Expression and Purification of Recombinant Outer Surface Protein D of *Borrelia Burgdorferi*

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Summary
To carry out the immunological experiments on the serum of Multiple Sclerosis (MS) patients, based on a correlation between *Borrelia burgdorferi* infection and contracting MS autoimmune disease the outer surface protein D (OspD) of the bacterium was expressed and purified. A clone containing the OspD gene in pET11a expression vector under the control of T7 promoter was transformed to the bacterial host *BL21* (*DE3*). Some of the colonies were selected for IPTG-induced expression. The colony with the highest amount of OspD was selected for large-scale expression. Large-scale protein purification was performed by the reversed phase HPLC with a C4 preparative column; ultimately, the expressed purified protein was confirmed by the Western blot technique.

Key words**:** *Borrelia burgdorferi*, OspD, expression, purification, HPLC

Introduction

Borrelia burgdorferi (*B.burgdorferi*) is one of the pathogenic spirochetes that cause Lyme disease in human. Lyme and Multiple Sclerosis (MS) have some common symptoms. On the other hand, some reports show immunological responses against *Borrelia* proteins in the serum of MS patients (Marrshall 1988, Burns 1999, Hausmann & Wucherpfennig 1997). Screening a human fetal spinal cord cDNA library, prepared in lambda gt11, by the serum of a MS patient showed some

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positive autoantigens, one of which was sequentially homologous with the OspD (Outer surface protein D) of *B.burgdorferi* spirochete (Sanati 1996). OspD is a cellular membrane protein available in the exterior layer of the bacterium. This protein, which has an important role in bacterial virulence, can be useful as an antigenic determinant in the diagnosis of a disease or the performing of cross-reaction tests. OspD has been identified and studied as an efficient lipoprotein in the pathogenic role of the *B.burgdorferi* (Garren *et al* 1998). Screening different isolates with the polymerase chain reaction (PCR) method showed that the OspD gene is present in 66% of European–Asian isolates and also in 25% of North American isolates (Marconi *et al* 1994). Since plasmids are able to move between bacteria of the same species, and even to move to other species of bacteria (Hinnebusch & Tilley 1993), there are important implications in having a plasmid, specially a plasmid that encodes a surface protein involved in antigenicity.

In this study we were produced the recombinant form of OspD in large amounts in order to perform some immunological experiments in MS patients. Also, we were encouraged by these data to conduct research on the correlation between the infection of *B.burgdorferi* and contracting MS autoimmune disease.

Materials and Methods

Vector. The cloned OspD gene in pET11a (Novagen, Madison's, WI, USA) expression vector under the control of T7 promoter, a gift by Dr. Ali Karami (PhD Thesis, Medical University of Copenhagen, Denmark), was used as a main source for protein production **(**pET System Manual, Norris *et al* 1992).

Microorganisms and media. The *BL21* (*DE3*) strain of *E.coli* taken from the bacterial strains collection of the National Research Center for Genetic Engineering and Biotechnology (NRCGEB), Iran was used as host. It was cultured in LB medium, incubated overnight at 37˚C, and then stored in an LB medium containing 30% glycerol at -70˚C. Competent cells were prepared from this host strain by the

calcium chloride standard method (Norris *et al* 1992) and were used freshly, or kept at -70˚C for future uses. The selective media were prepared by adding ampiciline (100µg/ml) or carbenicillin (50µg/ml).

PCR. *B.burgdorferi* plasmid DNA was purified by the alkaline lysis method (Norris *et al* 1992). The universal primers of pET11a vector were used to check the clone. A 50µl PCR reaction mixture containing 5µl 10X *Taq* DNA polymerase buffer, 1.5µl 10mM dNTPs mix, 2µl 50mM $MgCl_2$, 3µl of 5pmol/µl primers mix, 100ng plasmid DNA as template and 1.25U *Taq* DNA polymerase was subjected to amplification. A hot start process, 94˚C for 4min was followed by 35 cycles of 94˚C for 1min, 56˚C for 1 min and 72˚C for 1min. The last cycle was continued by a final extension at 72˚C for 10min.

