CLONING LasB GENE OF Pseudomonas aeruginosa EIASTE 10104-2AI IN E. Coli BL21 AND E. Coli DH5α AND INVESTIGATED THEIR EFFECT ON THE STRIPPING OF VERO CELLS

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ABSTRACT

Pseudomonas aeruginosa is a causative agent of various infectious diseases throughout the world. Our study aimed to determine and amplify LasB gene that encode to Metalloprotease (elastase) virulent factor by PCR and cloning into pGEX KG plasmid then expression in E. coli, also aimed to investigate the effect of P. aeruginosa supernatant that contains crude elastase on monolayer Vero cell line. This bacterium is capable of secreting Metalloprotease (elastase) with specific activity 191 unit /mg and decreased to 78 unit / mg after adding casein as an alternative to trypoton as nitrogen source in liquid production broth. The recombinant competent E. coli BL21 which contains pGEX KG-LasB was none inducible by IPTG and failed to express elastase. Also, taking into consideration all the steps that would make the success of the expression like changing the expression vector.

Keywords: Pseudomonas aeruginosa 10104-2ai, recombinant pGEX KG-LasB, metalloprotease (elastase), E. Coli DH5α, monolayer of Vero cells.

INTRODUCTION

Pseudomonas aeruginosa is an important gram negative, non-spore forming, motile and has rod shape, this bacterium found in a broad sort of habitats such as water or soil etc. (Lambert, 2002). It’s life-threatening as opportunistic pathogen causing a wide variety of serious infections include burn and wounds infections, urinary tract infections, skin and soft tissue infections, bacteremia and pneumonia (Gellatly and Hancock, 2001; Benie et al., 1999). LasB gene is one of major component of extracellular matrix in cancer cells, this work aimed to produce elastase of P. aeruginosa, by recombinant way and study their cytotoxicity effect on Vero cell line in vitro as a replacement strategy for clinical various medication to suppress tumor cell growth.

MATERIALS AND METHODS

Bacterial Strain: Two isolates belong to P. aeruginosa, were obtained from China Gene Bank as ampoule with code number 10105-4 and 10104-2ai, it has been cultured on nutrient agar (Oxoid) plate and activated in brain heart infusion broth (Oxoid) then incubated for 24-48 hrs. at 37°C in shaker incubator (HZQ). Bacterial strain was maintained on nutrient agar slant for 4-5 weeks and in LB broth supplemented with 20-25% glycerol (Shanghai chemical) at -70°C for long time preservation.

Metalloprotease assay: A single bacterial colony was inoculated in 10 ml of LB broth and incubated overnight at 37°C, 20μl of supernatant was transferred by micropipette to wells in skimmed milk
agar plate and incubated at 37°C for 24 hrs. when appearance of clear zone around the wells that indicator for protease production. In another step, 1 ml from the above incubated overnight culture was inoculated in 1000 ml of LB liquid and incubated for 24-48 hrs at 37°C with shaking at 180rpm. The crude protease was extracted from the bacterial culture by cooling centrifugation at 4°C with 10000g for 30 min. According to (Kunitz, 1947) method, the protease activity was assayed with some alterations by (Nakanishi et al., 1974), it briefly, 0.2 ml of crude enzyme solution was mixed well with 1.1 ml of (0.5%) casein (Oxoid) and then incubated in water bath at 37°C for 30 min. To stop this reaction 2.5 ml of 5% TCA (Trichloroacetic acid) that supply by (Amresco) was added and incubated again for 30 min at 4°C in refrigerator (Haier) or in ice water bath then centrifuged with cooling (Hitachi) at 10000g for 15 minutes. The blank solution was prepared by adding TCA before adding crude enzyme solution. The Standard curve of tyrosine was prepared with serial concentrations 10 to 100 micromole from stock tyrosine solution (Sigma) and incubated for 2-3 times, and then mixed well; the mixture incubated at 37°C for 30-60 min in water bath and then added 20 µl of proteinase K (20 mg/ml) and mixed well by vortex for 2-3 min and then the mixture was re-incubated at 65°C for 2 hrs in water bath, the cell debris was removed by centrifugation at 12000g. Finally, the pellet was removed but the supernatant contain DNA was kept in -20°C until used in PCR reaction. The purity and concentration of the extract DNA were determined by spectrophotometer at 260 and 280 nm respectively.

