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ORIGINAL ARTICLE

Cloning, expression and purification of the factor H binding protein and its interaction with factor H

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ABSTRACT

Background and Objective: *Neisseria meningitidis* is a leading cause of meningitis and sepsis worldwide. The factor H binding protein (fHBP) is a key virulence factor of *Neisseria meningitidis* that is able to selectively bind to human factor H, the key regulator of the alternative complement pathway, which it has important implications for meningococcal pathogenesis and vaccine design. The aims of present research were cloning, expression, purification of fHbp and confirmation of the interaction between serum factor H (fH) and produced factor H binding protein.

Materials and Methods: A 820 base pairs *fh*bp gene fragment was amplified by PCR and cloned into expression vector pET28a (+) in *Bam HI* and *SalI* restriction enzymes sites. Recombinant DNA was expressed in BL21 (DE3) cell. fHBP protein was purified by Ni-NTA agarose resin. Coupling of recombinant protein into CNBr activated Sepharose 4B resin was carried out for application in serum fH protein purification. (fH-fHBP) interaction was confirmed by SDS-PAGE and far-western blotting.

Results and Conclusions: SDS-PAGE results showed a 35 kDa protein band. 150 kDa fH protein was purified by designed Sepharose 4B resin. Far-western blotting confirmed (fH-fHBP) interaction and proper folding of factor H binding protein.

Keywords: *N. meningitidis,* FHBP, Cloning, FH protein, Far-western blotting.

INTRODUCTION

Neisseria meningitidis is a Gram-negative microorganism and an exclusive human pathogen that usually exists in an asymptomatic nasopharyngeal carriage state. However, *N. meningitidis* can cause devastating invasive disease, such as septicemia or meningitis, following penetration of the mucosal tissue, invasion of the bloodstream, and colonization of the meninges. The virulence of *N. meningitidis* is influenced by multiple factors: capsule polysaccharide and surface adhesive proteins expression (outer membrane proteins including pili, porins PorA and B, adhesion molecules Opa and Opc), iron sequestration mechanisms, and endotoxin (lipooli-

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gosaccharide, LOS) (1). fHBP was initially identified as a surface-exposed lipoprotein with unknown function named GNA1870 (genome-derived Neisseria antigen 1870) (2). The complement system plays an important role in innate immune defense against pathogenic microbes. Factor H is a 150-kDa soluble protein that is the main regulator of the alternative pathway (AP) which it is secreted in the nasopharnyx (3). Some microbes and viral pathogens such as *Echinococcus granulosus*, *Onchocerca volvulus* and *HIV*, avoid complement-mediated killing system by recruiting fH to their surfaces (4). Pioneering study demonstrated that the interaction of fH with the meningococcus GNA1870 (fHBP) is the principal of fH-binding meningococcal protein (5). Most (fH-fHBP) interaction studies have been done *in vivo* (5). Therefore we attempted to produce recombinant fHBP and investigate it *in vitro*. All together the production of recombinant proteins are one of the biotechnology special skills for drug or vaccine and nutrition science researches. Currently, recombinant proteins are found fundamentally in every medical testing and biological research laboratory (6). So the recombinant fHBP will be applied in medical sciences like the design of diagnostic kit or meningococcus vaccine preparation.

MATERIALS AND METHODS

Cloning of fHBP gene into pET 28a (+). The *fhbp* gene was selected based on the *fhbp* gene sequence in the GenBank accession nos. ACA52540.1. It was synthesized into pGH plasmid (Bioneer, Korea). The *fhbp* gene was amplified from pGH plasmid by specific primers. Amplification was carried out in 30 cycles of 40 sec at 94 °C, 40 sec at 64 °C and 1 min at 72 °C. The PCR product was confirmed by nucleic acid sequencing. Following the initial confirmation, PCR product and pET28a (+) (Novagen, USA) were digested with *BamHI* and *SalI* (Fermentas, Lithuania). Ligation was carried out with T4 DNA Ligase (Fermentas, Lithuania). The ligation reaction was transformed into *E. coli* Top10 competent cell (7). The recombinant plasmids were confirmed by colony PCR and restriction enzyme analysis.

