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Original article

OVEREXPRESSION OF A PUTATIVE *BRUCELLA MELITENSIS* ZINC PROTEASE IN *ESCHERICHIA COLI* INDUCES GROWTH ARREST AND CELL FILAMENTATION

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Summary

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While putative zinc protease genes have been identified in annotated sequenced genomes for many bacteria, no biochemical data have been presented to confirm that the gene product possesses protease activity. In this study, the gene encoding the putative novel zinc protease (GeneBank ID: AE008917.1) from *Brucella melitensis* 16M was cloned by direct PCR amplification from genomic DNA. Analysis of the deduced amino acid sequence encoded by an ORF of 927 bps revealed that this protease is a zinc metalloprotease belonging to Gluzincins family. Expression was achieved in *Escherichia coli* and the results demonstrated growth arrest and filamentation of *E. coli* cells as well as induced bacterial cell death. Proteolytic activity on skim milk and protease inhibitors effect assays confirmed that this putative protein is a member of the metalloprotease.

Key words: *B. melitensis,* cell death, cloning, protein expression, proteolytic activity, zinc protease

INTRODUCTION

Brucella spp. are Gram-negative, facultative intracellular bacterial pathogens that cause brucellosis, an infectious disease affecting animals and humans, inducing abortion and sterility in domestic mammals as well as chronic infections in humans known as Malta fever (Spink, 1956; Ficht, 2003).

The genome of *B. melitensis* strain 16M was found to have two circular chromosomes in which 3,294,931 bp were distributed. Of those two chromosomes, 3198 ORFs (open reading frames) were predicted (Halling *et al*., 2005). It was also discovered that on both chromosomes, there resided genes that encoded for DNA replication, protein synthesis, core me-

tabolism and cell-wall biosynthesis (all of which were considered "housekeeping genes") (DelVecchio *et al*., 2002).

Brucella functional genes have been established by phenotypic or biochemical analysis for only a minority (around 200) of the 3198 predicted genes. Screens for transpositional mutants attenuated in infection models yielded 184 mutants (Delrue *et al*., 2004), suggesting that these genes have a function in the infection process. Moreover, 688 genes corresponding to what appears in GenBank as hypothetical proteins, and most of them are conserved in at least one other α-Proteobacteria (Dricot *et al*., 2004). Most of these putative genes were identified by comparison with other pathogens and were classified according to their putative biological roles and molecular functions, but they have not been cloned or expressed *in vivo* like: *dsbA*, *pheB*, *vsrb* and *zinc protease* (Delrue *et al*., 2004).

Bacterial proteases, particularly those produced by pathogens act as toxic factors to their host (Miyoshi & Shinoda, 2000) and have been implicated in virulence and pathogenicity. Many of these bacterial proteases are zinc-containing metalloproteases (Hase & Finkelstein, 1993). Metalloproteases are widely distributed in nature and belong to four superfamilies or clans. All bacterial zinc metalloproteases belong to the zincins superfamily, which contains a conserved HEXXH motif. The two histidines are zinc ligands, and the glutamic acid is involved in enzymatic activity. The zincins can be subdivided, based on the position of the third zinc ligand (Miyoshi & Shinoda, 2000), into at least ten families, with three of the families containing bacterial metalloproteases: thermolysin, serralysin, and neurotoxins. Various human pathogenic microorganisms produce zinc metalloproteases which play an important role in pathogenesis (Miyoshi & Shinoda, 2000) such as *Clostridium* neurotoxins (Oguma *et al*., 1995), *Bacteroides fragilis* enterotoxin (Moncrief *et al*., 1995) and *Bacillus anthracis* lethal factor (Klimpel *et al*., 1994).