Effect of metalloprotease on Vero cells line: The Vero cells were obtained from the State Key Laboratory of Agricultural Microbiology of Huazhong Agricultural University-Wuhan-China. The cells were maintained according to (Balamurugan et al., 2006) briefly, in tissue culture flasks with DMEM (Dissolve 133.7 g of Dulbecco’s Modified Eagle Medium (GIBCO) in 800 ml of D.W. and dissolved by mixing; 3.7 g of NaHCO3 (Shanghai chemical) was added to adjust the pH to range 7.2-7.5. The volume was completed to 1000 ml by D.W and sterilized through filtration (0.2 mm membrane) and distributed in to small flasks). After the cell layer grown, it was washed gently with PBS for 1-3 times, and 0.25% of Trypsin-EDTA solution was added then incubated for 6-10 minutes at 36°C for trypsinization. The detached cells were suspended well by shaken gently and re-suspend the cells in 20 ml of DMEM supplemented with 10% fetal calf serum, 0.75 mM L-glutamine, 40 µg/ml gentamycin and 1 µg/ml amphotericin B (Oxoid) were added, then distributed in 96- well micro titer plate. After incubation at 37 °C in 5% CO2 incubator for 72 hrs., the old solution medium was replaced with 180 µl of new DMEM and then added 20 µl of crude metalloprotease to each well and re-incubated at 37 °C in 5% CO2 incubator for 24-96 hrs. The inverted microscope was used to determine any changes may occurred by protease compared with the control.

DNA extraction: Bacterial DNA was extracted by method described by (AL-Rubaii, 2009). Briefly, single bacterial colony was inoculated in 2 ml of LB broth and grown over night. Bacterial cells were collected by centrifugation at 12000g for 5 min at room temperature. The supernatant was turn off and pellet was washed with 1 ml of TNE- buffer by repeated pipetting 2-3 times and re-collect by centrifugation at 10000g for 3-5 minutes and decant the supernatant; The centrifuged tube contains pellet was pipetting gently with 300 µl of TNE-buffer supplemented with 2% Triton X-100 and added 30 µl of lysozyme (prepared immediately at 5 mg/ml) and mixed well. The mixture incubated at 37°C for 30-60 min in water bath and then added 20 µl of proteinase K (20 mg/ml) and mixed well by vortex for 2-3 min and then the mixture was re-incubated at 65°C for 2 hrs in water bath, the cell debris was removed by centrifugation at 12000g. Finally, the pellet was removed but the supernatant contain DNA was kept in -20°C until used in PCR reaction. The purity and concentration of the extract DNA were determined by spectrophotometer at 260 and 280 nm respectively.

