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Full Length Research Paper

Cloning, expression, purification and antigenic evaluation of hyaluronidase antigenic fragments recombinant protein of streptococcus pyogenes

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Streptococcus pyogenes **produce an extracellular hyaluronidase which is associated with the spread of the organism during infection. Enzyme hyaluronidase is capable of degrading hyaluronic acid. The aim of the present study was to clone and express antigenic regions of the hylA of *S.pyogenes* in *Escherichia coli.* The antigenic regions of hylA gene was inserted in pGEX-4T-1 vector. *E. coli* BL21 was transformed with hylA_pGEX-4T-1 and gene expression was induced by isopropyl-beta- D-thiogalactopyranoside (IPTG). The expressed protein was purified by affinity chromatography with GST sepharose resin. The integrity of the product was confirmed by western-blot analysis using sera of infected individual. Sera reactivity of infected individuals was further analyzed against the recombinant hyaluronidase protein. Our data shows production of recombinant hyaluronidase improved by pGEX-4T-1 in *Ecoli*.**

Key word: Hyaluronidase gene, cloning, expression of recombinant gene, antigenic region.

INTRODUCTION

Group A streptococcus (*Streptococcus pyogenes*) is an important species of gram-positive pathogenic extracellular bacteria. This bacteria can produce wide range of infectious diseases like, pharyngitis, tonsillitis, wound and skin infections, blood infections, scarlet fever, pneumonia, rheumatic fever, chlorea and glomerulonephritis (Kreikemeyer et al., 2004; Bisno et al., 2003; Guarner et al., 2006).

Symptoms may suggest a *S. pyogenes* infection, but diagnosis must be confirmed by culture and serological tests.The best test, for diagnosis of acute infection, is to collect sample from the infected area for culture. However, cultures are useless about two to three weeks after initial infection. Therefore, detection of the *S. pyogenes* antibody is used to determine if a streptococcal infection is present (Guarner et al., 2006; Hahn et al., 2005).

Serological diagnosis of group A streptococcal infections is based on immune responses against the extracellular enzymes streptolysin O, streptodornase B, hyaluronidase, DNase, and streptokinase. These enzymes induce strong immune responses in infected host (Guarner et al., 2006; Kreikemeyer et al., 2003).

The aim of the present study was to produce the recombinant antigenic regions of hyaluronidase enzyme in *Escherichia coli* and to evaluate the antigenicity of this protein by patients' sera.

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MATERIAL AND METHODS

Bacterial strains and plasmids

S. pyogenes strain ATCC 4283 was used as the source of chromosomal DNA for the polymerase chain reaction (PCR). *E. coli DH5á* (Stratagene) was used as the primary host and *E. coli, BL21* (Pharmacia) as host strain, for recombinant protein production. pGEX4T1 (Pharmacia) was used for production of recombinant protein.

Antigenic regions

To find out antigenic region, the sequence of hylA (*S. pyogenes*, accession number: EU078690.1] was submitted to ABCpred, BCPREDS, Bcepred and Emboss Antigenic web servers. (Kolaskar and Tongaonkar., 1990).

Primers were designed according to result sequences. The forward primer (5' GGATCCATCAAAGCAATTGAGAAA 3') starts from the beginning of the gene and contain *Bam*HI site. Reverse primer (5' TGAATTCCATCTGTTGACTATCAT 3') contain recognition site for *Eco*RI

DNA isolation and manipulation

Chromosomal DNA was prepared according to standard CTAB/NaCl method (Sambrook et al., 2001; Mahmoudi et al., 2010). After the pellet of 1.5 ml bacterial culture was resuspended overnight in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8), the bacterial cell was lysed by sodium dodecyl sulphate [SDS (10%)] and proteinase K (20 mg/ml), the chromosomal DNA was extracted by CTAB/NaCl solution (10%CTAB and 0.7 M NaCl). Cell debris and proteins were removed by mixing and washing two times with phenol/chloroform/isoamylalcohole (25:24:1) mixture. The tubes were centrifuged at 10000 RPM for 10 min at 4°C and the supernatants were transferred to a fresh tube. In this phase, protein was deposited and RNA and DNA remained in aqueous solution. DNA was precipitated by isopropanol and washed in ethanol (70%), air dried, and then suspended in TE buffer.

Gene amplification

PCR was performed in a 50 µl total volume containing 500 ng of template DNA, 1 µM of each primers, 2 mM Mg2+, 200 µM (each) deoxynucleoside triphosphates, 1X PCR buffer and 2.5 unit of pwo DNA polymerase (Roche). The following conditions were used for amplification: Hot start at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min (Sambrook et al., 2001).

Cloning of gene in bacterial expression vector

The PCR product was digested with *BamHI* and *EcoRI* and ligated to pGEX-4T-1. The vector was subjected to digestion procedure by the same restriction enzymes and T4 DNA ligase (Cinagen) at 16°C over night.