Protein expression. Single colonies of *BL21* (*DE3*) bacteria transformed by recombinant pET vector containing OspD gene (pETOspD), were picked up from cultured plates. Transformation of competent *E.coli* cells was carried out by heat shock method (Norris *et al* 1992). 10ml LB containing 50µg/ml of carbenicillin was inoculated and incubated at 37˚C with 200rpm shaking. The growth of culture was monitored by an optical density (OD) at 600nm of approximately 0.8–1. An aliquot (about 2ml) was removed from each sample as an uninduced control. IPTG was added to the final concentration of 1mM. To control the protein expression 0.5ml samples were removed every 30min. Bacteria were precipitated in each sample by centrifugation (5000*g* for 5min at 4˚C). Bacterial pellets were resuspended in the proper volume of sample solvent containing 2% SDS and 2-Mercaptoethanol, boiled and spun, and the supernatants were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The highest expressing colony was determined by this method and kept for large-scale protein expression.

Large-scale expression. A loop of high expressing bacterial colony stock was added to 10ml of LB medium containing 100µg/ml ampicillin and incubated at 37˚C with vigorous shaking (200rpm) approximately for 3h to an OD of around 0.8 at

600nm. This culture was used for inoculation of culture media. A large volume culture (1lit) was grown in 4-liter flask at 37˚C with shaking until the OD at 600nm reached 0.6. Then 10ml was excluded as an uninduced control. IPTG was added to the culture to a final concentration of 1mM. Shaking incubation was continued at 37˚C for 4h. Cells were precipitated by centrifugation (5000*g* for 5min at 4˚C) and resuspended in a lysis buffer containing 10mM Tris-Cl, 5mM EDTA, 1% NaCl and Triton X-100 pH7.5. The mixture was sonicated 4×20s in high power on ice with 60-s intervals, and then it was centrifuged (10000*g* for 10min at 4˚C). The supernatant containing recombinant OspD was stored at -20˚C.

OspD purification. The protein content of each bacterial colony was denatured by boiling in a sample solvent and isolated by centrifugation in supernatant before and after induction with IPTG. The proteins in the soluble phase were precipitated by a 12% TCA and, after being dissolved in a sample solvent, were run on 12.5% SDS-PAGE, along with proteins in the insoluble phase. Finally, the purification process was performed on proteins in the soluble phase. Soluble proteins were freeze-dried and then used for OspD purification by different methods. At first the ion exchange chromatography on DE52 column by an upward serial concentration of NaCl washing and gel filtration (size exclusion) on Sephacryl S300 were used. Finally the HPLC technique with a C4 reversed phase column was used (Sambrook *et al* 1989). HPLC separation was performed by an acetonitril mixture with 0.1% TFA (tri-fluoro acetic acid) from 0 to 100 (with a 95% gradient) in 30min. The isolation process was carried out in large scale on a preparative C4 column with 2.1×25cm dimensions (River *et al* 1983, Huhmer 1997). After lyophilization the collected fractions were analyzed by SDS-PAGE and silver nitrate staining.

Western blotting. After SDS-PAGE analysis, the proteins were transferred to a nitrocellulose membrane by semi–dry apparatus (LKB Pharmacia) to hybridize with an antibody probe. The membrane was soaked in 5% skimmed milk at room temperature for 2h with shaking to block the remaining binding sites. Pre–absorbed

anti–*Borrelia* serum (Harlow & Lane 1999) obtained from a Lyme patient was used to detect OspD. The serum was pre-incubated with *E.coli* antigens without OspD. The membrane was treated with anti-*Borrelia* serum for 2h at room temperature and overnight at 4˚C with gentle shaking. Rabbit anti-human antibody conjugated with horseradish peroxidase (HRP) was used as a secondary antibody (1:4000) to probe the membrane for 1.5h at room temperature. Finally, the membrane was developed using 4-Chloro naphtol and hydrogen peroxide as substrates (Doonan 1996).