Expression of the recombinant *fhbp* gene. *E. coli* BL21 (DE3) was used for protein expression as host with 50µg/µl kanamycin (Merck,Germany) in LB medium for selection, 0.5 mM IPTG (Isopropyl-beta-D-thiogalacto-pyranoside) (Merck,Germany) as inducer. Samples were collected 3, 5, 7, and 9 hours after induction. The cells were harvested, treated with lysis buffer (50 mM Tris base, 10% glycerol, 0.1%Triton X-100) (Merck, Germany) and the lysate was analyzed by SDS-PAGE and the quantity of the expressed protein was estimated by comparing the intensity of the protein bands.

Western blot analysis. Proteins resolved by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane (Wathman, UK). TBS buffer (Tris-Buffered Saline containing 3% BSA (Bovine Serum Albumin) (Sigma, USA) was used for blocking the membrane. The membrane immersed in 1:2000 dilution of ALP (alkaline phosphatase) conjugated anti His-tag monoclonal antibody (Abcam, UK) 2 hours at room temperature. Subsequently, it was visualized for color after development in NBT/BCIP substrate solution (Roche, Germany).

Purification of the protein. The recombinant protein purification was carried out by Ni-NTA column as specified by the manufacturer's instructions (Novagen, USA). The purified protein was subsequently analyzed by SDS-PAGE and western blotting using anti His-tag antibody.

Coupling of recombinant protein into CNBr activated Sepharose 4B and purification of fH serum protein. For studying of (fH-fHBP) interaction, purified fHbp protein was coupled to CNBr activated Sepharose 4B by the manufacturer's instructions (Novagen,USA). 500 µl of human serum was dialyzed by coupling buffer (NaHCO3 0.1 M, NaCl 0.5 M, PH 8). The affinity chromatography was performed for isolation of fH protein from serum sample. It was eluted by two different pH buffer (Glycin,NaCl, pH:2 and Diethanolamin,NaCl pH:11) (Merck,Germany).

Far Western blot analysis. The human serum proteins as factor H source, was separated under non-reducing conditions by 10% SDS-PAGE. The protein bands transferred to nitrocellulose membrane. After membrane blocking with 5% skim milk, the blots were immersed for 2 hours in purified buffered fHBP(PBS/0.5%Tween20/0.5% skim

milk). The eluted factor H was analyzed by western blotting using an ALP-conjugated anti-His tag antibody (Abcam, UK) (8).

RESULTS

The preparation of gene fragment. The *fhbp* gene was amplified with specific primers as shown in Fig. 1. The pET28a/fHBP plasmid was confirmed by specific PCR, universal PCR and restriction analysis (Fig. 2).

Fig. 1. Specific PCR product of *fhbp* gene: Lane M, 100bp DNA ladder ; Lane 1, 860bp PCR product.

Expression of fHBP recombinant protein. Recombinant protein expression was confirmed by western blotting (Fig. 3A). The optimum incubation time after induction was considered 3 hours (Fig. 3B).

Purification of the recombinant protein. Purification of the recombinant protein was carried out using Ni-NTA affinity column (Fig. 4A). The expected protein band was obtained in eluted fraction and confirmed by western blotting (Fig. 4B).

