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Molecular Cloning and Expression of a *Caenorhabditis elegans* Cathepsin B-Like Protease

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Abstract: The present study reports the cloning and expression of a *Caenorhabditis elegans* Cathepsin B-like Protease (CBP), with the objective of obtaining a recombinant enzyme bearing biochemical properties similar to natural CBP reported in the literature for *C. elegans* and parasitic nematodes. The gene was isolated by PCR from *C. elegans* cDNA, resulting amplicon was cloned into a baculovirus expression plasmid, insect cells were used for assembly of a recombinant baculovirus containing the *C. elegans* CBP gene. Thirty five and 45 kDa recombinant proteins were identified from baculovirus infected crude cells containing a His-tag antigenic marker identified by a specific polyclonal antibody in a Western blot assay, both of these recombinant proteins were capable of digesting gelatin in a SDS-PAGE-gelatin assay. Affinity chromatography purified fractions of this recombinant protease, were assayed for peptidase activity against synthetic fluorogenic peptides, including specific cathepsin B substrates and a caspase tetra peptide substrate, maximum cathepsin activity was detected at pH 6.0 for all synthetic substrates and total inhibition was achieved by cystein protease inhibitor E-64 but not by EDTA, pepstatin or PMSF protease inhibitors. Recombinant *C. elegans* cathepsin B-like protease can be obtained in large amounts from the infected insect cell culture.

Key words: Baculovirus, nematodes, recombinant enzyme, synthetic substrates, insect cells, cell culture, viral transfection

INTRODUCTION

Cystein proteinases are a class of lysosomal proteases classified as analogous to papain (Falcone *et al*., 2000), this peptide bound hydrolyzing enzymes belong to an important group of protein playing important physiological role in a wide range of organisms (Skuce *et al*., 1999). The functioning of a sub-group of CP known as caspases, mediate the programmed cellular death or apoptosis, an important process used as a defense mechanism against viral infection for most multicellular organisms (Vaux and Strasser, 1996). Another CP enzyme, known as Cathepsin B, shows an ominous side for human health, since this protease has been found to promote metastasis of human cancers by degrading the extra cellular matrix, thus assisting spreading of tumorous cells. Cathepsin B-like Proteases (CBP) are ubiquitous among metazoan organisms as well, they can be found as a conserved enzymes in several parasitic and free living nematodes including *Caenorhabditis elegans*, all nematodes show CBP significant activity during embryonic development (Britton and Murray, 2002), these proteins are expressed and secreted by nematodes intestine cells, regulating general catabolic functions and protein processing, CBP may be key to parasitic nematodes immunoevasion, excystment/encystment, ensheathing and tissue invasion (Selzer *et al*., 1999). Parasite cysteine proteases are usually immunogenic and have been exploited as serodiagnostic markers and vaccine targets (Miranda-Miranda *et al*., 2007, 2008), some researchers have proposed that this CBP expressing cells, may be a potential target for parasite control using immune or chemical based strategies (Jasmer and McGuire, 1991; Munn, 1997; Selzer *et al*., 1999; Knox and Smith, 2001; Sajid and McKerrow, 2002; Law *et al*., 2003). In accordance to this tendency, several studies highlight the importance of CBP as a target for the control of highly incident human and animal parasitic nematodes, including: *Haemonchus contortus* (Vervelde *et al*., 2002; Jasmer *et al*., 2001), *Ancylostoma duodenale* (Shompole and Jasmer, 2001), *Toxocara canis* (Falcone *et al*., 2000) and *Onchocerca volvulus* (Lustigman *et al*., 1996). During present study, a baculovirus expressed recombinant cathepsin B-like protease was obtained from *C. elegans* and its catalytic properties were tested for distinctive CBP

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activities. This study may provide a valuable tool to investigate these enzyme catalytic properties in relation to parasitic nematodes normal biological functions and nematode control strategies.

MATERIALS AND METHODS

This research was developed at the National Center for Disciplinary Research in Veterinary Parasitology, located at the city of Jiutepec, State of Morelos, México. The study started during the month of January 2008 and concluded in December of the same year.

