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Cloning and Expression of the *Streptococcus pyogenes* Hyaluronidase Gene in *E.coli*

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Abstract: ***Background:*** extracellular hyaluronidase of *Streptococcus pyogenes* is a spreading factor (hyaluronate lyase) which is composed of 868 amino acid protein with a molecular size of 99636 Da, the aims of cloning and expressing extracellular hyaluronidase of *Streptococcus pyogenes* in *E.coli* ***Methods:*** the hyal A gene was inserted in pTz57R/T vector. Subsequently, the vector was purified and than digested using *Sac*I and *Bam*HI restriction endonuclease. After inserting in pET32a vector, the recombinant plasmid was introduced in *E.coli* DH5α and transferred in the *E.coli* BL21 (DE3) pLysS bacteria, to monitor its expression by using IPTG and Protein analysis was carried out by SDS-PAGE electrophoresis. The new recombinant protein antigenicity was evaluated by Immunoblot analysis. ***Results:*** PCR product was a fragment with about 2608 bp, and high similarity with gene bank accession number af218838. Recombinant protein in expression vector pET32a was produced by 1mM IPTG. Several bands were seen in SDS-PAGE. The 113 kDa band belongs to Hyl A recombinant protein with His –tag, and other bands were 50-113 kDa in molecular weight range. This recombinant protein in western blotting was confirmed by using serum from mouse immunized with supernatant culture of S. pyogenes. ***Conclusion:*** several molecular weight of recombinant protein possibly is due to heavy weight of Hyl A protein or having two start codon in hylA gene. In other hands, western blotting results indicate that all bands of this recombinant protein have antigenic property, and can be used for diagnostic assay.

Keywords: recombinant, hyaluronidase, extracellular

INTRODUCTION

Hyaluronidase is an enzyme which is able to degrade hyaluronic acid, a major component of the extracellular matrix of body tissues, as well as being the major or sole component of the capsular material of certain bacteria (Berry A.M 1994; Baker JR 2002). Hyaluronidases are produced by different types of organisms and can be classified into three categories (Benchetrit LC 1977; Benchetrit LC 1978), the Testicular type hyaluronate-4-glycanohydrolases, the hyaluronate-3-glycanohydrolases produced by leeches , hookworms and the hyaluronate lyases or bacterial hyaluronidases (Cramer J.A 1994);(Gmachl M 1993). Various Gram-positive microorganisms including species of Streptococcus, Staphylococcus, Clostridium, Propionibacterium, Peptostreptococcus and Streptomyces produce hyaluronate lyase (Canard B 1994). All Gram-positive bacteria which produce hyaluronidase seem to be able to cause infections in animals. The degradation of hyaluronic acid and other glycosaminoglycans by these enzymes may cause bacteria to invade the tissues of an animal host (Berry A.M 1994). Possession of HA-degrading activities may also facilitate bacterial adhesion and colonization (Benchetrit LC 1984).The gram-positive bacterium S. pyogenes or group A streptococcus (GAS) is a versatile human pathogen, that produce several virulence factors that facilitate infection of human tissue (Hutchison SJ 1998) (JR 2004); (Watanabe N 1976) .These include extracellular proteins such as streptokinase, plasminogen, and the extracellular hyaluronate lyase, which can cleave the hyaluronic acid component found in virtually all tissues. Group A streptococci are able to producing two types of hyaluronidase, a bacteriophage associated enzyme and an extracellular hyaluronidase that is secreted from the cell (Hynes WL 1989); (Hynes WL 1994); (Hynes W.L 2000). Hyaluronidase genes from two S. pyogenes bacteriophages have been reported and evidence is provided that an extracellular hyaluronidase is produced by group A streptococci(Hynes WL 1995). only certain serotypes of group A streptococci Produce hyaluronidase and this production is a property of the strain rather than the serotype of the organism (Hynes W.L 2000). Hyaluronidase, likely increase penetration of chemotherapeutic drugs, so it was previously used as an adjunct in cancer treatment. Consequently it,may itself have intrinsic anticancer activity (Schulze C 2008); (Spruss T 1994). Hyaluronidase reduces human breast cancer xenografts in SCID mice (Shuster S 2002). In this study, recombinant hyaluronidase of *streptococcus pyogenes* in *E.coli* was produced.