Purification of serum fH protein. Serum factor H protein was purified using CNBr activated Sepharose 4B coupled to fHBP. It was analyzed by SDS-PAGE and a ≈150 KD protein was detected (Fig. 5)

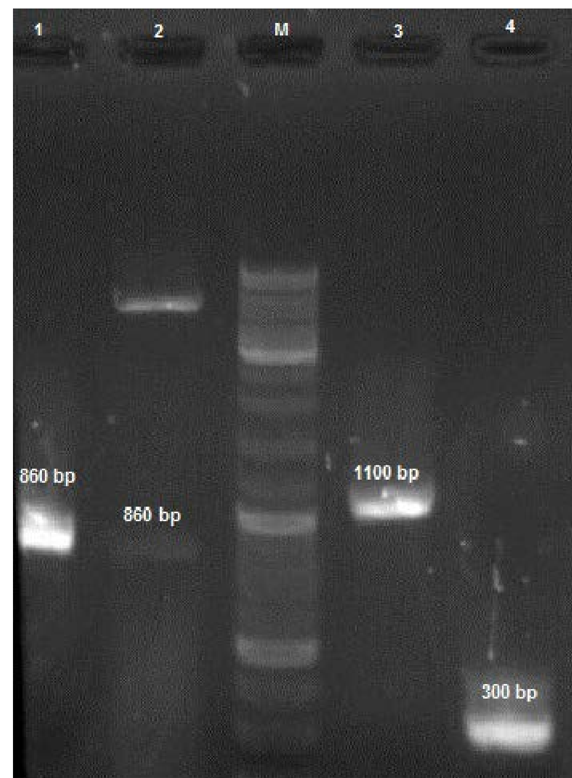

Fig. 2. Confirmation of gene cloning: Lane 1, specific PCR product of fhbp gene; Lane 2, double digestion of recombinant plasmid by *BamHI* and *SalI restriction enzymes*; Lane M, 100bp DNA ladder; lane 3, *fhbp* gene PCR product by plasmid universal primers; Lane 4, universal PCR product of pET 28a (+) as negative control.