In this paper, we report the sequence analysis of a novel putative zinc metalloprotease from *B. melitensis* 16M, the cloning and expression of this protease in *E. coli* host strains. Unexpected death of the *E. coli* cells during expression encouraged us to check the activity of this protease and investigate the effect of various IPTG concentrations and protease inhibitors on the production and activity.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

B. melitensis 16M was provided from Namur University – Belgium. *E. coli* BL21 (DE3) (Novagen, Germany) and BL21 (DE3) plysS (Stratagene, USA) were used as expression hosts. Plasmid pET-15b (Novagen, Germany) was used as a cloning and expression vector and pET-XYL1 vector (pET-15b plasmid containing xyl1 gene of *Scytalidium acidophilum*) was provided by Al Balaa *et al.* (2006), and was used as a positive control. All *E. coli* strains were grown at 37 ^{o}C in Luria-Bertani broth (LB) (Bio Basic, Canada] or LB plates solidified with 1.5% agar. When necessary, ampicillin (Applichem, Germany) was added at the concentration of 100 µg/mL. *B. melitensis* 16M was grown at 37 ^{o}C in *Brucella* broth (Acumedia, USA)

Preparation of DNA template and PCR

Genomic DNA of *B. melitensis* 16M were extracted by Wizard Genomic DNA Puri-

fication Kit (Promega, USA) A pair of specific primers Zp-PF: 5'-ATA TAT CAT ATG CGC TGG CAA GGC CGT-3' and Zp-PR: 5'-ATA TAT GGA TCC CTA AAC ATC GCC GC-3' were designed based on the sequence of putative zinc protease gene from *B. melitensis* 16M in GenBank (accession no. AE008917.1). The whole sequence of putative zinc protease gene was amplified by direct PCR using these primers which created the underlined unique *NdeI* and *BamHI* restriction sites respectively at each end of the amplified DNA fragment. The total volume per PCR was 25 µL containing 25 pmol of each of the two primers, 500 ng of extracted *B. melitensis* 16M genomic DNA, 1× HotStar HiFidelity PCR buffer and 1.25U HotStar HiFidelity polymerase (Qiagen, Germany). The cycling conditions were; one cycle of 95 ^{o}C for 4 min; 35 cycles of (95 ^{o}C for 60 s; 63 ^{o}C for 60 s; 72 ^{o}C for 60 s), then one cycle at 72 ^{o}C for 10 min. The PCR-generated DNA fragment was digested with *NdeI* and *BamHI* (Fermentas, Lithuania), and then ligated into pET-15b cleaved with *NdeI* and *BamHI*. The ligated plasmid was then used to transform *E. coli* BL 21 (DE3) and BL 21 (DE3) plysS. After ampicillin selection, several clones were picked up, the constructs were confirmed by sequence analysis and the constructed plasmid was designated as pET-Zp. Meanwhile pET-15b vector and pET-XYL1 were transformed and used as negative and positive controls, respectively.

DNA sequencing and computer analysis

DNA sequencing for pET-Zp construct was performed on an ABI 310 Genetic Analyzer using T7 universal primers of pET-15b plasmid to verify that zinc protease was indeed cloned into the pET-15b vector. Computer analysis of the complete nucleotide sequence and the deduced amino acids were done at the National Center for Biotechnology Information (NCBI) using the GenBank databases and the BLAST program for homology searches.

Expression of zinc protease in E. coli

Cultures of *E. coli* BL 21 (DE3) and BL 21 (DE3) plysS containing pET-15b, pET-XYL1 and pET-Zp were grown in 5 mL LB/ampicillin overnight, and 500 µL of the overnight cultures were used to inoculate 50 mL of LB/ampicillin. All cultures were grown in a shaking incubator at 37 ºC until optical density at 600 nm (OD_{600}) of approximately 0.5 was obtained. Protein expression was induced by isopropyl b-D-1-thiogalactopyranoside (IPTG) as an inducer. Various concentration of IPTG between 0.005 and 1 mM was used. The cultures were grown for an additional 4 h and OD_{600} was measured using spectrophotometer every hour. A mean value for each test was obtained by averaging the triplicate values.

Detection of protease activity on skim milk agar

Protease activity was determined using skim milk as substrate. Mid-exponential phase cultures were normalised to the 0.5 OD_{600} and spotted on LB 1% skim milk agar. The plates were incubated at 37 °C for 24 h and examined for clear zones surrounding the colonies.

Light microscopic observation

Growing cells were taken before and after 2 h of IPTG induction. After harvesting the cells by centrifugation, the supernatant was removed and cells were carefully resuspended in PBS buffer. Following this step, the cells were stained with Gram

stain and examined under Olympus DP70 light microscope.