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Methods:

Bacterial Strains And Vectors:

The principal strain employed in the present investigation was *S. pyogenes* strain ATCC 8668 and pTz57R/T cloning vector and pET32a expression vector.

Chromosomal Extraction and PCR Method:

S. pyogenes chromosomal DNA for using in PCR was extracted by the QuickExtract™ Bacterial DNA Extraction kit (Epicentre Biotechnologies). The hylA gene was amplified using a forward primer (5' ATGGAT CCGTGAATACTTATTTTTG3') and a reverse primer (5'AAGAATTCCTAA ATCCTTAAGTCTT 3') (Hynes WL 1992), (Hynes W.L 2000), which were manufactured by MWG company (Germany). PCR was carried out in 50 µl volumes containing 1 µl each deoxynucleotide (dATP, dTTP, dGTP, and dCTP), 2.5 µl each primer (10 pM), and 1 U of *Expand DNA* polymerase (Fermentas). Buffer was added as described in the manufacturer's instructions. Reaction mixes placed in an automated thermal cycler (eppendorf). Amplification conditions for primers were 5 min of Primary denaturation at 95°C, 30 cycles (30 s of Secondary denaturation at 95°C, 1 min of annealing at 50°C, and extension at 72°C for 1.5 min) and final extension at 72°C for 5 min. After amplification, the reaction mix was assayed on an agarose gel in the presence of 0.5 mg of ethidium bromide per ml. The hylA gene fragment obtained from PCR was purified by High Pure PCR Product Purification Kit (Roche).

Cloning:

The PCR product of hylA gene was digested by BamHI(Fermentas) and Sac1(Fermentas) and cloned into pTz57R vector.The ligated vector(pTz57RhylA) was transformed into E.coli DH5α cells and then they were grown at 37°C. Colonies were isolated, and their plasmid DNA was extracted by miniprep plasmid extraction, the extracted plasmid was assayed on 1% agarose(Merck) gel.

DNA Sequencing:

The constructed vector (pTz57RhylA) was sequenced by MWG Company (Germany) to confirm that the desired product had been obtained. Sequence data has been blast to GenBank, accession number AF218838.

Expression and Purification of Hyaluronidases:

The constructed vector (pTz57RhylA) was digested by BamHI (Fermentas) and SacI(Fermentas)and assayed on 1% agarose(Merck) gel. The fragment of hylA was extracted y gel extraction kit(Roche). The purified fragment was ligated in pET32a. The recombinant plasmid was introduced into E.coli DH5α and then transferred into BL21(DE3) bacteria to monitor its expression. The recombinant strain E. coli BL21(DE3)/ pLYS-S was grown overnight at 37°C in 2 mL Nutrient Broth medium containing ampicillin. Aliquot (1 mL) of this starter culture was transferred to 50 mL of the same medium in a 500 mL flask and incubated at 37°C with vigorous shaking. Expression was induced by adding IPTG to give a final concentration of 0.1 mM when the culture absorbance at 600 nm reached 0.5. Three hours after induction, the cells were harvested by centrifugation and the cell pellet was washed twice with 30 mL pH 7.5, 0.1 M phosphate buffer. The cell pellet was resuspended in the phosphate buffer and lysed by using an ultrasonic cell disruptor (Microson model XL200). The sample was in an ice-bath, and sonication was carried out in short bursts in order to avoid overheating the mixture. The lysate was centrifuged at 16,000g for 10 min at 4°C. Since the expressed recombinant protein fused with a 6×His tag at its C-terminal, the supernatant of the cell lysate was loaded onto a fast-flow chelating Sepharose (Amersham Pharmacia, Sweden) column for immobilized metal affinity chromatography (IMAC) purification following the protocol provided by the manufacturer (Lin B 1994).