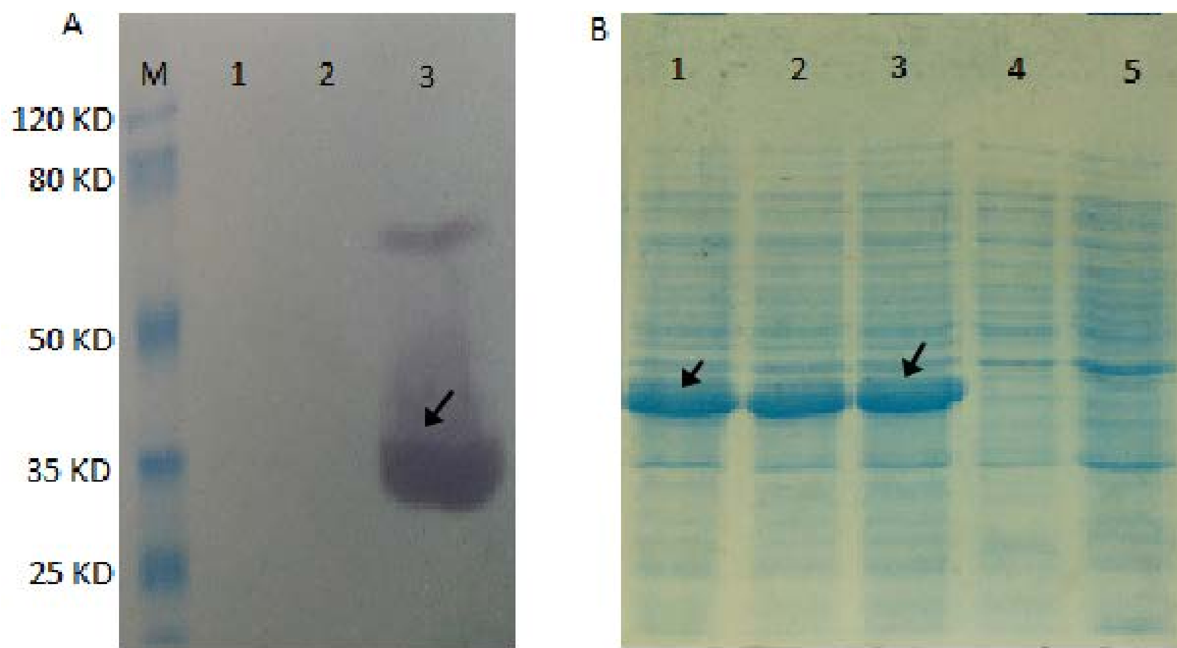

Fig. 3. SDS-PAGE and Western blot analysis of the recombinant protein expression at different times: (A) Lane M, molecular weight marker,Lane1, BL 21; Lane 2, 0 time; Lane 3, 3h after induction. (B) Lane 1,3h ; Lane 2, 5h ; Lane 3; 7h after induction with 0.5 mM IPTG. Lane 4, BL 21 cell; Lane 5, 0 time.

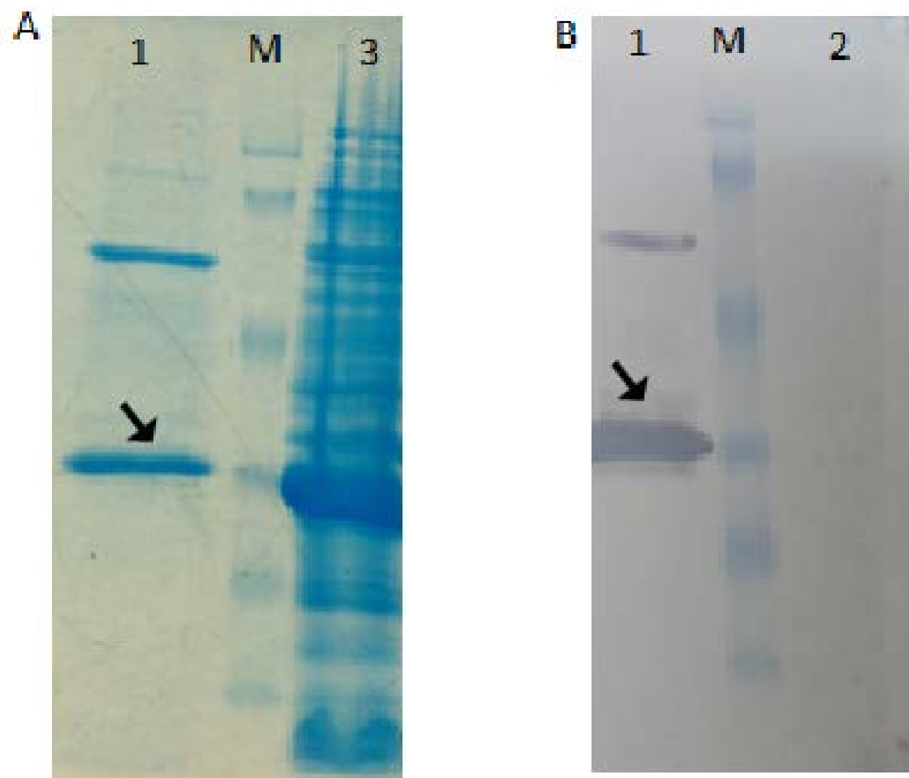

Fig. 4. SDS-PAGE and western blot Analysis of the purified recombinant protein after purification: (A) Lane 1, purified protein; 2; Lane M, molecular weight marker; Lane 3, bacterial lysate after 3h incubation. (B) Lane 1, purified protein; Lane M, molecular weight marker; Lane 2, BL21 cell lysate.