Results

Figure 1a shows the PCR products of two clones using the universal primers of pET11a vector. The size of the plasmid containing the OspD gene is 6364bp (this vector is 5677bp without an insert). This construct has two restriction sites for *Eco*RV and *Pst*I; one site in the OspD gene and the other one in the plasmid. The *Eco*RV cutting fragments are 4238bp and 2126bp, and the *Pst*I fragments are 4893bp and 1472bp. The extracted plasmids were examined by restriction digestion after being transformed to the *BL21* (*DE3*) expressing strain (Figure 1b).

a **b**

Figure 1. **a**) *PCR product of two clones of OspD gene by universal primers of pET11a vector); A) DNA size marker, B and C) PCR products*. **b**) *Restriction digestion analysis of pET11a–OspD; A) DNA size marker, B) Uncut pET11a-OspD plasmid (BL21 Host), C) EcoRV digested plasmid lane B, D) PstI digested plasmid lane B*

Figure 2 shows the protein expression of the three positive colonies. The colony no.4 has a relatively high expression of a 28.5KD protein in comparison with the uninduced one. The amount of expression was estimated by gel scanning method, which was 11.6% of the total bacterial proteins. Hourly sampling after induction with IPTG showed maximum expression in the fourth hour. The result of gel scanning showed that some expressed proteins were in soluble and some in the insoluble phase. Due to the relatively easier methods of working with the soluble phase and the high amount of proteins presented in this phase, the purification process was performed on soluble phase proteins. The quantity of the extracted OspD protein by the use of an anionic exchange DE52 column was very low; so size exclusion (gel filtration) chromatography on Sephacryl S300 beads was tried. Figure 2b shows the result of this purification.

a **b**

Figure 2. **a**) *SDS-PAGE of expressed OspD protein at different hours; A) total lysate of bacteria composed non recombinant pET11a, B) protein size marker (cytochrome C [12300], myoglobin [17200], carboanhydrase [30000], ovalbumin [45000]), C) uninduced pET11a-OspD control, D-G) expression at 1st to 4th hour after induction.* **b**) *Silver staining of size exclusion purified OspD; A) induced bacterial total protein, B and C) purified OspD protein (28KD), D) protein size marker.* b) *Silver staining of size exclusion purified OspD; A) induced bacterial total protein, B and C) purified OspD protein (28KD), D) protein size marker*

Finally, the reversed phase HPLC was used for the OspD extraction (Figure 3). It shows highly purified OspD proteins with a main sharp peak. Finally, the purified protein was analyzed by Western blotting (Figure 4).

Figure 3. *Silver staining of final HPLC isolated OspD; A) Protein size marker, B) Final extracted OspD, C & D) Two other HPLC fractions*

Figure 4. *Western Blotting of size exclusion purified OspD*

Discussion

OspD is one of the most antigenic proteins of *B.burgdorferi* spirochete. Expression of this protein in the prokaryotic system is a suitable method for preparing large amounts of OspD for various immunological experiments on the serum of MS patients. It expresses as the internal cytoplasmic system because it's gene has been cloned without any signal sequence, and also there is no pelB leader sequence in the vector for conducting the expressed protein to the periplasmic space of the bacteria.

In this study the presence of an insert with a size around 700bp was confirmed by PCR. *Eco*RV and *Pst*I restriction enzymes were chosen to check the presence of cloned genes in the plasmid for two reasons. First, the pET11a has only one restriction site for the enzymes and the second, cutting sites of the enzymes are in

the OspD cloned gene. Therefore, getting two fragments in the required sizes confirm the existence of the OspD gene in pETOspD. To achieve a higher level of confidence, direct methods such as Southern blot and DNA sequencing may be required.

There are different biochemical methods for isolating an expressed protein from bacteria. The best way is a combination of different types of chromatography; gel filtration for isolation according to the size of proteins, ionic exchange for isolation based on the average charge of proteins, and also the reversed phase according to the difference in hydrophobicity of different proteins. Our results showed that the reversed phase HPLC had the best efficiency (qualitatively and quantitatively) for the purification of OspD. The electrophoresis technique was employed to check the protein expression. A sample of molecular weight markers and a negative control (bacteria with a plasmid without the OspD gene) were run on the gel parallel with the positive clones. SDS-PAGE analysis of different expressing clones shows an increasing expression of protein around the molecular weight of the desired protein at different hours after induction. The final confirmation can be achieved by the Western blot technique. The assay was performed with the serum of a Lyme patient. Of course, before applying this serum, it was pre-incubated with *E.coli* antigens without OspD in order to eliminate any cross-reaction with any non-specific protein. The purity of the expressed protein was confirmed by the Western blot technique.

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