Protein Expression and Purification 109 (2015) 55–61

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Molecular and functional characterisation of a stress responsive cysteine protease, EhCP6 from *Entamoeba histolytica*

Anupama Ghosh [a,*], Sanghamitra Raha [b]

[a] Division of Plant Biology, Bose Institute, Centenary Campus, P1/12 C.I.T Road, Scheme VIIM, Kolkata 700054, India
[b] Integrated Science Education and Research Center (ISERC) and Department of Biotechnology, Visva-Bharati University, Santiniketan 731235, India

ARTICLE INFO

Article history:
Received 18 June 2014
and in revised form 2 February 2015
Available online 10 February 2015

Keywords:
Entamoeba histolytica
Cysteine protease
Stress response

ABSTRACT

Entamoeba histolytica cysteine protease 6 (EhCP6) is a stress responsive cysteine protease that is upregulated in response to heat shock and during pathogen invasion of the host tissue. In the present study an attempt has been made to express and purify recombinant EhCP6 in order to gain insights into its biochemical properties. The recombinant and refolded protein has been shown to undergo autoproteolysis in the presence of DTT and SDS to give rise to ~25 kDa mature form. The mature form of the protein was found to exhibit a protease activity that is sensitive to E-64, a specific cysteine protease inhibitor. *In silico* homology modelling of EhCP6 revealed that the protein exhibits conservation of almost all the major structural features of cathepsin-L like cysteine proteases. Further in vivo studies are needed to decipher the function of the protein in response to different stressed conditions.

Introduction

Entamoeba histolytica genome codes for approximately 86 proteinase genes. Among these about 50 members belong to cysteine protease family [1] with 37 members exhibiting structural similarity to C1 papain superfamily. *E. histolytica* cysteine proteases have been shown to play diverse roles related to pathogenesis of the parasite. For instance, the cysteine protease component of the secretory products of axenically grown virulent HM1:IMSS *E. histolytica* strains were found to induce human mast cell activation [2]. Another study led by Matthiesen and his colleagues showed that overexpression of specific cysteine protease genes ehcp-b8, -b9 and -c13 in non-pathogenic strain of *E. histolytica* could restore the pathogenicity of the trophozoites [3]. Cysteine proteases therefore constitute important virulence factors of *E. histolytica.* Besides pathogenicity, cysteine proteases have also been found to play role in the morphological differentiation of the parasite from invasive trophozoites to dormant infective cyst stage [4].

Out of this huge repertoire of cysteine proteases of *E. histolytica* only a few are expressed during axenic culture. Majority of the cysteine protease genes undergo an induction either in response to different stressed conditions or during host invasion [1,5]. *ehcp6* is one such cysteine protease gene whose expression has been found to be induced in response to heat shock and also during intestinal colonisation by the parasite indicating a specific role of the protease under these conditions [6,7]. Earlier studies revealed that approximately 90% of the cysteine protease activity in the lysates of cultured *E. histolytica* trophozoites is contributed by EhCP1, EhCP2 and EhCP5 whereas all the other cysteine proteases including EhCP6 are expressed in very low amounts in axenic culture [8]. Although during the recent past a number of studies have been done to shed light on the function of individual cysteine proteases of the parasite no specific studies have been made for EhCP6. In the present study we provide a biochemical characterisation of recombinant EhCP6 activity. In addition, based on an *in silico* model of the protease we provide a detailed structural analysis of the molecule.

Materials and methods

Strains, plasmids and media

Trophozoites of Entamoeba histolytica strain HM1:IMSS were grown at 37 °C in TYI-S-33 [9] media supplemented with benzylpenicillin and streptomycin. Cells were subcultured every 72–96 h as previously described [10]. For the purpose of cloning and expression of recombinant EhCP6 *Escherichia coli* strains DH10B and BL21 (DE3) were used. pET-30 Ek/LIC vector (Novagen) was used as an expression vector.

* Corresponding author.
E-mail address: ghosh.anupama@jcbose.ac.in (A. Ghosh).

http://dx.doi.org/10.1016/j.pep.2015.02.005
1046-5928/

Preparation of genomic DNA

Genomic DNA was prepared from *E. histolytica* trophozoites following a protocol as described in [10].

Cloning of ehcp6

The gene coding for the pre pro form of EhCP6 (GenBank accession number: XM_652272) was cloned within the Ligase Independent Cloning (LIC) site of the prokaryotic expression vector pET-30 Ek/LIC downstream of a 6X Histidine coding region following manufacturer's protocol. Briefly the gene was amplified from the genomic DNA of the parasite by PCR using a pair of forward and reverse primers, CP6LICfwd (GACGACGACAAGATGTTTGGTTTACTCTTTG) and CP6LICrev (GAGGAGAAGCCCGGTTATTTCTTTACTTCAGTAAC). The PCR product was then purified and treated with T4 DNA polymerase in the presence of dATP which was then hybridised with the vector. 100 ng of hybridised product was transformed into *E. coli* DH10B cells. Positive clones were verified by sequencing (Bangalore Genei). The recombinant plasmid preparations from the transformed *E. coli* DH10B were further transformed into *E. coli* BL21 DE3 codon plus bacterial cells for expression analysis.

