAAQGIPLEA QRLTGEPLVS VLRRSQSLFE VNSDPTPNFE QKIMDIKVNH QRLNL    75
Consensus   MKVLVLALCT VLCS.TGADE NAAQGIPLEA QRLTGEPLVA VLRRSQNLFE VNS.PTPNFE QKIMDIKVKH QKLNL    75

AAA29177    VVKNDPEPNE DIPEEVDPRE KFKCSTFV-I RDQANCGSCW AVSTAAAISD RICIATNGEK QVNISSTDIL TCCNP   148
M31112      MVKEDPDPEV DIPPSVDPRD VWKNCTTFVI RDQANCGSCW AVSTAAAISD RICIASKAEK QVNISATDIM TCCRP   150
EF371879    MVKEDPDPEV DIPPSVDPRD VWKNCTTFVI RDQANCGSCW AVSTAAAISD RICIASKAEK QVNISATDIM TCCRP   150
Consensus   MVKEDPDPEV DIPPSVDPRD VWKNCTTFVI RDQANCGSCW AVSTAAAISD RICIASKAEK QVNISATDIM TCCRP   150

AAA29177    QCGFGCGGGW SIRAWEVFVV EGVVSGGEVL TKGVCRPVPI HPCGHHGNDT VVGECPREAA TPPCKKKCQP GVKKI   223
M31112      QCGDGCEGGW PIEAWKVFIV DGVVSGGEVL TKDVCRPVPI HPCGHHGNDT VVGECRGTAP TPPCKRKCRP GVRKM   225
EF371879    QCGDGCEGGW PIEAWKVFIV DGVVSGGEVL TKGVCRPVPI HPCGHHGNDT VVGECRGTAP TPPCKKECRP GVRKV   225
Consensus   QCGDGCEGGW PIEAWKVFIV DGVVSGGEVL TKGVCRPVPI HPCGHHGNDT VVGECRGTAP TPPCKKKCRP GVRK.   225

AAA29177    FRMDKRQGKV AVGVEPKEEA IQREILRHGP VVASFAVYED FSLVKTGVYK HTAGALRGVH AVKMMGWGVD SKTKA   298
M31112      VRIDKRVGKD AVIVKQSVKA IQSEILRNGP VVASFAVYED FRHVKSGIYK HTAGELRGVH AVKMIGWGNE NNTDF   300
EF371879    VRIDKRVGKD AVIVKQSVKA IQSEILRNGP VVASFAVYED FRHVKSGIYK HTAGELRGVH AVKMIGWGNE NNTDF   300
Consensus   VRIDKRVGKD AVIVKQSVKA IQSEILRNGP VVASFAVYED FRHVKSGIYK HTAGELRGVH AVKMIGWGNE NNTDF   300
                      HEMOGLOBINASE                                S2
AAA29177    KYWLIANSWH NDWGENGVFR FIRGINDCEI EDTVAAGIVD VDSL                                      342
M31112      WL--IANSWH NDWGEKGVFR IIRGTNDCGI EGTIAAGIVD TES-                                      341
EF371879    WL--IANSWH NDWGEKGVFR IIRGTNDCGI EGTIAAGIVD TESL                                      342
Consensus   WL--IANSWH NDWGEKGVFR IIRGTNDCGI EGTIAAGIVD TESL                                      344
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Figure 1. Alignment of the *H. contortus* Cysteine Proteases. GenBank accession numbers of the displayed sequences have been indicated to the left. Positions having identical residues have been shaded. The hemoglobinase motif and S2 substrate binding site described in the text are shown. The amino acid sequence of CP reported herein was deduced from the nucleotide sequence and submitted to the Genbank (Genbank EF371879) and aligned with CP from *H. contoruts* reported in the Genbank (AAA29177 and M3112).

1.5 mM MgCl2, pH 8.3 buffer solution containing two units of Taq polymerase and 20 pg of *H. contortus* cDNA under the following PCR conditions: hot start 4 min at 94ºC, denaturation 1 min at 94ºC, annealing 1 min at 55ºC, elongation 1 min at 72ºC repeating the previous three steps for 35 cycles and a final incubation of 15 min at 72ºC. The PCR products were monitored by 1% agarose submarine electrophoresis gel in TBE buffer according to reported protocol (Sambrook and Russell, 2001). The PCR product was cloned in the baculovirus expression vector pBlueBac4.5/V5 His TOPO® according to the manufacturer protocol available on line (www.invitrogen.com). *E. coli* was transformed with the CP-expression vector and screened for the presence of positive clones, verified bacterial transformants were cultured further in 10 ml of ampicillin-Luria broth and a miniprep was performed according to the plasmid isolation commercial kit instructions Wizard® Plus available on line (www.promega.com).

