Recombinant Expression & Purification of EhCP6
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INTRODUCTION

1. Proteolytic Enzymes

Proteolytic enzymes catalyse the hydrolytic cleavage of peptide bonds linking two adjacent amino acid residues within a protein or a peptide molecule. However depending upon the positions of the peptide bonds being accessed by the protease the proteases can be classified as exopeptidases and endopeptidases which can be further divided into serine-, aspartic acid-, metallo- and cysteine proteases [1] based upon the identity of the active site residue. Like for instance a serine protease possesses an active site serine residue and an aspartic acid protease possesses an active site aspartic acid residue.

2. The Cysteine Proteases

Cysteine proteases are of wide occurrence in a number of different organisms ranging from viruses and prokaryotes to higher eukaryotes like plants and mammals including humans. This class of proteases has been found to participate in varied biological processes. Like for instance while human cathepsins are involved in protein breakdown within lysosomes, antigen presentation, proteolytic processing of proenzymes and prohormones, fertilisation, cell proliferation, differentiation and apoptosis [2, 3] cysteine proteases from the pathogenic bacteria and parasitic protozoans serve mainly as virulence factors. Like many other proteolytic enzymes most of the cysteine proteases are also synthesized as inactive precursors and need the cleavage of the N-terminal pro region for gaining full activity [4].

3. Cysteine Proteases from Entamoeba histolytica

Majority of the protozoal cysteine proteases belong to the C1 papain superfamily that also includes the mammalian cysteine proteases cathepsins B, C, K, L and S [5]. Likewise E. histolytica cysteine proteases are also not any exception. The parasite genome possesses 50 cysteine protease coding genes among which the majority is structurally related to the C1 papain superfamily with 37 members [6]. Besides these the genome also codes for C2 (calpain like proteases), C19 (ubiquitinyl hydrolase), C48 (Ulp1 peptidase), C54 (autophagin) and C65 (otubain) family members [6]. E. histolytica trophozoites release a large number of cysteine
proteases with molecular weights ranging from 16 kDa-96 kDa into the culture media [7]. Among these the predominant forms are 27 kDa-30 kDa mature enzymes. The amoebic cysteine proteases possess substrate specificity similar to that of the cathepsin B like proteases from papain family [8, 9]. Although most of them possess an optimum proteolytic activity within a broad range of pH from 5 to 9, majority of these enzymes are active in slightly acidic and neutral pH region [10].

Amoebic cysteine proteases are synthesized as precursor proteins with a 12-14 amino acid hydrophobic predomain signal peptide, a 78-82 amino acid prodomain, 216-225 amino acid catalytic domain and no C-terminal extension [7]. The preproenzymes are catalytically inactive and are thus processed into mature forms lacking both the pre and the prodomains. The predomain comprises of an N-terminal charged region, a central hydrophobic region and a polar C-terminal region. The prodomain on the other hand contains an ERFNIN motif [Glu-X3-Arg-X2-(Val/Ile)-Phe-X2-Asn-X3-Ile-X3-Asn] in most of the amoebic cysteine proteases. The ERFNIN motif is also a characteristic feature of cathepsin-H or cathepsin-L like proteases but not cathepsin-B like proteases [11]. Like all other cysteine proteases E. histolytica cysteine proteases also possess the key active site residues namely Gln, Cys, His and Asn within the C-terminal catalytic domain [12].

Among the 50 cysteine protease coding genes within the genome of E. histolytica only four of the corresponding proteins namely EhCP1, EhCP2, EhCP5 and EhCP112 have been purified and characterized successfully [13, 9, 14-16]. The data suggest that approximately 90%
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of the total cysteine protease activity present in the lysates of cultured *E. histolytica* trophozoites is based on the three cysteine proteases EhCP1, EhCP2 and EhCP5, whereas the genes coding for EhCP3, EhCP4, EhCP6, EhCP7 and EhCP112 are expressed at very low levels [17]. Besides, EhCP5 has been found to be the only amoebic cysteine protease that is also expressed on the parasite surface [16]. EhCP112 on the other hand has been shown to be involved in *E. histolytica* adherence, phagocytosis and cytolysis through the formation of EhCPADH complex in association with adhesin EhADH112 [18, 13, 19].

EhCP6 is one of the amoebic cysteine proteases that have been shown to have a very low expression during in vitro cultivation. However its expression increases many fold in response to heat shock [20] and also during intestinal colonisation by the parasite [21] thereby clearly indicating the role of the protease in the stress response as well as the pathogenesis of the parasite. This particular protease despite of its differential expression under above mentioned conditions that makes it a potential candidate cysteine protease for studying the stress responsive mechanisms of the pathogen, has not yet been studied in sufficient details.

So, our present study aims to investigate the expression and proteolytic activity of the protease. In contrast to what has previously been predicted we obtained not only a very good expression of the protease in vitro but also a significant amount of protease activity associated with the recombinant protein. Moreover we could also show the proteolytic maturation of the inactive precursor form of the protein to the fully active form. Further in vivo studies are however needed to decipher the role of EhCP6 in either the cellular stress response or the pathogenesis of the *E. histolytica* parasites.
RESULTS

1. Expression and Purification of Recombinant EhCP6

In order to produce and purify recombinant EhCP6, the gene encoding the protein was cloned within the ligase independent cloning site of pET30 Ek/LIC vector that directs the IPTG controlled T7 promoter driven expression of recombinant proteins. However the expression of the recombinant protein, when attempted in conventional *E.coli* BL21 (DE3) strain, failed completely. A subsequent analysis of the nucleic acid sequence of the gene revealed that all the arginines within the protein sequence of EhCP6 was actually encoded by the codon aga (fig 2.)

