

Cloning and Expression of S1 Subunit of Pertussis Toxin in *Escherichia coli*Abolfazl Khafri¹, Khosrow Aghaiypour^{2*}, Shahin Najar Peerayeh¹, and Reihaneh Ghorbani²

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Abstract

Bordetella pertussis is a gram negative bacterium that causes respiratory tract infection in human (whooping cough). Pertussis toxin (PT) is the main component of current acellular pertussis vaccine and the S1 (subunit1) is the main immunogenic part of it. Thus, S1 has been the target of many studies as a potent candidate of acellular vaccine against *Bordetella pertussis*, lacking the side effects of whole cell based ones. S1 gene was amplified and inserted in three expression vectors including pET-14b, pET-22b(+) and pAED4. The possibility and level of expression of these constructs were investigated in BL21 (DE3) strain of *Escherichia coli* (*E.coli*) as expression host. The highest expression was in pET-22b(+)-S1. Best expression achieved 6 hr post induction with 0.2 mM IPTG in LB broth containing ampicillin, at 30 °C with shaking (250 rpm). Recombinant S1 protein was observed in two distinct separated proteins with 28 and 31 kDa estimated molecular weight. In spite of toxicity of PT and S1 in the *E.coli*, considerable amount of S1 was expressed in *E.coli*. Two rS1 bands were detected on SDS-PAGE. Both were confirmed as S1 in western blot with specific monoclonal and polyclonal antibodies against pertussis toxin. Appearance of two distinct bands could be the result of leader peptidase activity or nonspecific peptidase from *E.coli* on recombinant S1. As the recombinant S1 is a suitable antigen for studies as a candidate acellular vaccine or development of ELISA for detection of *Bordetella pertussis*, further studies are underway.

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Introduction

Bordetella pertussis is a gram negative bacterium that causes respiratory tract infection in human (whooping cough). *Bordetella pertussis* has some important antigens such as pertussis toxin, adenylate cyclase, fimbriae, agglutinogens, Filamentous Hemagglutinin (FHA), pertactin and Outer Membrane Proteins (OMP). Pertussis toxin (PT) is also called lymphocytosis-promoting factor, histamine sensitizing factor, islet activating protein, and pertussigen⁽¹⁻³⁾.

Although pertussis is a vaccine preventable

disease, according to the World Health Organization (WHO), there are an estimated 50 million cases of pertussis per year worldwide, with approximately 300,000 leading to death⁽⁴⁾.

There are two different types of pertussis vaccine, whole cell and acellular vaccine^(1,4). Vaccination with whole cell of *Bordetella pertussis* causes some side effects e.g. a serious allergic reaction fever, prolonged seizures, decreased consciousness, lasting brain disease, or death. Thus, many reports have

been focused on acellular vaccine as a potent effective substitute, lacking the side effects. Pertussis toxin is thought to play a major role in the pathogenesis of whooping cough. It is also believed to be a major protective antigen, so is one of the major acellular pertussis vaccines ⁽⁵⁾.

PT can be divided into two different A and B parts. Part A is the enzymatically active S1 subunit while B is composed of S2, S3, (S4)₂, and S5 subunits. The B part binds to the receptor of eukaryotic cells and allows the entry of the enzymatically active S1 subunit into the cells ^(1,6,7). After internalization, S1 transfers the ADP-ribose moiety of NAD to the Gi regulatory component of the adenylate cyclase complex. The Gi ADP-ribosylation decreases the response to inhibitory agents of adenylate cyclase and increases the response of stimulatory agents ^(1,8-11).

In this study, as S1 is the most important immunogenic part of PT, it was cloned in suitable vectors and the recombinant S1 (rS1) was expressed in *E. coli* as a prokaryotic host cell. Finally, expressed rS1 was confirmed by monoclonal and polyclonal antibody against pertussis toxin.

Materials and Methods

Materials

Bordetella pertussis strain 18323 (ATCC 9797), a good producing PT and highly pathogenic strain (MAST, Germany), *E. coli* DH5α as cloning host, BL21 (DE3) (Novagen, USA) as expression host, pET-22b(+), pET-14b (Novagen, USA) and pAED4 as expression vectors, SmartTaq DNA polymerase, T4 DNA ligase, dNTPs (Cinagen, Iran), *Pfu* DNA polymerase, *Nde*I and *Xho*I (Fermentas, Lithuania), monoclonal antibody against pertussis toxin (Abcam, USA), polyclonal antibody against pertussis toxin (NIBSC, England) and HRP conjugated antibody against mouse IgG (Sigma, USA) were used in this study.