Moreover *E..coli DH5á* and *E. coli BL21* competent cells were prepared by calcium chloride method and were used for transformation of plasmid (Sambrook et al., 2001).

Gene expression

E..coli BL21 was transformed with pGEX-4T-1_ hylA and grown in 2 ml lysogeny broth (LB) broth supplemented with Ampicillin (100 µg ml^{-1}) at 37°C with agitation.

A colony which contained recombinant plasmid was cultured on shaking incubator for overnight at 37°C in 2 ml LB medium containing 100 µg ml^{-1} Ampicillin. The next day, 500 µl of culture was removed and inoculated in 50 ml LB broth (per liter contains: 10 g yeast extract (Difco), 20 g Bactotryptone broth (Difco), 0.2% (mass/vol.) glucose, 10 g NaCl, 1 g KCl, 0.5 g MgCl, 0.5 g CaCl, 100 mg ampicillin), incubated at 37°C with at 200 rpm shaking with vigorous agitation until the optical density reached to an absorbance of 0.5 to 0.8 at 600 nm.

Expression of the hylA protein was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubation was continued for further 4 h.

The expressed protein was purified using GST-sepharose column according to manufacture instruction (Pharmacia). The purified protein was dialyzed twice against phosphate-buffered saline (NaCl 140 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO 1.8 mM, pH 7.2) at 4°C over night. The quality and quantity of purified recombinant hylA protein was analyzed by SDS-polyacrylamide gel electrophoresis [SDS-PAGE (15%)] and Bradford methods, respectively (Sambrook., 2001).

Antigenicity of recombinant hyaluronidase and immunoblot analysis

Five acute phase patients sera, hylA antibody and normal human sera, received as a gift from Dr. Mossayebi (Immunology Department, the Arak University of Medical Sciences of Iran, Arak) was used as primary antibody. In western blot assay, normal human sera and hylA antibody were used as negative and positive control, respectively.

For western blot analyses, 0.5 µg of purified recombinant hylA protein was used per well. The gel was blotted on to polyvinylidine difluoride (PVDF Membrane, Roche) membrane using transfer buffer containing 25 mM Tris (pH = 8.3), 192 mM glycine and 20% methanol at 90 v for 1.5 h at 4°C. The blotted membrane was blocked with 2.5% (w/v) BSA in TBS buffer (0.5 M NaCl, 0.02 M Tris pH 8.5, 0.05% tween 20) for 1 h at room temperature. Membranes were incubated for 2 h at room temperature with diluted sera, 1:100 from patient's sera, normal sera and hylA antibody. After reactions with the primary antibody, the blots were washed three times with TBS and incubated with goat peroxidase conjugated with anti-human IgG (Bioscience, Cat No.GM5201-5) in 1:1000 dilution of TBS. The blots were then washed three times with TBS and reactions were developed by Diamino Banzidine (DAB, Roche) solution (- et al., 2010).

RESULTS

Antigenic regions

According to the result of three servers which was in accordance, amino acids sequence of 838 till 2320 were selected as a regions with high antigenic properties (Figure 1).

DNA amplification

The chromosomal DNA of *S.pyogenes* was extracted for amplification of hylA gene. The amplified fragment had the expected size of 1480 bp comparing to 100 bp DNA ladder.

MNTYFCTHHKQLLLYSNLFLSFAMMGQGTAIYADTLTSNSEPNNTYFQTQTLTTTDSEKKVVQPQQKDYYTELLDQWNSI
IAGNDAYDKTNPDMVTFHNKAEKDAQNIIKSYQEPDHENRTYLWEHAKDYSASANITKTYRNIEKIAKQITNPESCYYQDS
KAIAIVKDGMAFMYEHAYNLDRENHQTTGKENKENWWDYEIGTPRAINNTLSLMYPYFTQEEILKYTAPIEKFVPDPTRFR
VRAANFPPFEANSGNLIDMGRVKLISGILRKDDLEISDTIKAIEKVFTLVDEGNGFYQDGSLIDHVVTNAQSPLYKKGIAYTG
AYGNVLIDGLSQLIPIIQKTKSPIEADKMATIYHWINHSFFPIIVRGEMMDMTRGRSISRFNAQSHVAGIEALRAILRIADMSE
EPHRLALKTRIKTLVTQGNVFYNVYDNLKTYHDIKLMKELLSDTSVPVQKLDSYVASFNSMDKLALYNNKHDFAFGLSMFS
NRTQNYEAMNNENLHGWFTSDGMFYLYNNDLGHYSENYWATVNPYRLPGTTETEQKPLEGTPENIKTNYQQVGMTSLS
DDAFVASKKLNNTSALAAMTFTNWNKSLTLNKGWFILGNKIIFVGSNIKNQSSHKAYTTIEQRKENQKHPYCSYVNNQPVD
LNNQLVDFTNTKSIFLESDDPAQNIGYYFFKPTTLSISKALQTGKWQNIKADDKSPEAIKEVSNTFITIMQNHTQDGDRYAY
MMLPNMTRQEFETYISKLDIDLLENNDKLAAVYDHDSQQMHVIHYEKKATTFSNHNSHQGFYSFPHPVKQNQQQKLAHQ
GIAAKNNALNSHKIPHKRQRRLPRTGYQSSSLEFLGGALVASFNHITKPFRKKDLRI"

Figure 1. The result of antigenic fragments: Red sections are antigenic fragments.