DNA translation and protein sequence alignment

The isolated vector was thoroughly sequenced in order to verify the CP identity and correct open reading frame of the construction. The obtained DNA sequence was translated by using the ORF finder software on line (www.ncbi.nlm.nih.gov/gorf/gorf.html) and compared by protein multiple sequence alignment using Geneworks 2.45 software (Intelligenetics USA) against related sequences available on the GenBank (Accession numbers M31112 and AAA29177).

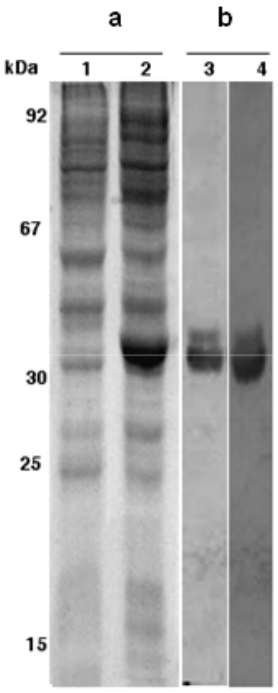

Figure 2. Recombinant Cystein Protease Expression in Sf9 cells.

(a) PAGE of: 1. Uninfected cells extract, 2 Recombinant Virus infected cells.
(b) Western Blot assays on PVDF membranes with infected Cells extract developed with: 3 anti His Tag antibody. 4 *H. contortus* infested sheep.

Recombinant virus generation

5 µg of the vector containing the CP gene oriented with the polyhedrine viral gene was co-transfected in a Sf9 cell line with nude Baculovirus DNA according to the instructions of the commercial Baculovirus transfection kit Bac-N-Blue™ available on line (www.invitrogen.com). Recombinant viruses were identified by expression of the Lac Z gene in transfected Sf9 cells covered with agarose-Xgal according to previous reports (Summers and Smith, 1987; Luckow, 1995).

Recombinant virus propagation and CP *in vitro* expression

Recombinant viral titer was raised by repeated infections of fresh batches of 10^6 Sf9 cell cultures in exponential growth until a level of 10^7 pfu ml^{-1} was reached according to a previous report (Summers and Smith, 1987), infected cells were harvested after 96 hrs post infection (hpi) and prepared for SDS-PAGE.

Polyacrylamide gel electrophoresis (PAGE) and Gelatin-PAGE

12.5% PAGE gels were used according to previously reported protocol (Laemmli, 1970) for expression analysis and western blot, 0.01% gelatin was copolymerized with the PAGE for enzyme activity according to previous report (Brady et al. 1999a; Brady et al. 1999b; Metayer et al. 2002; Manchenko, 2003), active proteases were identified by Coomassie staining.

Affinity chromatography purification

96 hpt cells were collected, washed and sonicated according to a previous protocol (Luckow, 1995); cells extracts were applied to Ni-agarose according to the protocol of commercial preparation available on line (www.invitrogen.com). Purified fusion proteins were assayed by PAGE, Gelatin-PAGE.

Western Blot assays

Immuno blots were performed on PVDF membranes according to the protocol described previously (Towbinet et al. 1979) all commercial antibodies were purchased from Santa Cruz Biotechnology Inc. (www.scbt.com). Electrophoretically transferred Sf9 cell samples were reacted with the presence of a 1:5000 dilution of PBS of a rabbit anti His-tag antibody, and a 1:5000 dilution of a secondary antibody conjugate anti rabbit IgG-AP. A mixture of ten sera samples from experimentally *H. contortus* infested sheep were used in a 1:5000 dilution in PBS, a 1:5000 PBS dilution of a secondary AP-conjugated

Table 1. Mean percentage of inhibition of protease activity from extracts of recombinant *H. contortus* Cysteine Protease.