Expression and purification of recombinant EhCP6

500 ml of LB was inoculated with 5 ml of an overnight culture of *E. coli* BL21 (DE3) cells harbouring the recombinant plasmid. The resulting culture was grown at 37 °C till OD_{600} reached 0.6. The expression of recombinant EhCP6 was then induced by 1 mM IPTG and the cells were further grown for 3 h at 37 °C. Subsequently the culture was harvested by centrifugation at 4500*g* for 30 min and the resulting pellet was resuspended in 5 ml of lysis buffer containing 50 mM Tris, 300 mM NaCl, pH 8.0 supplemented with 2% Triton X-100, 1 mg/ml lysozyme and 1Xprotease inhibitor cocktail (Pierce). Following an incubation of 30 min at 4 °C the cells were sonicated on ice using an Ultrason 250 sonicator (LabPlant, England) till a fairly clear non-viscous lysate was obtained. Sonication conditions include short bursts of 30 s each at a power amplitude of 60%. In order to prevent heating of the probe and the sample a time lapse of 1 min was practised between each successive burst. The lysate was then centrifuged at 16,000*g* for 30 min at 4 °C. The resulting pellet containing the inclusion body was washed twice with lysis buffer. The purified inclusion bodies thus obtained were solubilised in 2 ml of inclusion body solubilising buffer (8.0 M urea in lysis buffer) supplemented with 10 mM imidazole. After solubilisation the suspension was centrifuged at 16,000*g* for 30 min to precipitate any particulate matter. Recombinant EhCP6 was then purified from the supernatant using Ni^{2+}–NTA resin (Qiagen) following manufacturer's recommendations for purification under denaturing conditions. Briefly the solubilised inclusion bodies from approximately 500 ml bacterial culture were subjected to 0.5 ml of packed Ni^{2+}–NTA resin column equilibrated with the inclusion body solubilising buffer. The column was subsequently washed with 10 volumes of inclusion body solubilising buffer supplemented with 25 mM imidazole. The protein was then finally eluted with elution buffer (500 mM imidazole in inclusion body solubilising buffer) and analysed on 12% SDS–PAGE.

Refolding of purified recombinant EhCP6

Refolding of the purified recombinant EhCP6 was done by rapid dilution method. The eluted protein was diluted to a final concentration of 5 μg/ml in the refolding buffer (50 mM Tris pH: 6.8, 150 mM NaCl, 5% glycerol, 0.5 mM oxidised glutathione, 3 mM reduced glutathione) at 4 °C. The refolded protein was concentrated 200 times (final volume of 500 μl containing 0.5 mg of full-length protein) (Table S1) to a final conc of 1 mg/ml using a centricon with a cut off of 10 kDa and stored at 4 °C till further use.

Processing of recombinant EhCP6

Prior to processing of refolded and concentrated EhCP6, the protein was dialysed against 50 mM Tris, pH: 6.8. The concentration of the dialysed protein was then adjusted to 1 mg/ml. Processing of the full length recombinant refolded EhCP6 was induced by incubation in 50 mM Tris, pH: 6.8 containing 0.05% SDS and varying concentrations of DTT (5, 7 and 9 mM) for 30 min. The processing products were then run on an SDS gel and visualised through silver staining of the gel. For subsequent analysis of the activity associated with the processing products in-gel protease assay was performed. For quantification of the enzymatic activity associated with the matured form of EhCP6 a second affinity chromatography was performed on the products of EhCP6 maturation experiment. This step yielded 0.25 mg (Table S1) of a partially purified matured form of EhCP6 that is about 25 kDa in size. The enzymatic activity of the partially purified active form of the protease under different conditions of pH and temperature was then performed using azocasein assay.

In gel protease assay (gelatin zymography)

In gel protease activity of the recombinant refolded EhCP6 was determined in 12% SDS–PAGE copolymerised with 0.1% gelatin as previously reported [11]. Briefly 30 μl of samples representing 1 μg of total protein were mixed with 10 μl of 4X Laemmli buffer and incubated at 37 °C for 10 min. 20 μl of each sample was then run on substrate gel. After separation of the recombinant protease the gel was incubated in 2.5% Triton X-100 for 1 h followed by an overnight incubation at 37 °C in the activation buffer containing 100 mM sodium acetate, pH: 5.2, 1% (v/v) Triton X-100 and 20 mM DTT. The gel was subsequently stained with coomassie brilliant blue. For cysteine protease inhibition assay 1 μg of purified protein was incubated in 100 μl of elution buffer supplemented with 10 μM E-64 at 37 °C for 10 min. The proteolytic activity was then assayed using gelatin zymography as described above.

Assay of protease activity

The protease activity of EhCP6 was determined by a spectrophotometric assay using azocasein as the substrate as previously described [11]. Briefly 1 μg of partially purified, refolded and processed EhCP6 was incubated in 500 μl of 0.1 M citric acid–0.2 M Na_2HPO_4, pH 7.0 buffer containing 6.5 mg/ml azocasein at 37 °C for 10 min. Reactions were stopped by addition of 500 μl of 20% (w/v) trichloroacetic acid (TCA) and incubated at 4 °C for 10 min. Samples were centrifuged at 10,000*g* for 15 min and absorbance of the supernatants were immediately determined at 440 nm. For calculating the specific activity of the protease the value of $E^{1\%}$ was considered as 35. One unit of proteolytic activity was defined as amount of enzyme releasing 1 μg of azocasein per min.

Determination of optimal pH and temperature of EhCP6 enzymatic activity

Optimal pH of EhCP6 activity was determined as described previously [11] by incubating the purified and refolded protein at 25 °C for 1 h in buffers with pH ranging from 3.0–10.0. 0.1 M citric acid–0.2 M Na_2HPO_4 buffer with pH 3.0–7.0 was used for assaying acidic condition. 0.1 M Tris HCl pH 8.0, 0.2 M glycin-0.2 M NaOH buffer pH 9.0 and 10.0 were used for assaying basic conditions. For determining optimal temperature of EhCP6 activity 1 μg of