Bordetella pertussis culture and genomic DNA extraction

Bordetella pertussis was cultured in Bordet Gengou agar for 3 days at 37°C. The bacteria were harvested in PBS and then were heated at 56°C for 30 min. Genomic DNA was extracted from pellets by DNA extraction kit (high pure template DNA extraction kit, Roche Applied Science, Germany) according to the manufacturer's instruction.

Primer designing and amplification of S1 gene

PT DNA sequences of *Bordetella pertussis* from Genbank was retrieved and aligned by DNAMAN software (Version 4.13). On the basis of *Bordetella pertussis* 18323 DNA sequences (GenBank: AJ506996.1), two specific primers were designed with Oligo software (version 5) for amplification and isolation of the S1 full length. The upper and the lower primers were flanked by *Nde*I and *Xho*I restriction sites, respectively. These enzymes allow cloning of the gene in pET system vectors. The sequences of the primers were:

*Nde*I_Bor_S1 (Forward):

5-GAATTCATATGCGTTGCACTCGGGC-3

*Xho*I_Bor_S1 (Reverse):

5-CCGCTCGAGGAACGAATACGCGATGCTTT-3

PCR was carried out in Mastercycler gradient (Eppendorf, Germany) using *Pfu* DNA polymerase. The PCR reactions were carried out in 50 μl containing: 1 μl purified *Bordetella pertussis* genomic DNA, 5 μl 10X PCR buffer (100 mM Tris-HCl, 15 mM MgCl₂ and 500 mM KCl), 200 μM dNTP, 0.2 μM of both primers and 2 U of *Pfu* DNA polymerase. The initial denaturation step was at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, 1 min annealing at 64°C and 1 min elongation at 72°C. The final elongation step was performed at 72°C for 10 min.

Cloning of amplified S1 gene

Amplified PCR product was purified by high pure PCR product purification kit (Roche Diagnostic, Germany). Both of S1 fragment and pET-22b(+) were double digested with *Nde*I and *Xho*I. After cleaning with PCR product purification kit (Roche Diagnostic, Germany), they were ligated to

each other by T4 DNA ligase at 22°C. DH5 α was transformed with the ligation mixture via heat shock. The positive clones were screened on ampicillin/LB agar. The recombinant S1-pET-22b(+) plasmid was purified by high pure plasmid purification kit (Roche Diagnostic, Germany).

Ligation mixtures were transformed to *E.coli* DH5 α . All of insertion steps of S1 gene are done individually for all of the mentioned vectors. The S1 gene that had been inserted in purified plasmid pET-22b(+) was sequenced by MWG Company (Germany). The sequence was compared with *Bordetella pertussis* 18323 S1 gene sequences (GenBank: AJ506 996.1) for homology by BLAST analysis. The vectors were transformed in BL21 (DE3) as expression host by heat shock method.

Expression of recombinant S1 (rS1)

Expression of recombinant S1 was in LB broth and optimized by addition or adjusting of some component such as glycine, glycerol in LB broth, use of different incubation temperatures (4°C, 25°C, 30°C, 37°C) or different concentrations of IPTG (0.1 to 1 mM) as inducer.

In brief, 50 ml of media were inoculated by 1.5 ml of an overnight fresh culture of expression host. Induction was carried out when culture had reached the OD of 1 at 600 nm. Zero, 3 and 6 hrs and overnight samples immediately were centrifuged at 4°C at 12000 rpm for 5 min. Pellets were resuspended in 2x SDS-PAGE sample buffer and stored at 50°C. Expression of different mentioned vectors were studied in *E.coli* BL21 (DE3) as expression host. All of samples (pellets and supernatants) were run by SDS-PAGE electrophoresis following by Coomassie Brilliant Blue for pellets and silver nitrate for supernatants.

Western blot assay

The SDS-PAGE pellet of BL21 (DE3) with rS1-pET-22b(+) were wet transferred on nitrocellulose for 1 hr at 100 volt. Monoclonal and polyclonal antibodies against pertussis toxin were used as the primary antibody and rabbit HRP conjugated IgG against

mouse IgG as secondary antibody. 4-chloro-1-naphthol was used as substrate for visualization of bands.