Synthesis and amplification of lasB gene by PCR: Primers for lasB gene responsible for elastase expression was designed on the basis of whole sequence according to NCBI reference sequence under code NC_002516.2 from base 4168762 to 4170706. The suitable primers were designed in order to be constructed with (5Kb) pGEX-KG vector (obtained from the State Key Laboratory of Agricultural Microbiology of Huazhong Agricultural University, Wuhan, China). The 29 oligonucleotides forward primer was computerize designed by software based on start codon for ATG with BamHI restriction site 5’-CG GGA TCC ATG AAG AAG GTT TCT AGC CCTT-3’, while the 28 oligonucleotides of revers primer was 5’-A CGC AAG CTT TTA CAA CGG GCT CGG GCA-3’, it was designed based on terminal site TAA with restriction Hind III; the expected size of amplicon was 1497 bp. The lasB gene was amplified using PCR technique for (35 cycles) includes pre-denaturation at 95°C for 5 min, denaturing at 95°C for 30 s, annealing at 60°C for 1 min and extension at 72 °C for 1 min. The
PCR products was applied to electrophoresis in a 0.8% agarose gel, stained with ethidium bromide and band was observed under ultraviolet light eliminator in a gel documentation system. T4 DNA ligase was used to ligation between amplified gene and pGEX-KG vector which was already restricted by BamHI and Hind III restriction enzymes (Takara). The recombinant plasmid was sub-cloned into E. coli DH5α and extracted after cultured in LB broth supplemented with 100 µg/ml ampicillin for 18 hrs at 37°C. To confirm the cloning, the constructed pGEX KG-lasB vector was re-digested with same endonucleases at 37°C for 4 hrs and observed double restriction digestion by agarose electrophoresis. **Expression of lasB gene in E. coli:** To express the elastase enzyme that encoded by lasB gene, the recombinant expression vector pGEX KG-lasB was transformed in to competent E. coli BL21 ED3 by the chemical CaCl2 method. Positive clones were identified by colony PCR and double restriction digestion. Detailed protocols according to (Sambrook and Russell, 2001). The transformed E. coli BL21 ED3 and E. coli DH5α harboring pGEX KG-lasB were cultured in 2 ml LB broth containing 100 µg/ml ampicillin and incubated at 37°C overnight with vigorous shaking at 200-250 rpm and then sub-cultured 1 ml inoculums to 100 ml fresh LB broth containing the same concentration of ampicillin and re-incubated at 37°C for 2 hrs with shaking. The expression was induced by adding 1 µ/l/ml of (1 mM of IPTG). Cells were harvested at 1, 2, 4, and 6 hrs by cooling micro-centrifuge at 10000 g for 30-60 seconds. The pellets were re-suspended in 250 µl of 2X Laemml protein sample buffer and mixed well by vortex and then heated at 100°C for 5 minutes and allowed to cool at room temperature. The supernatant was transferred to a fresh labeled tube and stored in freezer at -20°C until analyzed by SDS-PAGE. The negative control was prepared by the same method using cells but without IPTG induction.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE):** The molecular weight of recombinant elastase band in SDS-PAGE (12% polyacrylamide) was determined approximately according to (Garfin, 1990) method with some modification; briefly, 150 µl of sample (elastase) was mixed well with 25µl of loading buffer, the mixture was heated in boiling water bath for 10 minutes and then cooled. The sample and control were loaded in each lane of the gel. The gel was run for 3-4 hrs. After electrophoresis stopped, the gel was stained with staining buffer containing Coomassie Brilliant Blue R-250- Acetic acid iso-propyl alcohol for 4 hrs with shaking, and then washed with De-staining solution containing acetic acid-methanol-water (10:25: 65, v/v/v) respectively for at least 3-4 hrs. with slow shaking. The destaining solution was changed many times until clear bands appear. Finally, the gel was fixed in 7% acetic acid. The Western blotting was carried out according to (Sambrook and Russell, 2001) and the expression of the protein was detected by using 6His Tag Rabbit Polyclonal antibody (Thermo, USA).