![Fig 2. Nucleic acid and corresponding amino acid sequence of the EhCP6 gene. All the arginine residues are marked and as shown are encoded by the aga codon.](image)
Since conventional *E. coli* BL21 (DE3) strains possess limited number of Arg-tRNA against aga codon the expression of the recombinant protein was then tried within the *E. coli* BL21 (DE3)-codon plus-RIL strain possessing extra copies of Arg-tRNA genes which thereby yielded significant expression of the protein when the transformed cells were induced with 1 mM IPTG at 37°C for 3 h. As expected the recombinant EhCP6 was obtained as a 40 kDa His tagged protein only in the induced transformed *E. coli* cell lysates but not in the uninduced samples (fig 3a). The molecular weight was also in good agreement with the theoretically calculated 40 kDa. The expression was further confirmed by western blot of the induced and uninduced cell lysates with anti His antibody which also indicated a band at around 40 kDa only in the induced sample but not in the uninduced sample (fig 3b).

![Fig 3. Expression and purification of recombinant EhCP6. (a) Purification of recombinant His tagged EhCP6 protein. Lane 1: protein molecular weight marker, Lane 2: Uninduced transformed *E. coli* BL21 DE3 codon plus cell lysate, Lane 3: Cell lysate from transformed *E. coli* BL21 (DE3) induced with 1mM IPTG at 37°C for 3 hours, Lane 4: Inclusion body solubilised in 50 mM Tris pH: 8.0, 300 mM NaCl, 8 M urea and 10 mM imidazole, Lane 5: Purified recombinant EhCP6. (b) Western blot analysis with mouse anti His antibody to check the expression of](image)
recombinant His tagged EhCP6. Lane 1: uninduced *E. coli* BL21 (DE3) codon plus cell lysate, Lane 2: Cell lysate from *E. coli* BL21 (DE3) induced with 1mM IPTG for 3 hours.

2. Refolding and the protease activities of the recombinant EhCP6

When a substrate gel was run with the recombinant refolded EhCP6 to detect any protease activity available in the purified protein, a clear band was noticed at the 25 kDa region but not at the 40 kDa region (fig 4a) indicating that the full length protein does not possess the cysteine protease activity. Moreover a SMART domain analysis of the primary sequence of EhCP6 protein showed that it possess a Cl peptidase domain of 217 amino acid residues (molecular weight ~ 24 kDa) at the C-terminus of the protein. Probably the activity band observed in the substrate gel corresponded to this active fragment of the recombinant protein. But unfortunately the 25 kDa band was not visible in the coomassie stained gel of the purified recombinant refolded EhCP6 (fig 4a). So, an attempt was taken to induce the processing of the full length protein by subjecting the protein to mild denaturing conditions which have previously been shown to aid the processing of recombinant EhCP5 [17] and EhCP112 [22]. Finally the processing of the protein was evidenced at varying concentrations of DTT in combination with 0.05% SDS. The processing products became visible at 1 mM to 9 mM DTT but not at or above 10 mM DTT. Silver staining of the gel showed three bands, the full length 40 kDa, an intermediate ~32 kDa band and a 25 kDa band (fig 4b). Moreover to confirm that the activity band was associated with a cysteine protease the purified recombinant refolded EhCP6 was incubated either in the absence or in the presence of a specific cysteine protease inhibitor E-64 and run on a substrate gel a coomassie staining of which showed no clear band corresponding to E-64 treated sample but a band was found to be associated with the sample that was not treated with E-64 (fig 4c) confirming the cysteine protease activity of the purified recombinant protein.
Fig 4. Activation of purified recombinant refolded EhCP6. (a) Lane 1: coomassie stained gel showing purified recombinant refolded EhCP6, Lane 2: Gelatin zymogram of purified recombinant refolded EhCP6. (b) Processing of purified recombinant refolded EhCP6 induced by varying concentrations of DTT in combination with 0.05% SDS, lane 1-6: 0-5mM DTT respectively, lane 7: 7mM DTT, Lane 8: 9mM DTT and lane 9: 10mM DTT. (c) Effect of E-64 on the cysteine protease activity of EhCP6, gelatin zymogram of recombinant refolded EhCP6 either in the presence of E64 (lane 1) or in the absence of E-64 (lane 2).
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DISCUSSION

EhCP6 is one of the cysteine proteases that have been shown to get up-regulated during heat shock treatment to the *E. histolytica* trophozoites [20]. Although it has been speculated that degradation of the damaged proteins generated during the heat shock treatment to the trophozoites is one of the probable functions of the protein [20], EhCP6 has never been shown previously to have a cysteine protease activity either in vivo or in vitro. Within the scope of the present study we could show a significant activity associated with EhCP6 that is also sensitive to the specific cysteine protease inhibitor E-64. Moreover the activity was found to be associated only with the ~25 kDa fragment after refolding of the recombinant EhCP6 from the inclusion body. When compared to the quantity of the full length protein in the refolded sample the amount of the 25 kDa fragment was negligible and was not even visible within the coomassie stained gel of the purified recombinant refolded EhCP6. Besides, an attempt to induce the processing of the full length protein to the active fragment yielded very meager amount of the processed product. Keeping all these observations in mind it seems that EhCP6 definitely possess an activity atleast in vitro associated with the C1 peptidase domain of the protein but processing of the full length protease to the active C1 peptidase domain probably needs some in vivo components (some other protease) in addition to its very negligible autoproteolysis activity.

Further in vivo studies however are needed to characterize the function of the protease in response to different stresses including heat shock and to decipher its activation mechanisms. Moreover a study of EhCP6 in relation to the induction of apoptosis-like cell death in *E. histolytica* by different agents such as G418 [23], Nitric oxide [24] and hydrogen peroxide [25] has the potential to yield significant information regarding the cell death machineries of the parasite.
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