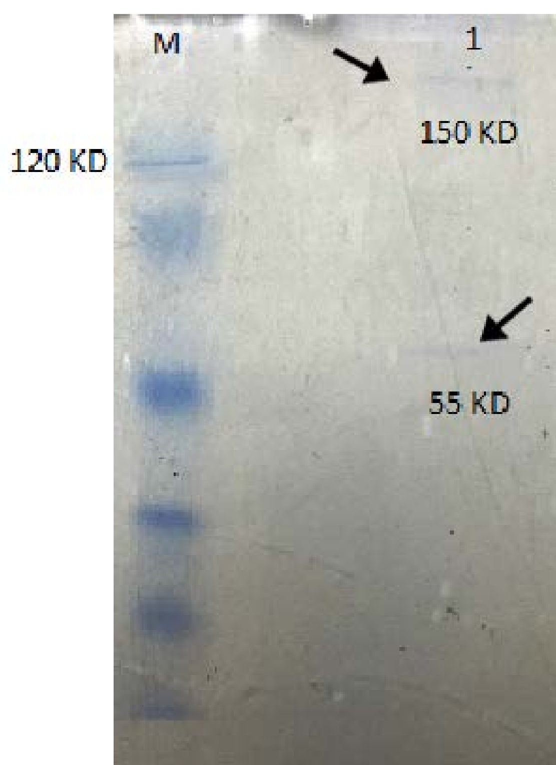

Fig. 5. Western blot analysis of the purified fH protein by affinity chromatography: Lane M, molecular weight marker, Lane 1, purified factor H protein

Far Western Blot analysis. fH-fHBP interaction was determined by anti-His tag antibody. A band with approximate size of 150 KD appeared on the membrane (Fig. 6).

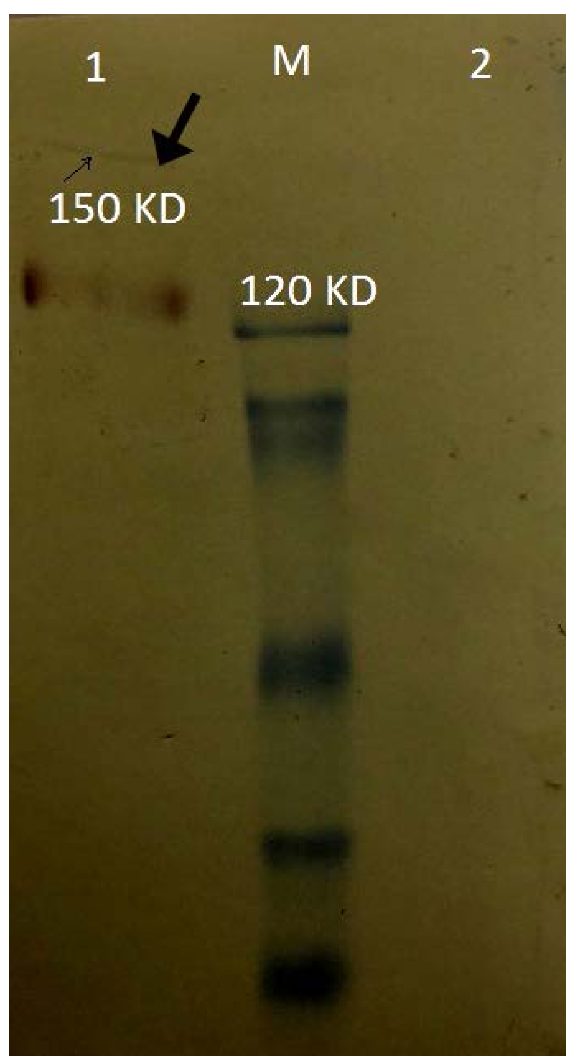

Fig. 6. fH-fHBP binding confirmation by Far western blot analysis:
Lane1, confirmed fH-fHBP complex with anti his tag antibody; Lane M, molecular weight marker; Lane 2, mouse serum as negative control.