**RESULTS**

The two isolates of *P. aeruginosa* under code 10105-4 and 10104-2al when grown on skim milk agar, a clear zone around the colonies was observed with 0.75 and 2.5 mm respectively, its indicating protease production, the largest clear zone bacterium was selected to complete this study. The protease specific activity was 191 unit /mg when cultured in LB broth, but it was decreased to 78 unit/mg when casein was added instead of Trypton as nitrogen source. The elastase gene lasB of *P. aeruginosa* 10104-2al was amplified from the genomic DNA by PCR using designed forward and reverse primers which containing restriction sites for BamHI & Hind III, the product showed one band about 1500 bp in the electrophoresis gel (figure 1), and then inserted in double digested pGEX-KG by same digested enzyme, the new recombinant pGEX-KG-lasB expression vector showed one band about 7000 bp in electrophoresis (figure 2), the recombinant vector introduced into E. coli DH5α to confirm cloning and the results showed double bands, the heavy band represented linear pGEX-KG while light band is linear LasB gene (figure 3). To show the expression of LasB gene protein, the recombinant vector was transformed into both E. coli BL21 ED3 and E. coli DH5α and then allowed to grow on LB agar containing ampicillin, the positive growth cloned cells that resist ampicillin were selected and their recombinant plasmid size was showed about 7000 bp which indicate the correct gene was inserted. the induced recombinant E. coli BL21 containing pGEX-KG-lasB did not show response to expression (figure 4), while induced recombinant E. coli DH5α showed weak band in SDS-PAGE (figure 5), the target gene expression was still few until after 6 hrs compared with control, but the Western blotting results was negative. The nucleotide sequence of the lasB gene was appeared the presence ATG.
start codon and TAA stop codon in open reading frame of 1497 bp with G+C about (63.79%). It was shown 12 to 16 bp upstream from the start codon ATG represented putative ribosome binding or Shine-Dalgamo sequence. The bacterial supernatant that containing crude elastase was investigated against Vero cell line and the result was shown detaching, changing in morphology from spindle shaped to round and shrived cells observed, these changes were followed by gradual destruction and cytotoxic activity on monolayer cells then complete damaged after 96 hrs causing the death of the cell culture (figure 6).

Figure 1: Gel electrophoresis demonstrated amplification lasB gene by PCR. Lane M: DNA marker DL 2000bp, Lane1 & 2: represented amplified target gene.

Figure 2: Gel electrophoresis demonstrated recombinant expression vector pGEX-KG-LasB 7000bp. Lane M: DNA marker DL 15000bp. Lane1 & 2, repeated sample for ligation form of pGEX-KG 5000bp with target Las B gene 1497bp.

Figure 3: Confirm recombinant expression vector. Lane M1: DNA marker DL 2000 bp. Lane M2: DNA marker DL 15000 bp. Lane1 & 2: repeated sample for double digested of recombinant vector by BamHI and HindIII restriction enzymes, the supreme band 5000 bp is digested recombinant vector, lower band 1497 bp is target LasB gene. Lane 3: Control undigested vector pGEX-KG 5000 bp.

Figure 4: SDS-PAGE profile. Lane M: Standard molecular weight for proteins. Lane 1: induced E. coli BL21 (ED3) containing pGEX-KG vector without LasB gene, it was shown bold band represented Glutathione S-transferase (GST) that naturally protein that can be expressed in E. coli with full enzymatic activity (positive protein expression control). Lane2: lyses of induced E. coli BL21 (ED3) containing recombinant vector pGEX-KG-lasB.

Figure 5: SDS-PAGE analyses. Lane M: molecular weight of standard proteins. Lane 1,2: repeated sample for lyses E. coli DH5α that does not contain pGEX-KG expression vector, no bands shown (negative control of protein expression). Lane3: lyses E. coli DH5α that contain pGEX-KG expression vector but without induced by IPTG as control. Lane4: lyses of induced E. coli DH5α by IPTG containing pGEX-KG-lasB, slightly new band was shown.
DISCUSSION