SDS-PAGE and Immunoblot Analysis:

SDS-PAGE analysis was carried out under reducing conditions using a 12% polyacrylamide gel. A Mini protein II gel system (Bio-Rad Laboratories, Richmond, CA) was used to run the gels, which were then stained with Coomassie blue.

For Western blot analysis, proteins were transferred from the SDS-PAGE (12% [wt/vol] polyacrylamide) gel to 0.2 µm pore size nitrocellulose membranes (Bio- Rad). Membranes were probed, first with a polyclonal mouse antibody (mice were immunized by supernatant culture of *S. pyogenes*) and then with rabbit anti-mouse IgG conjugated to horseradish peroxidase (SBA). Detection of HylA protein was achieved upon development with the substrate 4-chloro-1-naphthol chromogen and H2O2 (Sigma-Aldrich) (Jedrzejas M.J 1998).
This study was approved by the Ilam University of Medical Sciences/Ilam Ethical Committee.

Results:

PCR was used successfully to amplify a gene fragment encoding the sequence of hyl A. PCR product has about 2607bp on 1% agarose gel electrophoresis (Figure-1,A; lane 1). For preliminary confirmation, the

fragment was subjected to digestion by XhoI. Digestion with XhoI yielded 1136 and 1471 bp fragments, (Figure-1, B, lane 2). Ligation of PCR product in pTz and pET32a was confirmed by SacI and BamHI digestion. The sequencing of the PCR product fragment revealed complete homology at the nucleotide level to *hyl A* gene in NCBI.

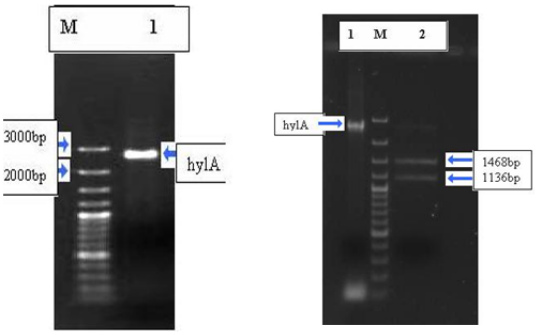

Fig. 1: A-Electrophoresis of PCR product on 1% agarose gel (lane 1), B-PCR product (lane 1) digested by XhoI (lane 2).

Expression, Purification:

The recombinant protein from *E. coli* BL21 (DE3) harboring pET32a containing the coding sequences of hylA gene was detected in cell lysates and appeared at about 110 to 50 kDa on SDS-PAGE gel (Fig. 2-A). The recombinant HylA protein was collected and further purified by using HIS KIT .Purified recombinant HylA protein appeared at about 50 to110 kDa (Fig. 2-A).