Cell growth in the presence of protease inhibitors

The *E. coli* BL21 (DE3) containing pET-15b was cultured in LB broth containing 100 µg/mL ampicillin and 0.5 mM IPTG to induce protease expression. The following inhibitors were tested for their ability to inhibit protease activity through expression; 1mM EDTA (ethylene-diaminetetra-acetic acid) which used as metalloprotease inhibitor (positive inhibitor) and 100 mM PMSF (phenylmethyl-sulphyonyl) used as serine protease inhibitor (negative inhibitor). These inhibitors were added one hour after IPTG induction, and cell growth was monitored by OD_{600} (a mean value for each test was obtained by averaging the triplicate values).

RESULTS

Cloning and sequence analysis of zinc protease gene

Specific primers were designed for amplification of the complete ORF of zinc protease gene from extracted genomic DNA of *B. melitensis*. The PCR product of 927 bp was ligated into *NdeI-BamHI* cut expression pET-15b vector. The resultant recombinant plasmid was named as pET-Zp and introduced into *E. coli* BL 21 (DE3) and BL 21 (DE3) plysS strains. The plasmid DNA was extracted and confirmed by PCR amplification using the designed primers and by digestion with *BamHI* and *NdeI* restriction enzymes. The reaction products were examined on agarose gel.

The nucleotide sequence of the above-mentioned fragment (927 bp) was analyzed and revealed 100% homology between this gene and standard zinc protease gene outlined in GenBank with accession number AE008917.1. Analysis of the sequence revealed a complete ORF extending from an ATG codon to a TAG stop codon, which encodes a protein with 308 amino acid residues (Fig. 1).

Expression analysis of zinc protease in E. coli

- Zinc protease expressed in *E. coli* induces bacterial death

The *E. coli* strains were transformed with the plasmids pET-15b, pET-XYL1 and pET-Zp. A single colony of each plasmid was inoculated in LB medium at 37 °C and 0.5 mM IPTG was added to induce protein expression by the transformed plasmids. The bacterial cells containing pET-15b and pET-XYL1 grew well, while the bacterial cells expressing zinc protease ceased the growth as well as induced bacterial cell death, two hours after expression induction (Fig. 2).

- Expression of zinc protease induced bacterial cell morphology change

The morphological change of the *E. coli* BL21 (DE3) cells following zinc protease expression was examined using light microscope. The cells were filamentous as compared with the control cells that carried the pET-15b vector alone. At the beginning of expression, the cells exhibited the typical spherical shape of *E. coli* (Fig. 3B). Three hours after 0.5 mM IPTG induction expression, the cells morphology changed, such as unusually elongated shape (Fig. 3B), and eventually lead to cell death 4 h after induction. In contrast, the *E. coli* cells containing the pET-15b vector alone showed normal morphology (Fig. 3A).