Inhibitor[b]	Substrates[a]		
	YVAD	FR	RR
E-64	98	100	100
EDTA	33	45	22
Pepstatin	0.0	0.0	2.1
PMSF[c]	0.0	2.3	14.4

[a]Peptide substrates FR-AMC, RR-AMC, and YVAD-AMC.
[b]Means percentage of inhibition (n = 5), compared to non-inhibited controls.
[c]Phenylmethylsulfonyl fluoride.

antibody anti sheep IgG also was used. Chromogenic reactions were performed with a 5 mM Bromo Chloro Indolyl Phosphate and 7mM Nitro Blue Tetrazolium in Tris-HCl 50 mM pH 9 buffer.

Protease synthetic substrate assays

Inhibitors andsynthetic substrates were aquired from BIOMOL (www.biomol.com) Cells extracts were homogenized in PBS containing 1% Triton X-100. Protein concentration of lysates was determined by Bradford colorimetric assay (Bradford, 1976). The extracts were stored in 200 µl aliquots at -20ºC. Protease activity was determined using the di- and tetrapeptide substrates, Phe-Arg-Amino Methyl Coumarin (FR-AMC), Arg-Arg-Amino Methyl Coumarin (RR-AMC) and the caspase substrate tetrapeptide Tyr-Val-Ala-Asp-Amino Methyl Coumarin (YVAD-AMC), at 50 µM in Tris HCl 50 mM adjusting pH from 2 to 8 according to the required experimentally conditions. Reactions were performed in 1.5-ml volumes with 50 µg of affinity purified recombinant CP and incubated at 37ºC; reactions were evaluated at 30 min intervals for 2 hrs. Inhibition studies were performed in Tris HCl 50 mM pH 6 with the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µM E-64, 1 µM pepstatin, 5 mM EDTA. Absorbance readings were taken at 358 nm in a Unico UV 21 Spectrophotometer. The amount of Amino Methyl Coumarin released during the reaction was calculated estimating a Molar Extinction Coefficient of 72, 000 Mol^{-1} (Khatyr et al. 2002).

RESULTS AND DISCUSSION

A 1100 base pair (bp) PCR product was obtained and cloned into the expression vector, proved to be a CP by DNA sequence analysis, it was submitted to the GenBank and the entry number EF371879 was assigned to it. Sequence comparison of the obtained sequence EF371879 (Figure 1) reveals 72% amino acid identity to GenBank reported sequences M3112 and AAA29177, shearing similar features such as a signal sequence at the amino terminus (Cox et al. 1990), as well as a hemoglobinase moiety and the S2 substrate pocket (Chan et al. 1999; Yatsuda et al. 2003; Yatsuda et al. 2006). Selected bacterial transformats produced a CP-vector oriented with the Polh promoter and His-tag according to sequence verification. Transfected Sf9 cell line expressed the Lac Z marker by blue staining the cells as indication of infection with a recombinant baculovirus. This marker was also used for titration and selection of the recombinant virus achieving a level of 10^{-7} pfu ml^{-1} after five passes of reselection and viral purification. The 6X His-Tag-CP fusion protein was identified by western blot using His-tag polyclonal rabbit antibody by detecting the His-tag fusion protein bands on transfected cellular extracts not detected on untransfected cells (Figure 2). His tagging facilitates the identification of the recombinant expressed protein by western blot using an antibody which during our experimental work helped on the identification of His tagged bands in the infected Sf9 cells extracts, although an unexpected higher mass band was identified by the antibodies (Figure 2b), probably due to misfolding of part of the recombinant CP during preparation of the PAGE samples. The Histidine tag affinity for Ni was also useful in the purification procedure and a double band with protease activity of approximately 37 kDa was obtained. Protease activity demonstrated activity against synthetic substrates with values at 82, 43, and 17 pmol AMC min^{-1} μg^{-1} for FR-AMC, RR-AMC and YVAD-AMC respectively (Figure 3). Protease activity was not detected in uninfected cells extracts (Figure 3a), or medium supernatant from infected cells despite the signal peptide within the cloned gene, this unexpected result may be due to the powerful protease inhibitory properties of the fetal bovine serum in the insect cell medium used. The activity on synthetic substrates was highest around pH 6.0 on FR-AMC and RR-AMC with an optimal pH range of about pH 5 to 7. Activity against YVAD-AMC was lower at all pH ranges assayed but consistent with caspase activity Amino acids at the homologous position, lining the S2 substrate pocket (depicted in Figure 1), in CP of other organisms determine substrate specificity (Chan et al. 1999; Sajid and McKerrow, 2002). Site mutagenesis has demonstrated that a glutamic acid residue at S2 position supports the typical cathepsin B-like activity of hydrolyzing substrates Phe-Arg-AMC (FR-AMC) and Arg-Arg-AMC (RR-AMC), whereas a hydrophobic residue results in cathepsin L-like specificity leading to hydrolysis of only FR (Chan et al. 1999; Sajid and McKerrow, 2002). Purified CP was inhibited by E-64 which is consistent with CP activity despite the lack of a glutamic acid at the S2 pocket; protease activity against YVAD under mild acidic conditions is inconsistent for caspases, which have neutral pH requirements (Alnemri, 1997). Inhibitory effects of pepstatin and E-64 indicated that the majority of the activities against FR, RR, and YVAD substrates from recombinant CP are due to a Cysteine Protease (Table 1).