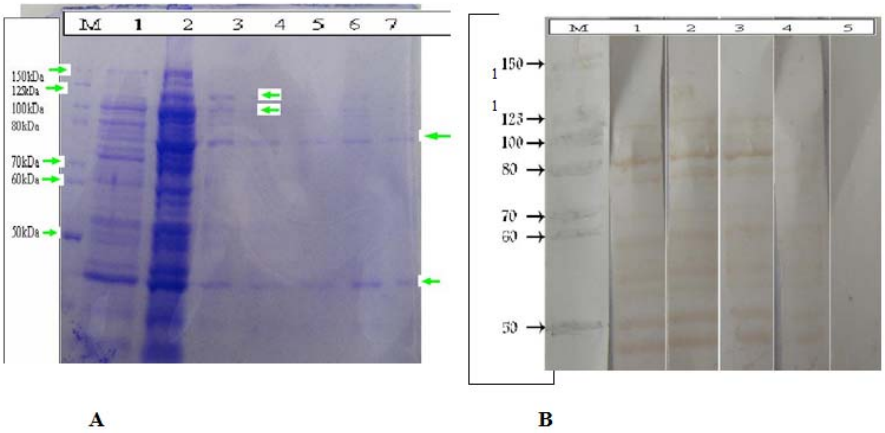

Fig. 2: A, SDS-PAGE of *E.coli* BL21 (DE3)/ pLYS-S cell lysate harboring pET32a in uninduced and IPTG-induced conditions. Arrows indicate bands of the recombinant proteins. M: protein marker, 1: Uninduced proteins of *E.coli* BL21 (DE3)/ pLYS-S harboring pET32a-hylA, 2: IPTG-induced proteins of *E.coli* BL21 (DE3)/ pLYS-S harboring pET32a-hylA, 3, 4, 5: purified proteins of induced *E.coli* *E.coli* BL21(DE3)/ pLYS-S harboring pET32a-hylA . B Western blots analysis of recombinant Hyal A protein using polyclonal mouse antibody (mice were immunized by supernatant culture of streptococcus pyogenes), M: Marker, 1,2,3,4 recombinant protein , 5, negative control.

Western Blotting of Fusion Protein:

Serum from immunized mouse was used for blotting (Figure.2-B). The result shows that HylA protein can be detected. This suggested that the recombinant HylA protein was successfully produced by *E.coli* harboring pET32a with *hylA* gene.

Discussion:

S. pyogenes extracelullar hylorunidase enzyme is coded by chromosomal hylA gene. This enzyme was first purified in 2000 as a 99636 Da , Cleavage of the proposed signal peptide results in an extracellular protein of 95941 Da. Comparison with other bacterial hyaluronidases, indicates strong similarities with the genes from

Streptococcus pneumoniae, Streptococcus agalactiae and Staphylococcus aureus. The sequence of *S. pyogenes* hyl A gene is 2607 bp and Hyl A protein contains 868 amino acids (Hynes W.L 2000).

It is said that *S. pyogenes* extracelullar hylorunidase enzyme, in fact spreads infection(Starr C R 2006), similar to other animal hyalorunidase such as scorpion and snake , that causes spreading of toxin(Gmachl M 1993; Spruss T 1994). Application of hyaluronidase used as subdermal spreading of vaccine, local anesthetic drugs and facillator of tissue penetration of drugs for chemotherapy of tumor(Spruss T 1994). In this research, hyl A gene was cloned and expressed in *E.coli*,, amplification of hylA showed a fragment with about 2607bp(fig.1, A) this indicates that hyl A gene has been properly produced. Primarily, PCR product was established by XhoI digestion, (fig.1, B) sequencing results indicate similarity of PCR product with genebank. In sum, these results confirmed PCR product is hyl A gene.

Induction was carried out by IPTG 1mM (fig.2, A lane 2). The molecular weight of recombinant protein HylA was 40-113 kDa. In other hands, purified protein by Ni-NTI (fig.2, A, lanes 3,4,5) determined the molecular weight of recombinant protein (HylA) approximately 113 kDa with signal peptide and His-tag protein. Other bands were denatured forms of recombinant protein. Previous researchers reported that extraction and purification methods affect weight of hyaluronidase. HILL, et al revealed the molecular weight of hyluronidase of S.pyogenes after extraction in SDS-PAGE was 50Kd, and after purification by filtration was 70kDa(HILL 1976). In other hands, the molecular weight range of cattle testis was 61-62kDa(Phelps 1981). The results showed extacelular hyaluronidase to be a large exported protein about 99,000 Daltons as an enzyme, similar to hyaluronidase of other organisms(Canard B 1994). several molecular weight of recombinant protein is possibly is due to heavy weight of Hyl A protein or perhaps it has two start codon in hylA gene. Western blotting analysis, indicated similarity with purified protein pattern, i.e. Bands of purified recombinant protein conscious is similar to western bands. Serum from Mouse immunized by S.pyogenes can detect recombinant protein, this shows, that the recombinant protein has antigenic property. In other hand antigenic property of this protein of strain ATCC 1447 S.pyogenes may be different from other type of group A streptococcus. This is the first report of cloning, expression and purification of extacelular hyaluronidase of *Streptococcus pyogenes* in E.coli. At present, stability of recombinant protein, enzymatic effect, design a ELISA kit for comparing with ASO test under investigation.

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