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         M  R  W  Q  G  R  R  Q  S  D  L  V  E  D  L  R  G  E  Q  G
  1   ATGCGCTGGCAAGGCGTAGGCAAAGCGACAATGTTGAAGATCTGCGCGGTGAGCAGGGT
         G  V  V  G  G  G  F  G  R  G  G  G  F  G  G  P  G  F  R  I
 61   GGTGTGGTTGGCGGCGGCTTCGGCCGCGGGGGTGGTTTCGGCGGCCCCGGCTTCAGGAT
         V  R  G  G  G  I  S  G  I  L  I  L  V  V  M  F  F  V  L  R
121   GTGCGCGGCGGTGGAATTTCCGGCATTCTCATTCTCGTCGTCATGTTCTTCGTGTTGCGC
         A  V  G  I  D  P  M  P  I  L  F  G  D  G  S  M  S  P  T  V
181   GCGGTTGGCATCGATCCGATGCCCATCCTCTTTGGCGACGGCAGCATGAGCCGAACCGTG
         Q  T  Q  G  T  G  R  T  V  A  E  G  G  T  V  A  N  D  E  T
241   CAGACGCAAGGCACGGGCCGCACGGTGGCTGAAGGCGGCACTGTCGCCAATGACGAGA
       T  Q  F  A  R  T  V  L  A  E  T  E  D  V  W  S  G  I  F  Q
301   ACGCAGTTCGCCCGCACCGTTCTGGCCGAGACAGAAGATGTCTGGAGCGGTATTTTCCAG
         S  R  G  Q  T  Y  T  P  P  T  M  V  L  F  S  D  Q  V  R  S
361   TCGCGCGGCCAGACCTATACGCCGCCGACGATGGTGCTGTTTTCCGATCAGGTCCGTTCG
         A  C  G  Y  A  S  A  A  S  G  P  F  Y  C  P  G  D  R  K  L
421   GCCTGCGGCTATGCTTCGGCTGCTTCCGGTCCGTTCTACTGTCCCGGCGACCGTAAGCTT
         Y  I  D  L  S  F  Y  K  E  L  A  N  R  F  G  A  S  G  D  F
481   TATATCGACCTCAGCTTCTACAAGGAGCTTGCCAATCGCTTTGGCGCTTCGGGCGATTTC
         A  Q  A  Y  V  L  A [H  E  V  G  H] H  V  Q  N  L  L  G  I
541   GCGCAGGCCTATGTTCTGGCGCATGAAGTGGGCCACCATGTCCAGAATCTTCTGGGCATC
         L  P  K  F  N  Q  M  R  Q  Q  M  S [E] A  Q  A  N  Q  T  S
601   CTGCCGAAATTCAACCAGATGCGCCAGCAGATGAGCGAGGCGCAGGCCAACCAGACGTC
         V  R  V  E  L  Q  A  D  C  L  A  G  V  W  G  H  Y  T  D  Q
661   GTGCGCGTCGAGCTTCAGGCCGATTGCCTTGCGGGCGTATGGGGCCACTACACAGACCA
         K  G  I  L  E  A  G  D  L  E  E  A  L  W  A  A  H  Q  I  G
721   AAGGGCATTCTGGAAGCGGGCGACCTTGAGGAAGCCTTGAACGCGGCGCATCAGATCGG
         D  D  T  L  Q  R  R  S  Q  G  Y  V  V  P  E  S  F  N  H  G
781   GATGACACATTACAGCGTCGCAGCCAGGGCTATGTCGTACCGGAAAGCTTCAACCATGGC
         T  S  A  Q  R  A  K  W  F  Q  R  G  F  D  S  G  Q  L  G  S
841   ACCTCCGCGCAACGCGCCAAGTGGTTCCAGCGTGGGTTCGATAGCGGTCAGCTTGGATC
         C  D  T  F  S  G  D  V  *
901   TGTGACACGTTCAGCGGCGATGTTTAG
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Fig. 1. Nucleotide and amino acid sequences of Zp. Arrows indicate the location of PCR primers (F5' and R3') used to the amplification of full-length Zp and construct the Zp expression vector. Translation initiation codon ATG is shown at the start of the sequence. Asterisk indicates a stop codon. The deduced amino acid sequence is given above the DNA sequence and the conserved zinc motif HEXXH, 21aa , E shared by metalloproteases of the Gluzincins family is boxed.

Detection of protease activity on milk agar

Zinc protease possessed proteolytic activity as predicted from the amino acid sequence, *E. coli* BL21 (DE3) was transformed with a pET-Zp plasmid, and the proteolytic activity of the recombinant *E. coli* BL21 (DE3)/pET-Zp was detected on a 1% skim milk agar plate (SMA). *E. coli* cells harbouring pET-15b as a negative control did not show any hydrolysis of casein (Fig. 4).

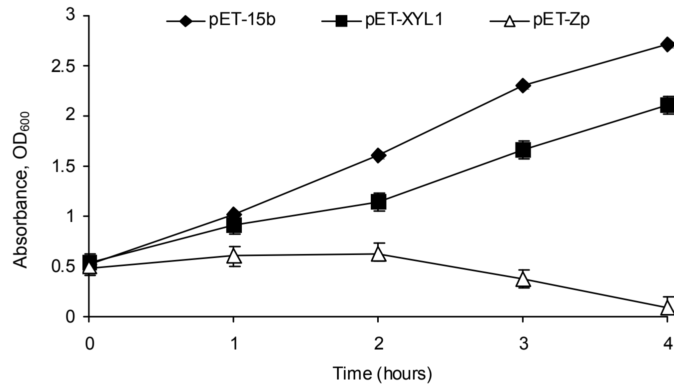

Fig. 2. *E. coli* cells containing pET-Zp lost viability 2 h after IPTG induction, while others (negative and positive controls) were still viable. Cell growth was monitored by OD_{600}.