Results

Genomic DNA isolation and PCR amplification of S1 gene

Genomic DNA from 72 hrs grown *Bordetella pertussis* cells was extracted successfully. The quality and purity of the extracted DNA in Nanodrop and also on agarose gel electrophoresis were good. S1 gene was isolated by PCR amplification using the designed primers. The optimized PCR amplification isolated an intense single 810 bp band (Figure 1). The fragment contained *NdeI* and *XhoI* overhangs on the 3' and 5' of the gene, respectively to ligate it in accurate direction for expression.

Cloning and sequencing of S1 gene

Both amplified fragment and pET-22b(+) were purified and digested with *NdeI* and *XhoI* for ligation to each other. The ligated plasmids were selected on the basis of ampicillin resistance. Plasmids which were extracted from positive clones showed two fragments, one about 810 bp (inserted S1 gene) and the other one about 5400 bp (plasmid without inserted S1 gene) in digestion with *NdeI* and *XhoI* (Figure 1).

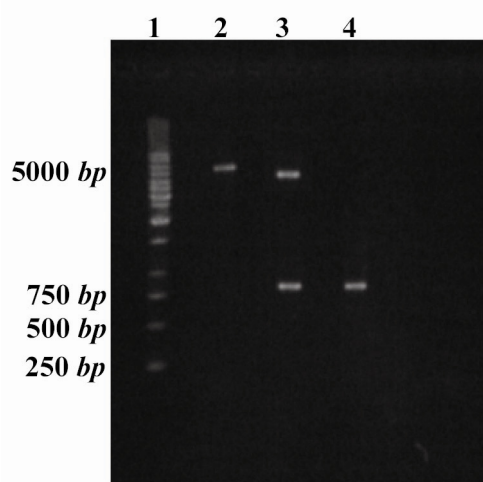


Figure 1. Agarose Electrophoresis in 1.5% gel, lane 1 1Kb ladder (Fermentas, Lithuania.), lane 2: pET-22b(+) with inserted S1 after digestion with *NdeI*, lane 3: pET-22b(+) with inserted S1 gene after double digestion with *NdeI* and *XhoI*, lane4 PCR product of S1 gene

Restriction analysis of the isolated fragment with *Nco*I, an extra confirmation test for the isolated S1 gene, produced two expected fragments; a 730 bp and 80 bp and confirmed the accuracy of S1 sequence (data not shown). This restriction analysis was used to control and trace the gene in all steps of purification, digestion and ligation.

Constructed vectors were extracted, purified and sequenced bidirectional to have the highest possible accuracy. Nucleotide sequencing of inserted gene had 100% homology with nucleotide sequence of *Bordetella pertussis* 18323 S1 gene (GenBank: AJ506996.1).

Expression of recombinant S1 (rS1)

S1-pET-22b(+) construct was able to express S1 in relatively high level, enough to be observed obviously on SDS_PAGE with Coomassie Brilliant Blue staining (Figure 2). Cell extract and supernatant were studied to trace any possible expression. Two distinct bands did exist in cell extract with 28 and 31 kDa estimated bands. No secretory expressed protein was observed in the gel electrophoresis of supernatant. In order to indicate any possible expression, the supernatant SDS-PAGE gel was re-stained by silver nitrate because of its higher sensitivity. No obvious extra cellular expression was observed (data not shown). By optimization of culture conditions the best condition for expression rS1-pET-22b(+) in *E.coli* BL21 (DE3) were:

LB broth as media, 30°C incubation temperature, 250 rpm rotation, 6th hr culturing and 0.2 mM IPTG as inducer. In these conditions two distinct and separate molecular weight proteins (about 28 and 31 kDa) were detected (Figure 2). S1-pET-14b and S1-pAED4 constructs were not able to express S1 in enough level (Figure 2).

Western blot assay

Both of the different bands (about 28 and 31 kDa) were confirmed as S1 in western blot analysis (Figure 3). This test was repeated with polyclonal primary antibody against PT instead of monoclonal primary antibody