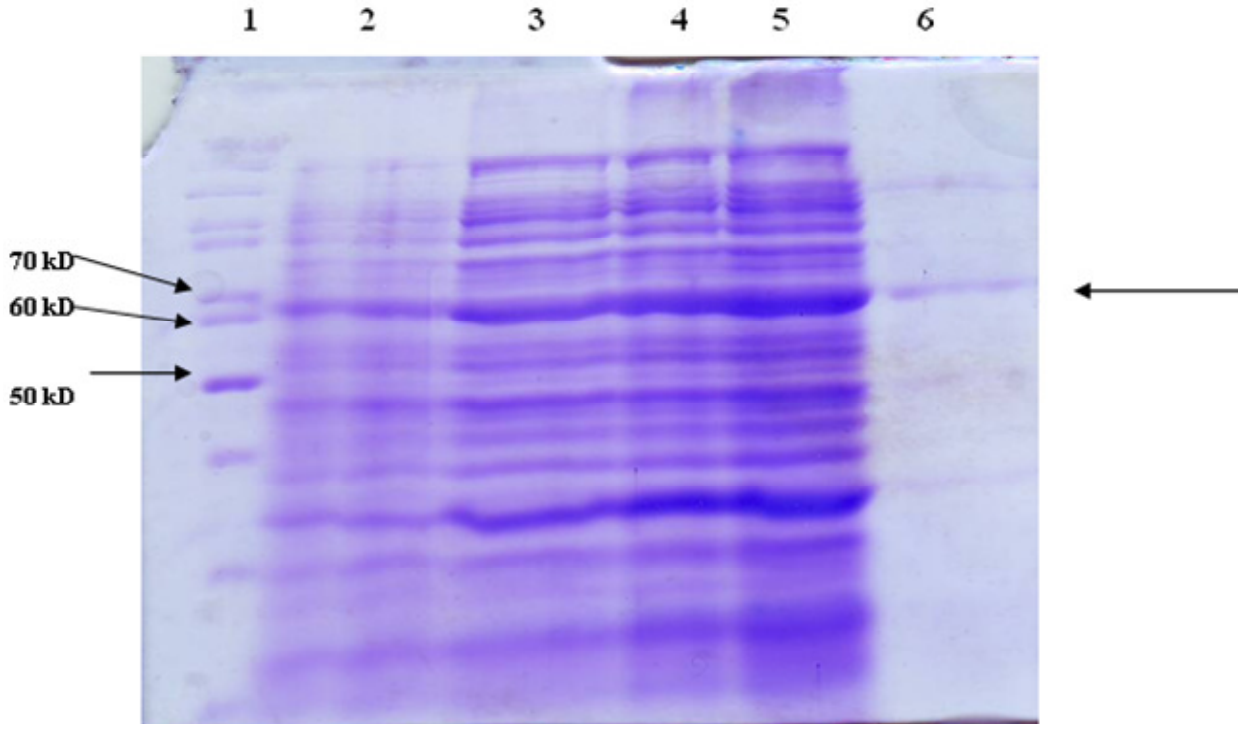

Figure 2. Expression of recombinant antigenic fragments of hyaluronidase protein and its purification. Lane 1, Protein marker; Lane 2, pGEX4T1- hylA before induction; Lanes 3 - 5, pGEX-4T-1_ hylA; Lane 6, elution of recombinant fragment of hyaluronidase protein.

The recombinant plasmid (pGEX-4T-1_hylA) was sequenced. The sequencing result was confirmed by comparing with databases and using basic local alignment search tool (BLAST) software (data not shown). To confirm the transformation of pGEX-4T1- hylA into *E.coli* BL21, PCR reaction and enzymatic digestion with *BamH*I and *EcoRI* were performed.

Expression and purification of recombinant protein

pGEX-4T-1_hylA in *E. coli* BL21 was induced and the expressed protein was purified by GST-sepharose column (Figure 2). SDS-PAGE analyses showed the expected molecular weight of near 60 kDa for recombinant protein. The concentration of recombinant protein was assayed after purification and calculated to 470 mg purified protein per liter of the initial culture.