Monitoring of E. coli BL21 cell growth using different amounts of IPTG

The effects of the inducer (IPTG) concentration on culture growth rate during expression of zinc protease recombinant protein were evaluated to determine if the expression of zinc protease was killing the bacteria. Each host strain culture was induced with five IPTG concentrations (0.005, 0.05, 0.1, 0.5 and 1 mM) at an OD_{600} of 0.5, and at temperature of 37 °C for 4 h. Bacterial growth was slightly inhibited when the final concentration of IPTG was at 0.005 mm and 0.05 mM, while the cell growth was ceased at 0.5 mM and 1.0 mM of IPTG concentration (Fig. 5).

Effect of protease inhibitors on cell growth of E. coli during expression

The effects of two inhibitors on the culture growth rate during expression of zinc metalloprotease were evaluated. Addition of protease inhibitors into culture medium after induction affects the level of protease produced by *E. coli* cells and reveals that the produced protease was insensitive to PMSF (negative inhibitor), but inhibited by EDTA (positive inhibitor) (Fig. 6).

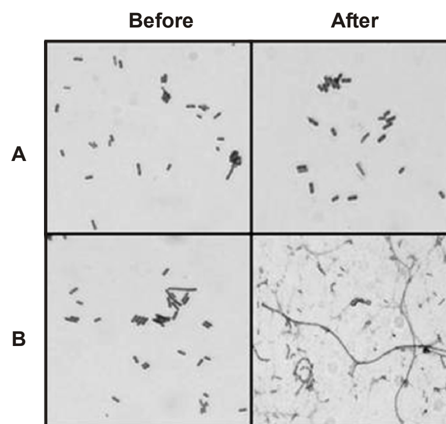

Fig. 3. Morphological change in *E. coli* BL21 induced by zinc protease. Expression of zinc protease was induced with 0.5 mM IPTG, samples were taken before induction and 3 h after induction. **A.** *E. coli* cells containing pET-15b vector. **B.** *E. coli* cells containing pET-Zp vector. The morphological change was monitored by light microscope (OLYMPUS DP 70).

Fig. 4. Proteolytic activity using skim milk agar plate. Cultures containing pET-15b and pET-Zp plasmids were placed on LB agar containing 1% skim milk and incubated at 37°C for 24 h. A clear zone around the *E. coli* BL21 (DE3)/pET-Zp colony was detected differently to *E. coli* BL21 (DE3) / pET-15b.

DISCUSSION

In this study, the nucleotide sequence encoding the putative zinc protease from *B. melitensis* strain 16M was identified by the cloning, sequencing and expression methods. The isolated gene consisted of 927 bp was cloned into pET-15b vector and the resultant plasmid was named as pET-Zp. Nucleotide sequence of the 927bp *NdeI-BamHI* fragment of pET-15b was determined by sequence analysis.

The gene that we designated as *Zp* encodes a novel unusual type of polytopic membrane protein. Classification analysis of the amino acid sequence deduced from this gene demonstrated that this protease is a zinc metalloprotease that contains a consensus metalloprotease metal binding site of the HEXXH type (Rawlings & Barrett, 1995). Sequence analysis of *Zp* predicted that it encoded a 308 amino acid polypeptide with an active site (HEVGH). BLASTing, the protein sequence deduced from ORF zinc protease against the NCBI databases predicted the protease to be a