In this study the extracellular product of P. aeruginosa isolates showed hydrolysis activity on skim milk agar that indicating metalloprotease (elastase) production, the LB broth that containing yeast extract and trypton was recommended to large scale production; It can be concluded that yeast extract and trypton may support the growth of bacteria and encourage protease activity combatively with casein as nitrogen source. Many articles reported that skim milk agar and LB broth contain an easily digestible protein were suitable for cultured, detection and production of extracellular elastase from different bacteria such as P. aeruginosa (Raftari et al., 2013) Aeromonas hydrophila (Cascón et al., 2000) and Serratia marcescens (Braunagel and Benedik, 1990). Among virulence factors that suggested for pathogenic P. aeruginosa to cause damage in infected organs is elastase. It is one of the most important factors which called pseudolysin or LasB protease (Matsumoto, 2004). Because much less is known about this important virulent factor ,we try to produce it by DNA recombinant method and reach to highest amount of the elastase in order to continue to know more about the physiological functions, biochemical characterization, role in pathogenesis action as well as the genetic and sequence analysis in addition to proposal medical application for this enzyme such as digestive aid and as anti-cancer inhibitor to suppress tumor cell growth, for that, the responsible gene Las B of P. aeruginosa 10104-2al was amplified by PCR technique. the PCR product was showed one band only (~ 1500 bp) in analyzed the electrophoresis gel after restricted by BamH I and Hind III (figure 1), The expression vector pGEX-KG 5k bp was digested with same restriction enzymes to obtained restricted plasmid and then combined with amplified target gene together by ligase enzyme to construct pGEX-KG (Las B) expression vector, the new pGEX-KG (Las B) showed excess in size ~7000 bp as one band in electrophoresis (Figure 2). The cloned Las B gene in pGEX-KG was confirmed by run in PCR again and restriction analysis, the PCR product of Las B gene from new constructed pGEX-KG (Las B) was showed one band with ~1500 bp. The restriction analysis to recombinant pGEX-KG (Las B) by BamHI and HindIII was showed two bands heavy band ~ 5000 bp and another light band ~ 1500 bp respectively in the electrophoresis gel (figure 3), these results indicate that the Las B gene (1497 bp) was cloned to express vector pGEX-KG 5000 bp successfully. Although the competent host E. coli DHα is used to increase the copies of expression vectors, it was used in this study along with another competent host E. coli BL21 DE3 for overexpression of recombinant proteins (elastase). Expression of inserted target gene is under the control of the tac promoter, which is induced by Isopropyl-beta-D-thiogalactopyranoside (IPTG). pGEX-KG vector has own lacIq gene (it was engineered) which encoded to specific repressor protein that able to bind with the operator region only of the tac promoter and lead to stop of expression until induction by IPTG, thus maintaining tight control over expression of the inserted foreigner gene. The new constructed expression vector pGEX-KG(LasB) was introduced to competent host cells E. coli DH5α by transformation way and
grown in LB agar supplemented with ampicillin; a few visible colonies resistant to ampicillin were observed, each colony represents a clone of transformed cells and indicated that carry target gene on the constricted vector. pGEX-KG(LasB) was introduced again to E. coli DHα and E. coli BL21 colonies and induced by IPTG for expression, but GST protein was fused with recombinant protein (elastase). GST is the gene encoded to glutathione S-transferase which act as tag promoter in expression pGEX-KG vector. GST gene using as marker for purification of the fusion protein, the result in this study was really surprising because did not appear elastase activity in both supernatant and lyses of induced E. coli BL21 DE3 cloned (figure 4), but in the supernatant of induced E. coli DHα a weak expression was observed until after 6 hrs of stimulation and detected as light band in SDS-PAGE with molecular weight about 50-55 KDa. To give suitable explain for this result it may attributed to an expression of a new protein 33 KDa fused with 26 KDa of GST protein (figure 5) but this expression gave negative determination by western blotting method due to decreased in concentration. The molecular weight of elastase was estimated about 33 kDa by (Kesse et al., 1992; Jose et al., 2017) while it’s about 34 KDa by (Draper, 2017). There is no available information about using pGEX-KG vector in cloning and expression