Cathepsin B gene isolation: The mRNA was isolated from 50,000 *C. elegans* nematodes strain N2 according to the protocol of the commercial kits totally RNA and RNAquos 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 by using the forward primer 5'-ATGAAGACGTTGCTCTTCCT-3' and the backward primer 5'-GTCATCGTAGACGTGGCGGC-3' designed from GENEBANK sequences (access numbers L39894 and NM_171708). The 10 pmol of each primer were used in a 20 μL total reaction volume in a 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 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 94°C, denaturation 1 min at 94°C, annealing 1 min at 58°C, elongation 1 min at 72°C repeating the earlier three steps for 35 cycles and a final incubation of 15 min at 72°C. The bands were monitored by 1% agars submarine electrophoresis in TBE buffer according to reported protocol (Sambrook and Russell, 2001). The PCR product was cloned in the baculovirus expression vector pbluebac 4.5v5 histopo according to the manufacturer protocol available on line (www.invitrogen.com). *E. coli* was transformed with the CBP-expression vector and screened for the presence of positive clones, verified bacterial transform 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). The isolated vector was thoroughly sequenced in order to verify the CBP identity and correct open reading frame of the construction. The obtained DNA sequence was translated and compared by protein multiple sequence alignment using Geneworks 2.45 software (Intelligenetics USA) against related sequences available on the Genbank. Five microgram of the vector containing the CBP gene oriented with the polyhedrin baculoviral promoter was co-transfected in a Sf9 cell line with nude Multiple Nuclear Polyhedro Virus (AcMnPV) DNA according to the instructions of the commercial baculovirus kit Bac-and-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 earlier reports by Luckow (1995).

Recombinant virus propagation and CP *in vitro* expression: Recombinant viral titer was raised by repeated transection of fresh Sf9 cell cultures in exponential growing until a 10^7 pfu was reached according to as earlier report by Summers and Smith (1987), transfected cells were harvested after 96 h post transfect ion (hpt) and prepared for SDS-PAGE according to earlier report by Luckow (1995), a Western Blot was performed on PVDF membranes according to the protocol described earlier by Towbin *et al.* (1979) in the presence of a commercial rabbit antibody anti His-tag and a secondary antibody conjugate anti rabbit IgG-AP.

Polyacrylamide gel electrophoresis (PAGE) and gelatin-PAGE: 12.5% PAGE gel was used according to previously reported protocol (Laemmli, 1970) for expression analysis and western blot (Towbin *et al.*, 1979), 0.01% gelatin was copolymerized with the PAGE for detection of gelatinase activity according to earlier reports by Brady *et al.* (1999a, b), Metayer *et al.* (2002) and Manchenko (2003) active proteases were identified by Comassie staining.

Affinity chromatography purification: Ninety six hpt cells were collected, washed and sonicated according to a earlier 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 according to earlier reports by Brady *et al.* (1999a, b), Metayer *et al.* (2002) and Manchenko (2003).

Protease synthetic substrate assays: Cells extracts were homogenized in PBS containing 1% Triton X-100. Protein concentration of lysates was determined by using a commercially prepared Bradford colorimetric assay (www.BIORAD.com). The extracts were stored in 200 μL aliquots at -20°C. Protease activity was determined using the di and tetra peptide substrates, Phe-Arg-Amino Methyl Coumarin (FR-AMC), Arg-Arg-Amino Methyl Coumarin (RR-AMC) and the caspase substrate tetra peptide Tyr-Val-Ala-Asp-Amino Methyl Coumarin (YVAD-AMC), at 50 μM concentrations in tris HCl 50 mM

adjusting pH from 2 to 8 according to the required experimental conditions. Reactions were performed in 1.5 mL volumes with 50 μg of affinity purified recombinant CP and incubated at 37°C, reactions were evaluated at 30 min intervals for 2 h. Inhibition studies were made in tris HC1 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