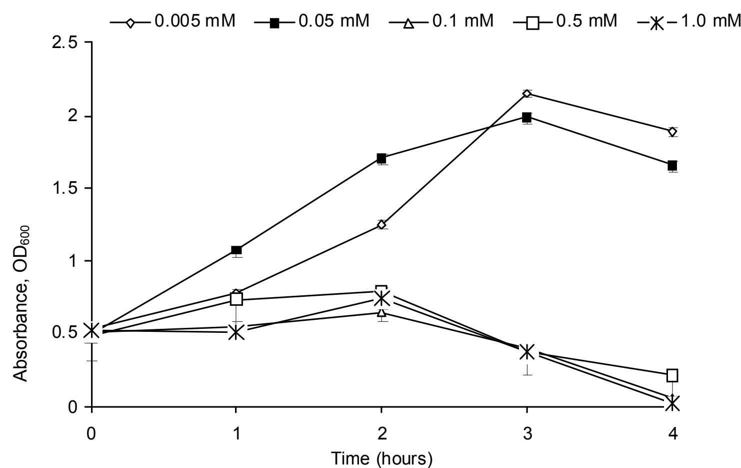

Fig. 5. *E. coli* strains containing zinc protease were grown in LB broth and expression of zinc protease was induced by adding the indicated amounts of IPTG. When the IPTG was at 0.005 mM and 0.05 mM, bacterial growth was slightly inhibited after 3 h, while other IPTG concentrations stopped bacterial growth within 2 h of induction.

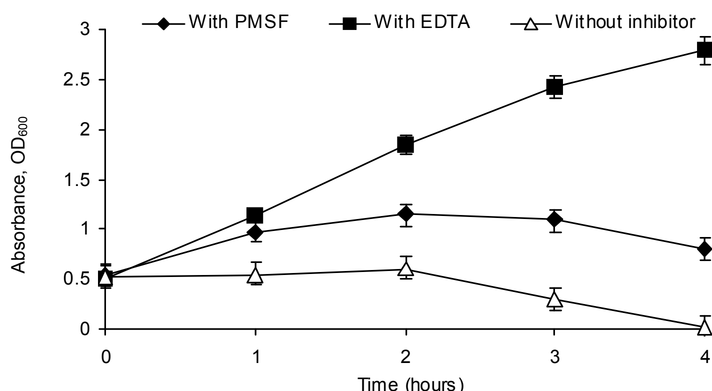

Fig. 6. Effect of protease inhibitors on the cell growth of *E. coli* BL21 (DE3) was determined by measuring the cell growth rate after induction. Addition of PMSF to culture medium showed a decrease of bacterial growth rate in contrast to EDTA which showed an increase of bacterial growth rate in the culture media.

member of the zinc proteases as it includes a zinc metalloprotease HEXXH-E consensus motif (HEVGH, 21aa, E) between amino acids 188 and 213 (Fig. 2). It is noted that three active site residues are required to co-ordinate a zinc cation (Vallee & Auld, 1990a). The histidine residues of the HEXXH motif function as the first and second zinc ligands whilst the location of the third zinc ligand, which is usually a glutamic residue (E), can vary (Vallee & Auld, 1990b). Based on the location of the third zinc ligand, bacterial zinc metalloproteases with the HEXXH sequence can be subdivided into three families (Miyoshi & Shinoda, 2000). We found the potential third zinc ligand of Zp was located 21 bases downstream from the second histidine of the zinc binding motif (HEXXH, 21aa, E). Therefore, Zp likely belongs to the Gluzincins family of zinc metalloproteases (Vallee & Auld, 1990b). A list of HEXXH metalloproteases compiled by Rawlings & Barrett (1995) includes metalloprotease families and the families were grouped in clans.

Sequence analysis suggested that our zinc protease belongs to the metalloprotease clan MA which is characterized by the motif HEXXH+E that contains a downstream glutamic acid (E ~ 20 residues later) residue that completes the metal-binding site. This site has been defined more extensively as *abx*HE*bb*H*bc* in which *a* is most commonly valine or threonine; *b* is an uncharged residue; **c** is hydrophobic; and *x* can be any amino acid except proline (Rawlings & Barrett, 1995). The corresponding sequence in zinc protease protein is *VLA*HE*VG*H*HV*, which matches the extended consensus precisely with exception of the *b* residue which corresponded to an uncharged residue (Fig. 2). Nonetheless, given the enormous variability in the sequence of metalloproteases, it is not difficult to imagine change residues in *b* position, especially since zinc protease is postulated to be a novel hydrophobic metalloprotease (Rawson *et al.*, 1997). However, to acknowledge, *in silico* approaches determine that our protease appears to be a hydrophobic

transmembrane protein [http://bioinf.cs.ucl.ac.uk/psipred]. Numerous studies demonstrated that the zinc-binding motif, HEXXH+E is known to be highly conserved in zinc-metalloproteases, one of these studies showed that this motif was present in the deduced amino acid sequence of Pap6 at positions 330–354 (Teo *et al*., 2003).