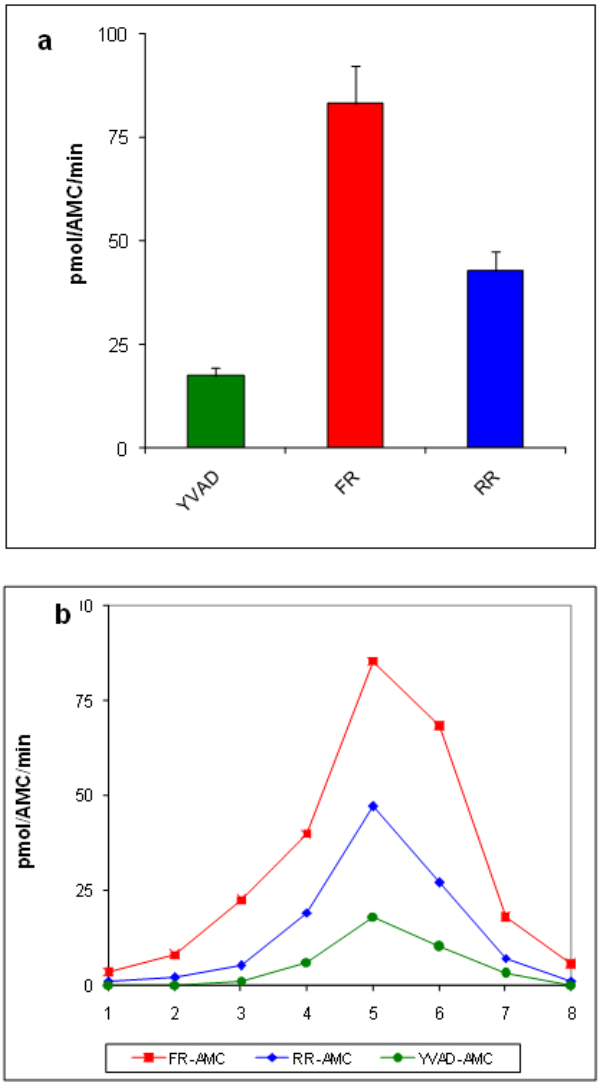

Figure 3. Protease activity of *H. contortus* recombinant purified CP.

(a) 50 µg aliquots of purified fraction, were assayed at pH 6.0 at 37ºC for 2 hrs. Synthetic substrates YVAD-AMC, FR-AMC and RR-AMC were used at 50 µM. Enzyme activity was expressed as picomoles of AMC $min^{-1}µg^{-1}$. Means of five assays are presented, bars represent standard deviations.

(b) pH effects on *H. contortus* recombinant CP protease activity. Protease activities were assayed at pH 2.0-9.0 with FR-AMC, RR-AMC, and YVAD-AMC. Means of assays are shown.

H. contortus CP is important for the physiology of the parasite and several studies focus on the application of CP and other types of proteases for immunodiagnostics and immunotherapy of ruminant Haemonchosis. Another potential niche for recombinant CPs, which may have a very promising perspective, is *in vitro* assessment of protease inhibitors as potential antihelminthic compounds (Chan et al. 1999; Selzer et al. 1999).

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