A 1260 base pair (bp) amplicon was obtained and cloned into the expression vector, with the gene ORF oriented with the plasmid Polh promoter. The vector-CBP correct construction was verified by DNA sequence (Data not shown). Successful baculovirus transection of the insect Sf9 cell line was corroborated by the expression of the Lac Z marker staining the cells with a blue color in the presence of X-gal (Fig. 1). Lac Z marker was also used for titration and purification of the recombinant virus achieving a 10^7 plaque forming units (pfu) after five passes of reselection and recombinant viral purification. Recombinant proteins were monitored by SDS-PAGE protein electrophoresis on crude cells extracts from infected cells compared to uninfected cells (Fig. 2a). The His-Tag-CBP fusion proteins were identified by western blot using an anti His-tag polyclonal rabbit antibody as four bands with a molecular mass between 35 to 60 kDa. On baculovirus infected cellular extracts, but not detected in uninfected cells (Fig. 2b). Gelatin SDS-PAGE digestion assay showed gelatin degrading proteases with an approximate mass of 35 and 45 kDa (Fig. 2c). In the baculovirus infected crude cells extracts, however this proteolytic activity was absent in the uninfected crude cells. The His-Tag affinity for Ni allowed the isolation of the recombinant protease activity from crude infected cell extracts and this purified fractions of the recombinant proteases were tested against synthetic substrates. *Caenorhabditis elegans* recombinant CBP optimum proteolytic activity showed a pH range of 5 to 7 when tested against different synthetic substrate (Fig. 3a), however activity was highest at pH 6.0 on the dipeptide FR-AMC and RR-AMC, showing a catalytic rate of 93.2 and 42.7 pmol AMC^{-1} min^{-1}, respectively (Fig. 3b). Activity against YVAD-AMC was somehow lower at the same pH conditions ranking at 17.3 pmol AMC^{-1} min^{-1}. Uninfected control cell extracts CBP activity were in all

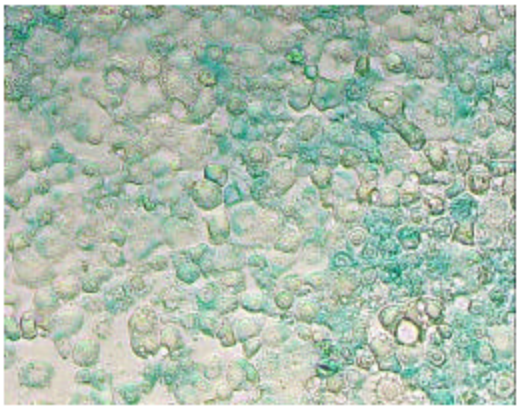

Fig. 1: Sf9 insect cell culture transfected with recombinant baculovirus expressing Lac Z marker. Only baculovirus infected cells expressed the Lac Z marker or β-galactosidase, staining the cells with a greenish-blue color in the presence of the chromogenic β-galactosidase substrate X-gal

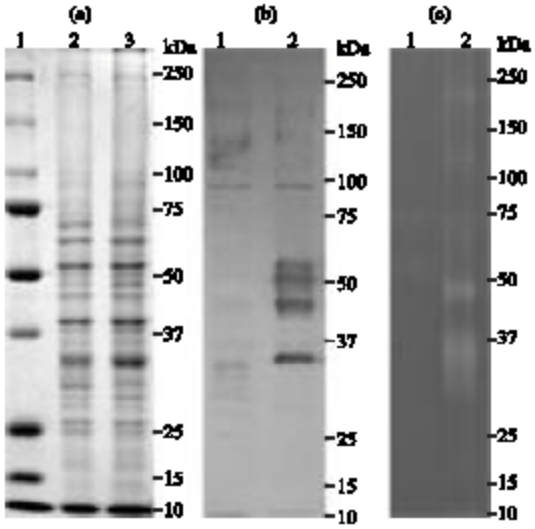

Fig. 2: Protein SDS-PAGE, western blot and gelatin digestion activity assays. (a) Crude protein extracts of uninfected (lane 2), compared to infected Sf9 insect cells at 96 hpi (lane 3), (b) Western blot assay of uninfected cells (lane 1) compared to infected cells (lane 2) and (c) gelatin digestion assay after SDS PAGE of uninfected cell extracts (lane 1) compared to infected cells extracts (lane 2)

cases bellow 4 pmol AMC^{-1} min^{-1}, this comparative data showed statistically significant difference ($p<0.0001$ unpaired t-test) proteolytic activity between infected and uninfected cells. E-64 protease inhibitor effectively blocked proteolytic activity against synthetic dipeptide