Immunoblotting analysis

To determine the antigenicity of recombinant hylA protein in patients' sera, the recombinant hylA protein was assayed by western-blot analysis. Figure 3 shows the specific interaction between patients'serum, human normal sera and hylA antibody with purified recombinant hylA protein. Human normal sera were used as a negative control

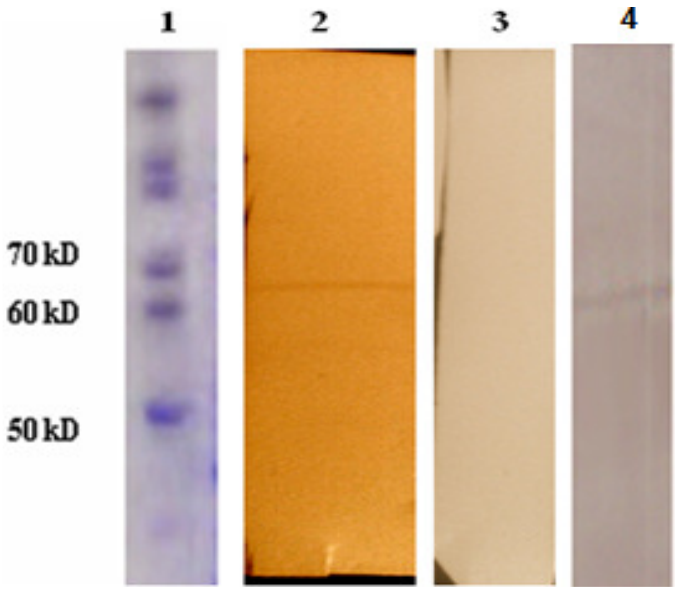

Figure 3. Western blot analyses of hyaluronidase recombinant antigenic fragments using patient sera. Lane 1, Protein marker; Lane 2, western blotting by patients' sera; Lane 3, western blotting by human normal sera (negative control), Lane 4, western blotting by hylA antibody (positive control).

and hylA antibody used as a positive control.

DISCUSSION

In this present study, we have shown that antigenic regions of streptococcal hyaluronidase might be detected by infected sera. Our data shows that recombinant antigenic regiond of streptococcal hyeluronidase protein can be produced by pGEX-4T-1 expression in *E.coli*. This antigenic protein could be recognized by sera in infected human.

Hyaluronidase is an extracellular enzyme capable of degrading hyaluronic acid. Bacterial hyaluronidases enzyme are produced by various gram-positive microorganisms including species of Streptococcus, Staphylococcus, Clostridium, Propionibacterium, Peptostreptococcus and Streptomyces (Hynes et al., 2000; Hynes and Walton, 2000).

Diagnosis of *S.pyogenes* is based on isolation of streptococcus in culture or serological tests. Serological detection of *S. pyogenes* infections is based on immune responses against the extracellular products streptolysin O, streptodornase B, hyaluronidase, DNase, and streptokinase, which induce strong immune responses in infected host (Cunningham, 2000).

Anti-streptolysin O (ASO) is the antibody response most often examined in serological tests to confirm preceding group A Streptococcal infections. The ASO titer is ordered primarily to determine whether a previous *S. pyogenes* infection has caused a post streptococcal disease, rheumatic fever or glomerulonephritis.

Antibodies to streptolysin O are produced in approximately 75 to 80% of *S. pyogenes* infections, but are usually not seen in cutaneous infections caused by group A streptococcal infections. False positive results may occur from hypercholesterolemia in ASO test. If an anti-streptodornase B or antihyaluronidase assay is performed on sera, the patient with at least one positive antistreptococcal enzyme titer that rises to 95% (Steer et al., 2009).

Production of this enzyme has been already done by the Moradkhani et al. (2011), but due to heavy molecular weight (140 KD), the protein was degraded. Hence in this study, part of hyaluronidase protein with suitable antigenic properties which had been determined with bioinformatic methods was used for detecting antibody. Therefore the protein obtained in this study is much smaller than normal hyaluronidase protein, with the same antigenic properties (Moradkhani et al., 2011). This recombinant hyaluronidase can be used for detecting antihyaluronidase antibody.

In this study, we have cloned and expressed antigenic regions gene that encode hyaluronidase under the control of Trp promoter in optimized condition. Present data shows that recombinant antigenic regions of hyaluronidase can be detected as an antigen by serum in patients. Therefore, recombinant antigenic region of hylA protein has same epitopes with natural form of this antigen. Recombinant antigenic regions hylA also seemed to be a promising antigen for the serologic diagnosis of *S.pyogenes* infections.

Conclusions

Data indicates that antigenic regions of recombinant hyaluronidase protein from *S. pyogenes* were recognized by patient sera. It can be concluded that antigenic regions of hyaluronidase have antigenic property that can be further used for diagnostic purpose.

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