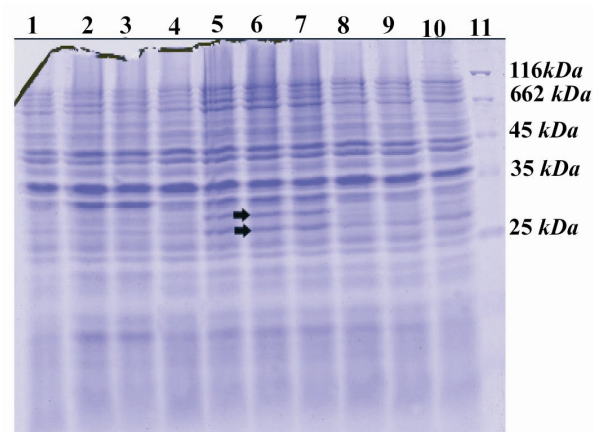


Figure 2. SDS-PAGE Electrophoresis of the expressed rS1 in BL21(DE3): lanes 1,2 and 3: extracts from cells harboring pET-22b(+) without S1 just before induction(0 hour), 3rd hours and 6th hours after induction, lanes 4: extract from cells harboring rS1-pET-22b(+) before induction(0 hour), lanes 5, 6 and 7: three separate clones 6th hours after induction, lane 8 Cell extracts rS1-pET-14b clones from inserted vector 6th hours after induction, lane 9 and 10 cell extracts of different clones of rS1-pAED4 6th hours after induction, lane 11 MWM (Fermentas, Lithuania).

against PT and both mentioned bands were detected (data not shown).

Discussion

Pertussis vaccine has a crucial role in the control of highly contagious whooping cough disease. It has enough importance to attract many scientists. Thus, many studies have been done on bacteriology, physiology, im-

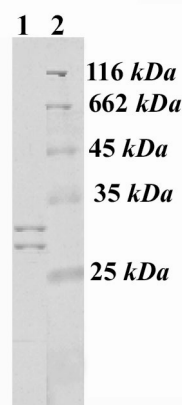


Figure 3. Western Blot assay by monoclonal antibody against pertussis toxin as the primary antibody and HRP conjugated antibody against mouse IgG as the secondary antibody. Lane 1 Cell extracts from cells harboring rS1-pET-22b(+) 6th hours after induction with IPTG (two distinct rS1 with a little different molecular weights), lane 2 MWM (Fermentas, Lithuania)

munology and pathology of this bacterium.

Currently, two types of vaccines including whole cell and acellular vaccines are being used worldwide. Developed and some of the developing countries are switching to use acellular one; because of the side effects of whole cell vaccines. S1 subunit, one of the most important immunogens of pertussis toxin (an essential components of acellular pertussis vaccine), can be used as a good candidate for this purpose. Some studies attempt to express the recombinant S1 in different hosts such as *E.coli* ^(2,9,12,13). However, because of the toxic nature of this protein its expression is relatively a tedious work.

In previous studies, old generation strains of *E.coli* have been used for expression of S1, but quality (suitable immunogenicity) and quantity of their rS1 production hasn't been satisfying ^(2,9,12,13).

In this study, new engineered strain of *E.coli* capable for better expression and folding of recombinant immunogenic proteins is used. New expression hosts have been engineered to contain better promoter for producing high level of recombinant proteins. Many enzymes and genes were knocked out in recent expression cells, to achieve proper folding or prevent degradation.

Comparison (BLAST) of nucleotide sequence of inserted rS1 gene in the Gene Bank, showed 100% homology between amplified rS1 gene and the native S1 gene in *Bordetella pertussis* strain 18323(ATCC 9797). Thus, amino acid sequence (primary structure) of expressed protein (rS1) was completely similar with native S1 protein in *Bordetella pertussis* 18323(ATCC 9797). Both SDS-PAGE bands (rS1) were confirmed using monoclonal and polyclonal antibodies against pertussis toxin by western blot analysis. So, they were rS1 but with different molecular weight.

Two explanations could be the reasons for expression of rS1 in two different bands with about 3 *kDa* difference. The construct does have leader or signal peptide sequence (102 *bp*) in the beginning of amplified S1 gene (similar to native S1 gene in *Bordetella per-*

tussis). Because of 85% homology between the leader peptide of *Bordetella pertussis* and *E.coli* ⁽¹²⁾, probably this leader peptide was recognized and digested by *E.coli* specific leader peptidase partially. Thus, two different rS1 did appear on SDS-PAGE electrophoresis.

H. Pande et al and H. Carbonetti et al in two separate studies reported that the S1 subunit is unstable against non specific intracellular proteases ^(14,15). H. Pande et al study indicated the maximum instability did exist between 30°C to 37°C ⁽¹⁴⁾. Thus, this non specific digestion could be the second possible hypothesis for observing two distinct bands detectable with anti PT antibodies.

Conclusion

In future, we are going to evaluate the immunogenicity of these fragments (*in vivo* and *in vitro*) in order to investigate the possible protectivity as candidate immunogens for acellular pertussis vaccine. In a parallel study the antigenicity of them as a specific marker of anti pertussis antibodies could be investigated for detection of any current or previous pertussis infection.

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