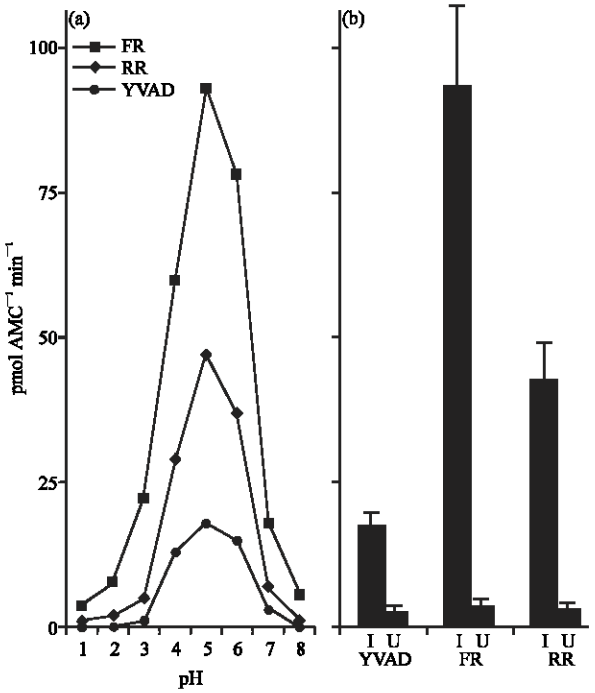

Fig. 3: *Caenorhabditis elegans* recombinant purified CBP protease activity against fluorogenic synthetic peptidic substrates. (a) pH effects on recombinant CBS were assayed at pH 1.0-8.0 with FR-AMC, RR-AMC and YVAD-AMC. Fifty microgram aliquots of purified fraction were assayed at pH 6.0 at 37°C for 2 h. Synthetic substrates were used at 50 μM and (b) CBP peptidase activity under optimal pH conditions 50 μg aliquots of purified fraction (I) and of uninfected cell extracts controls (U), were assayed at pH 6.0 at 37°C for 2 h. In both experiments enzyme activity was expressed as picomoles of AMC min^{-1} μg^{-1}. Means of five assays are presented, bars represent standard deviations

Table 1: Mean percentage of inhibition of protease activity from extracts of recombinant *C. elegans* Cathepsin B-like Protease

	Substrates[a]		
Inhibitor[b]	YVAD (%)	FR (%)	RR (%)
E-64	76.0	97.0	88.0
EDTA	22.0	38.0	19.0
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: Percentage of inhibition (SD) (n = 5), compared to non-inhibited controls, c: Phenylmethylsulfonyl fluoride

FR-AMC and RR-AMC affecting 97 and 88%, respectively of the recombinant CBP proteolysis (Table 1). Caspase tetra peptide YVAD-AMC peptidase activity was inhibited to a 76 % in the presence of E-64 (Table 1). EDTA inhibition was partial with all synthetic substrates ranking at 22% for YVAD-AMC, 38% for FR-AMC and 19% for RR-AMC (Table 1). No significant inhibitory effects were found when Pepstatin and PMSF were used.