DISCUSSION

Neisseria meningitidis is an obligate human pathogen and important cause of septicemia and meningitis that it is known a major cause of morbidity and mortality worldwide. To cause disease, the bacterium must successfully survive in the bloodstream where it has to avoid being killed by host innate immune mechanisms, particularly the complement system (9). Most *N. meningitidis* strains studied to date, express fHBP, with levels of expression that vary significantly among isolates (2). Humans clear meningococci via both the classical and alternative complement pathways (10). Factor H is a 150-kDa glycoprotein typically presents in human plasma at concentrations of 300 to 500 µg/ml (11). fH is the main inhibitor of the alternative complement pathway and a key discriminator between host and pathogen cells. Many pathogens, however, have evolved to evade this alternative complement pathway by binding fH on the bacterial surface. Although fH binding proteins have been identified in many pathogens, the sequence and structure of fHBP are unique to *N. meningitidis* and a few other neisserial species (12). Interestingly, fHBP only binds human fH, which may be one explanation of the species-specificity of meningococci for the human host (13). The ability of fHBP to bind human fH with high affinity has implications not only for its role as a

virulence factor *in vivo*, but also it can be as a target antigen for vaccination against meningococcus. Anti-fHBP antibodies can elicit protection by two mechanisms: direct complement-mediated killing of the bacterium and blocking fH binding to the bacteria to increase the susceptibility of the bacterium to be killed by the alternative complement pathway (5). Several studies have shown that inhibition of fH binding to fHBP results in increased susceptibility of the bacteria to complement-mediated bactericidal activity (14). The main objective of present study was producing of recombinant fHBP and fH-fHBP interaction study *in vitro*. Confirmation of this step, can be used in the field of vaccine and specific antibodies production. Prokaryotic systems are powerful tools for production of this recombinant protein. *Escherichia coli* is one of the best micro organisms of choice in this field. It is a factory and well-established micro organism for the most popular expression platform. Recombinant DNA is extensively used in biotechnology, medicine and researches (15). For this reason, pET expression system has been developed for the cloning and expression of recombinant proteins in *E. coli* based on the T7 promoter (16). So, successful sub cloning of 820 bp fHBP gene into pET 28a (+) expression vector was carried out by enzymatic digestion. Western blotting technique using Anti-6X His-tag antibody confirmed 35 kDa molecular mass recombinant protein expression. Purified protein by Ni-NTA agarose resin showed two distinct monomer and dimer bands on gel and western blotting. There were two ways to check (fH-fHbp) interaction. One of them was designing of new affinity chromatography which resin binds to recombinant fHBP. CNBr activated Sepharose 4B is able to coupling with some proteins. Therefore, by activation of resin according to manufacturer's protocol, fHbp was coupled into resin. fH is a ligand for fHBP (5) and it has typically 150-kDa glycoprotein presents in human serum at concentrations of 300 to 500 μg/ml (11, 17). Because of unique structure of this recombinant protein and specific coupling condition, its purification will be very specific. Rickard Nilsson in 2013 described an affinity chromatography system, based on the HVR of the M5 protein, allowing efficient, single-step purification of fH from human serum (8). Another way to check fH-fHBP interaction was far-western blotting. The binding of fH to surface of sero groups A, B, and C of *N. meningitidis* strains were detected by FACS and Far-Western blot analysis (18). Far-western blotting (WB) was derived from the standard WB method to detect protein-protein interactions *in vitro*. In Far WB, proteins in a cell lysate containing prey proteins are firstly separated by SDS or native PAGE, and transferred to a membrane, as in a standard WB. The proteins in the membrane are then denatured and renatured. The membrane is then blocked and probed, usually with purified bait protein (s). The bait proteins are detected on spots in the membrane where a prey protein is located, if the bait proteins and the prey protein together form a complex. Compared with other biochemical binding assays, Far WB allows prey proteins to be endogenously expressed without purification, and determines whether two proteins bind to each other directly (19, 20). So, to demonstrate fH-fHBP interaction, we successfully used Far-Western blotting. In present study, human serum fH protein was used as a prey protein and purified recombinant fHBP as a bait one. Recombinant fHBP consists of 6X-His tag peptid, which is detectable with conjugated Anti-6X His-tag antibody.

Based on the results of this study, considerable amount of recombinant fHbp protein was expressed in *E. coli* BL21 (DE3). It was used for designing a proper chromatography column to purify a large amount of the factor H protein.

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