of the Las B gene in to E. coli, but many articles reported used pET-32a, pET22b and pET2-8b system respectively for cloning the responsible genes Las B or Las A for elastase encoding (Raftari et al., 2013; Zhu et al., 2015; Draper, 2017). Thus, this study to enhancing the inappropriate pGEX-KG vectors for cloning Las B gene and may incomplete cleavage of GST protein by thrombin. Some authors have recognized in their reports to overlap many reasons in a few or no expressions includes: i) high number of rare codons in competent E. coli may include and cause inactivity for protein, these codons have been related with translation errors in E. coli such as arginine encode by CGA/AGG/CGG/AGA, while glycine encode by GGA, isoleucine encode by AUA but leucine and proline encode by CUA and CCC respectively. ii) Expression of toxic protein or after induction in competent host own pLysE or pLysS genes that tension regulation of expression systems using the T7 promoter. The lysozyme encoded by these genes, which binds with and inactivate function of T7 RNA polymerase and caused expression failure. (AL-Rubaii, 2009, Rosano, et al., 2014). iii) incorrect folding occur in recombinant target proteins due to contain disulfide bonds and may lead to inactivity. iii) drooping in the function of T7 RNA polymerase may attributed to few or loss number of plasmid or mutations that arising on the plasmid or chromosome (Vethanayagam and Flower, 2005). Among the P. aeruginosa virulence factors, there are several enzymes may cause modifying in euakrtyotic proteins and matrix components such as phospholipases(Ostroff et al., 1989) alkaline protease (Horvat and Parmely, 1988) ribosylating enzymes, exotoxins A and S (Iglewski and Kabat, 1975, Coburn et al., 1989) and elastase (Bejarano et al.,1989).In this study we used Vero cell line monolayer to investigate the ability of P. aeruginosa elastase to disassemble and demolition cells; The results showed detaching and changing in morphology of normal Vero cells from spindle shaped to round and shrived cells observed after was treated with elastase of P. aeruginosa 10104-2aI; these changes were followed by gradual devastation, cytotoxic activity and complete damage in monolayer of cells after 96 hrs. causing death of the cell culture (Figure6). These morphological changes can be defined as cytopathic effects (CPE) and can be useful to classify cytotoxicity. Jose and his co-workers were observed the zinc dependent elastase of P. aeruginosa MCCB 123 has cytotoxicity role on Hep2 cells (Jose et al., 2017) and ready to fracturing the endothelial barrier and decreased levels of vascular endothelial (VE)-cadherin system (Golovkine et al., 2014). The destruction of Vero cells in our results may attribute to the ability of P. aeruginosa 10104-2aI metalloprotease (elastase) to break down the extracellular matrix components including collagens, laminin, fibronectin, proteoglycans, elastin, etc. these data compatible with Tamura and his co-workers results were confirmed that metalloprotease aid to beat the barriers and entrance of pathogens to the host cells, spreading and causing expanded tissue damage (Tamura et al., 2017, Casilag et al., 2016) In another hand, the ability P. aeruginosa 10104-2aI elastase to interactions and degradate these barrier compounds may effect on cancer cells and become naked ,these unclothed cells are left alone vulnerable to attack by the immune system. The enzymes are widely used for the treatment of variety diseases such as leukemia, inflammation, skin ulcers, digestive disorders, cardiovascular diseases, Fabry’s disease, Parkinson’s disease, Pompe’s disease, pancreatic disorders, celiac disease, etc (Mane and Tale, 2015). But, there are
many bacterial enzymes have been tried to use in the treatment of cancer such as L-Asparaginase (Bhargavi and Jayamadhuri, 2016) Chitinase (Fusetti et al., 2002) Collagenase (Ostlie et al., 2012) Nattokinase (Hsia et al., 2009) Serratopeptidase (Rothschild, 1991) Argnase, Tyrosinase and Glucosidase (Kaur and Sekhon, 2012) Glutaminase (Spiers and Wade, 1976) Zinc Metalloprotease (AL-Rubaii, 2009) Hyaluronidase (Scodeller et al., 2013, MCTee et al., 2014) chondroitinase (Denholm et al., 2001). In conclusion we report P. aeruginosa 10104-2a elastase may employed and provide chance to treatment of cancer cells as alternative or enhancing anti-tumor drug through degradation of extracellular matrix of tumor mass and become unarm. Also, we conclude pGEX-KG expression vector is unsuitable for cloning the long gene such as LasB gene of elastase with a view to expression in competent cells E. coli due to may interference factors.

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