Sequence homology analysis reveals that zinc protease appears to be a novel enzyme displaying less than 85% identity with the metalloproteases of bacterial species. The best homologues are with putative zinc protease of *Ochrobactrum anthropi* (83%), *Mesorhizobium loti* (77%), *Rhizobium leguminosarum* (75%), *Agrobacterium sp.* (75%) and *Sinorhizobium meliloti* (74%).

The work presented in this paper demonstrates that the expression of the zinc protease from *B. melitensis* in *E. coli* had a detrimental effect on the host cells. Expression of this protease *in E. coli* BL21 (DE3) was lethal within 2 h of induction with 0.5 mM IPTG. The cells were filamentous as compared with the control cells that carried the pET-15b vector alone. SDS-PAGE analysis was unable to demonstrate the successful expression of zinc protease once stained with Coomassie blue, this may perhaps be due to protease undergoing internal auto-cleavage during expression because the expression level of zinc protease is low compared to that of control, which did not affect the growth of *E. coli*. Otherwise, various metalloproteases have been successfully expressed in *E. coli* which did not undergo death during expression (Teo *et al*., 2003; Zhang *et al*., 2007). *Bacillus cereus* zinc metalloprotease gene (*BCcalY)* was cloned and expressed. The (*BCcalY)* gene contains an ORF of 794 bp encoding a polypeptide of 265 amino acids. Plasmid pET-100D harbouring the complete *BCcalY* gene was transformed into *E. coli* BL21 star ^{TM} (DE3) plysS to express the recombinant enzyme. The transformant *E. coli* was aerobically cultured in LB broth containing ampicillin (50 µg/mL) at 37 °C for 4 h. SDS-PAGE was performed and there are no noticeable differences apparent upon staining with Coomassie blue (Myers, 2005).

The function of this putative protease was confirmed by proteolytic activity on 1% skim milk agar plate. Casein is a large insoluble protein which exists in milk and gives milk its characteristic white, opaque appearance. *E. coli* BL21 (DE3)/pET-Zp showed a clear halo zone indicating that the protease hydrolyzed casein, whereas *E. coli* BL21 (DE3)/pET did not show any hydrolysis.

No expression on LB broth medium was observed using SDS-page gel, despite the activity of our zinc protease that was determined on a solid medium in the presence of casein as a substrate. Therefore, the effects of different IPTG concentrations on *E. coli* cell growth during expression were evaluated. Cell growth was inhibited according to the amount of IPTG added. When the IPTG was at 0.005 mM and 0.05 mM, bacterial growth was slightly inhibited after 3 h, while the addition of 0.5 mM and 1.0 mM of IPTG caused bacterial growth that ceased 2 h after induction. Almost all *E. coli* cells containing pET-Zp lost viability after IPTG induction while others were still viable. Although we attempted expression in *E. coli* BL21 (DE3) plysS which provides tighter control for expression of toxic proteins, we got the same results. Therefore, we can consider this protease as a toxic protein because high levels lead to cell death as measured by decreased

optical density and colony-forming ability (Fozo *et al*., 2008).

Furthermore, enzyme inhibitor analysis showed that this enzyme is most likely a zinc metalloprotease as it was inhibited by the zinc-specific metal chelators such as EDTA and was unaffected by a serine protease inhibitor PMSF. We have shown the effects of these inhibitors on the *E. coli* growth cells rate during zinc protease expression. *E. coli* cells that express zinc protease in the presence of EDTA were grown satisfactorily because this inhibitor is classified as a general metalloprotease inhibitor and its chelation of the metal ion required catalytic activity (Auld, 1995). So it affected zinc protease activity and the bacterial cells did not die, and its growth rate was very similar to that seen with *E. coli* cells containing pET-15b (Fig. 2), indicating completely zinc protease inhibition. The addition of PMSF (negative control) (Syngkon *et al*., 2010) showed decrease of bacterial growth rate in contrast to EDTA 2 h after expression induction. Even using EDTA during expression, the protein was not detected by Coomassie blue staining. This result supported the suggestion that EDTA effectively inhibits zinc protease activity against a foreign substrate, without affecting internal auto-cleavage (Bowen *et al*., 2003). On the other hand, few studies have shown that expression of metalloprotease can induce bacterial cell death or toxicity, and these proteases are generally toxic to the cell and thus difficult to express and purify (Siddiqui *et al*., 2007).