DISCUSSION

The obtained amplicon cloned into the expression vector, proved to be a peptidase C1A cathepsin by a BLAST algorithm DNA sequence analysis available on line (www.ncbi.nih.gov), with significant alignment to reported Gene bank *C. elegans* sequences: L39894, NM_171708, NM_001129641, NM_001029255 (www.ncbi.nih.gov), these sequences were originally reported as Cathepsin B-like cystein proteases (Larminie and Johnstone, 1996), which gave us confidence to proceed with the cloning procedure in the baculovirus system with previous *E. coli* transformation. Bacterial transformants produced a CBP-vector oriented with the vector Polh promoter and in sync with the ORF of the His-tag according to DNA sequence verification (data not shown), a required condition for *bonafide* expression of *C. elegans* in insect cells. Transfected Sf9 cell line expressed the Lac Z marker by blue staining the cells as indication of infection with a recombinant baculovirus (Fig. 1). This marker was useful 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 according to previous reports (Summers and Smith, 1987; Luckow, 1995). The His-Tag-CBP fusion protein identified on baculovirus infected cellular extracts, but not detected on uninfected cells (Fig. 2b), gave us an indication of *bonafide* expression of the *C. elegans* CBP in insect cell culture since an aberrant peptide sequence would not be detectable by the polyclonal antibody (Luckow, 1995). His-Tag facilitated the identification of the recombinant expressed protein by western blot using an antibody which during our experimental work helped on the identification of proteases (Fig. 2b), however, unexpected higher mass bands were identified by the polyclonal antibodies, something attributed to misfolding of part of baculovirus recombinant proteases during preparation of the PAGE samples (Fig. 2b) (Brady *et al*., 1999a; Chan *et al*., 1999). Protease activity was found against copolymerized gelatin in a SDS-PAGE assay (Fig. 2c) (Metayer *et al*., 2002), this activity coincided with at least two of the His-Tagged bands identified by the polyclonal antibody (Fig. 2c), demonstrating that at least two of the His-Tagged recombinant proteins, were not properly folded to accomplish an enzymatic activity, this observation has been reported earlier in other recombinant proteases

(Law *et al*., 2003; Chan *et al*., 1999). Neither His-Tagged bands, nor gelatin digestion activity, were found in uninfected cell extracts, providing further confirmation of a successful recombinant *C. elegans*-CBP expression in the baculovirus infected insect cell line. Purified CBP peptidase activity against synthetic substrates: FR-AMC and RR-AMC, is consistent with CBP activity (Larminie and Johnstone, 1996) and excludes other types of related proteases, digestion of YVAD-AMC, although marginal denotes a close relationship between CBP and caspases (Vaux and Strasser, 1996). A translation of the cloned CBP into its amino acid sequence by ORFinder algorithm available on line (www.ncbi.nih.gov), shows a CBP characteristic feature S2 substrate pocket (data not shown), S2 pocket of other organisms determine substrate specificity (Chan *et al*., 1999; McKerrow, 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 (Sajid and McKerrow, 2002; Chan *et al*., 1999). Present CBP was inhibited by E-64 which demonstrates irreversible blockade of a cystein residue at the CBP active site (Chan *et al*., 1999; Alnemri, 1997), inhibitory effects of pepstatin EDTA and PMSF indicated that the majority of the activities against FR-AMC, RR-AMC and YVAD-AMC substrates from *C. elegans* recombinant CBP are consistent with the catalytic properties of reported CBP (Alnemri, 1997; McKerrow, 1999). The experimental data presented drove us to conclude that recombinant cathepsin B-like protease obtained from insect cell culture, produced a functional protease with catalytic properties similar to the analogous proteases already reported from parasitic as well as free living nematodes (Jasmer and McGuire, 1991; Munn, 1997; Selzer *et al*., 1999; Knox and Smith, 2001; Sajid and McKerrow, 2002; Law *et al.,* 2003), The experimental data presented here, lead us to conclude that recombinant cathepsin B-like protease obtained from insect cell culture, produced a functional protease with catalytic properties similar to the analogous proteases already reported from parasitic as well as free living nematodes (Jasmer and McGuire, 1991; Munn, 1997; Selzer *et al*., 1999; Knox and Smith, 2001; Sajid and McKerrow, 2002; Law *et al.,* 2003), the described experimental procedure provide a method for the catalytic assessment of protease inhibitors and specific antibodies destined to be used as anthelmintic compounds in future experiments, this approach can be used in the design of new strategies for the control of parasitic nematodes specially those posing a treat to crops and domestic animals in tropical and subtropical countries.

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