Bacteria are commonly used for the production of heterologous proteins, and when proteins are greatly overexpressed the bacteria could stop growing and die (Dong *et al*. 1995). This is thought to be due to accumulation of gratuitous proteins and starvation response. Consequently, the starvation response continues until the bacteria commit suicide by, among other things, destroying their ribosomes (Kurland & Dong, 1996). This type of death perhaps does not appear to be the mechanism responsible for zinc protease induced bacterial death, because our positive control pET-XYL1 was still viable after induction in the same condition, so we suppose that this might be due to the expression of zinc protease.

On the other hand, over the last few years it has been realised that lower organisms can undergo programmed cell death that is similar, if not identical, to apoptosis (Ameisen, 1996). Apoptosis, or programmed cell death, is an important mechanism for multicellular organisms to remove unwanted or dangerous cells (Raff, 1992). Experiments have shown that proteases play a significant role in activation of apoptosis. The mechanisms of this activation, in particular, of endonucleases are still unknown. As it has been shown in many models of apoptosis, a proteolytic step is required prior to DNA cleavage by endonucleases (Squier *et al*., 1994). Therefore, proteases seem to play a principal role in initiating the programmed cell death. Apoptosis may be induced by exogenous proteases injected into a cell (external activation pathway) or triggered upon activation of intracellular proteases by certain stimuli (internal activation pathway) (Sukharev *et al*., 1997). Our data demonstrated that the expression of this protease was the reason for the death and morphological changes of *E. coli* cells by monitoring the effects of various IPTG concentrations and protease inhibitors on zinc protease production and activity.

Microscopy of cultures during protease expression showed that *E. coli* cells containing pET-Zp vector exhibited filamentous morphology and under the light

microscope the induced bacteria looked like 'snakes'. Filamentation is a common response in which bacteria replicate but incompletely divide, leading to long slender chains that resemble fungal hyphae (Sutton *et al*., 2011). This phenomenon has been implicated in bacterial survival during exposure to various stresses (Justice *et al*., 2008). FtsZ protein is an essential protein for cell division and in most strains is solely responsible for the filamentation that occurs as part of the SOS response inhibiting cell division (Rothfield & Garcia-Lara, 1996). Since expression of MsFtsH protein (zinc metalloprotease of *Mycobacterium smegmatis*) in *E. coli* was toxic and resulted in growth arrest and filamentation of cells (Anilkumar *et al*., 2004), then we suggest that our zinc protease targeted this essential protein (FtsZ) causing cell filamentation, then cell death in *E. coli.*

CONCLUSION

In conclusion, we have cloned and expressed a novel putative zinc metalloprotease from *B. melitensis* 16M in *E. coli* cells host strains using standard expression vector. Sequence analysis revealed that this protease belongs to metalloprotease class. Unexpected death of the cells and changing of the morphological shape during expression impelled us to identify the proteolytic activity and evaluate the effects of various IPTG concentrations and protease inhibitors on production and activity of this protease. Proteolytic activity on skim milk by formation a clear halo around the colonies, and protease inhibitors effect, confirmed that this putative protein belongs to metalloprotease class.

The cloning of putative zinc protease gene from *B. melitensis* and the expression in *E. coli* in view to identify its encoded enzyme reveals the presence of this gene and enzyme activity in *Brucella*. Proteases play an important role in a variety of biological processes ranging from digestion and metabolism to hormone activation and apoptosis. Furthermore, various non-human proteases facilitate important cellular processing from many human pathogens and thus, proteases are often important drug targets. The understanding of the roles played by proteases and their inhibitors might provide insight into the pathogenesis of these conditions and suggest novel therapeutic strategies.

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