EXPRESSION AND PURIFICATION OF EPITOPE FLAGELLAR ANTIGEN H:1,2 (FljB1) FOR DIAGNOSISTIC *SALMONELLA* KIT DEVELOPMENT

Nguyen Thi Trung, Do Thi Huyen, Truong Nam Hai

Institute of Biotechnology, Vietnam Academy of Science and Technology

Received May 15, 2010

1. INTRODUCTION

Salmonella typhimurium is one of the two main *S. enteritica* serovars causing food poisoning for the human [24]. Until recently, the most common cause of food poisoning by *Salmonella* is due to *S. typhimurium*. The disease is characterized by diarrhea, abdominal cramps, vomiting and nausea, and generally lasts up to 7 days. Unfortunately, in immunocompromized people, that is the elderly, young, or people with depressed immune systems, *Salmonella* infections are not treated with antibiotics [23].

Salmonella typhimurium is primarily associated with cattle but it has spread to a range of food animals, including pigs, sheep and poultry. Chickens that survive the initial infection are lifelong carriers of the *S. typhimurium* and have no sign of an illness, which are very difficult to demonstrate by microscopic examination specifically. Currently, no drug or vaccine is available to clear the *S. typhimurium* completely or prevent the chickens from the *S. typhimurium* infection. Due to the widespread occurrence of *S. typhimurium*, diagnosis and prevention of that disease is important in areas of both farm and factory. We must anticipate that it may become more widespread in the environment and hence throughout the food chain. We must regard to an 'emerging' pathogen and monitor the situation accordingly [22]. Therefore, it is necessary to develop a reliable, sensitive, specific, and inexpensive immunodiagnosis kit to detect both acute and latent infections with the *S. typhimurium*.

For immunodiagnosis, sensitivity, specificity, and cost mainly depend on the antigen. Native crude antigens can non-specifically react to test sera, and preparation on a large scale is very complicated and laborious. These results are partially responsible for the limitation of the complement fixation test (CFT) and have hundred the development of the enzyme-linked immunosorbent assay (ELISA) [1, 14, 19]. Hence, it is quite plausible to use recombinant antigens in detection of *S. typhimurium* infection [15, 20].

Flagella surface antigen plays an important role in host cell recognition, attachment and penetration by *S. typhimurium* [4, 10, 16]. They are, hence, logical targets of host immunue responses [2, 3, 6, 13]. There are two flagella surface antigens (FljB and FliC) in *S. typhimurium* [10, 17]. They are good candidate for a diagnostic regent for the detection of antibody against *S. typhimurium* [12]. The aim of the present study is to construct an ELISA kit for specifically detecting the *S. typhimurium* infected chicken. Firstly, a truncated region of FljB (FljB1) gene encoding only the epitope of flagella antigen was amplified employing template from DNA

genome of *S. typhimurium*, cloned into pET-22b(+) expression vector and then expressed in *E. coli* BL21(DE3). The recombinant strain harbouring pET-FljB1 was investigated in different cultivation conditions such as incubation temperature, induction time, post-induction time, IPTG concentration to improve the protein expression. The recombinant FljB1 protein was purified by affinity column and evaluated for its immunodiagnosis potential by Western blot analysis.

2. MATERIALS AND METHODS

2.1. Materials

E. coli BL21 (DE3) [F-ompT hsdSB (rB- mB-) gal dcm (DE3)] was used as a host cell for the expression of recombinant protein.

S. enteritidis and *S. typhimurium* (donated by National Institute of Veterinary Research, Vietnam) were used for creation of experimentally *S. enteritidis* and *S. typhimurium*-infected chickens respectively.

The pETFljB1 plasmid was used to transform FljB1 gene into *E. coli* BL21 (DE3) strain.

Luria-Bertani (LB) medium (w/v) containing 0.5% yeast extract, 1% tryptone and 1% NaCl [11] was used in seed culture and recombinant protein expression.

Sera samples were collected from 2 chickens experimentally infected with *S. typhimurium* and *S. enteritidis* respectively in 30 days post-infection and 2 healthy chickens were obtained from the Institute of Veterinary Research, Vietnam.

2.2. Methods

2.2.1. Expression analysis

A single colony of *E. coli* BL21 (DE3), harbouring the pETFljB1 vector, was grown in LB medium containing 50 µg/ml ampicillin overnight. The overnight culture of cells was diluted 1:50 in 50 ml LB medium with 50 µg/ml ampicillin in a 250 ml flask and inoculated at certain conditions according to the optimization purpose.

After fermentation, cells were harvested by centrifugation at 5,000 rpm for 25 min, and then resuspended in washing buffer (50 mM sodium phosphate buffer pH 7, 500 mM NaCl) and lyzed by sonication. After centrifugation at 13,000 rpm for 30 min, the supernatant was taken out as the soluble protein sample. The protein sample was checked on 12.6 % polyacrylamide gel (SDS-PAGE).

2.2.2. Purification of recombinant FljB1

The target protein (FljB1) was easily purified by Hitrap-chelating column (Phamacia Biotech, Sweden) due to its His Tag. The affinity column filled with chelating resin was pre-treated and equilibrated with binding buffer (50 mM sodium phosphate buffer pH7, 0.5 M NaCl). After sample loading and equilibration, the His-tagged fusion protein was eluted by the elution buffer (20 mM sodium phosphate buffer pH7, 0.5 M NaCl, 250 mM Immidazole, pH 7) at 1 ml/min by linear gradient by explore FPLC system.

2.2.3. SDS-PAGE and Western Blot

The samples were boiled for 5 min in a sample buffer (Fermentas, Germany) and subjected to SDS-PAGE standard 12.6%. For Western blot analysis to determine antigencity of FljB1 (binding to natural antigen), proteins were transferred from gel to PVDF membranes (Sigma, USA) by Transfer apparatus (Bio-rad, USA) in Blotting buffer (1.441% glycine; 3% tris; 20% methanol). The blots were incubated with serum samples diluted 1:100 in TTBS (Tris-HCl 1 M pH7.5 25 ml; NaCl 5 M 10 ml; Tween 20, H_2O 500 ml) for 60 min at room temperature and then washed with TTBS three times, 10 min each time; then with TBS (Tris-HCl 1 M pH7.5 25 ml; NaCl 5 M 10 ml, H_2O 500 ml) 5 times, 5 min for each. The membranes were incubated with horseradish peroxidase-conjugated rabbit anti-chicken immunoglobulin G antibody (Sigma, USA) diluted 10^{-5} in TTBS for 60 min. After three washes with TTBS and five washes with TBS, the membranes were exposed to a substrate solution containing 4 chloro 1 napthol (0.2 mg/ml), 17% methanol, 0.03% H_2O_2 in 30 ml TBS to develop the colour and visualize the specific antigen bands.

3. RESULTS AND DISCUSSION

Recently, some soluble expression strategies are introduced to express functional proteins efficiently. The downstream purification procedure will become simpler because the soluble protein is always accurate-folded, and the refolding procedure will be avoided. Therefore, soluble expression is an attractive alternative for heterologous expression in bacteria. Fusion expression and optimization of cultivation condition are the strategies widely used [5, 21]. In this work, the *fljB1* gene was cloned into pET22b(+) vector and expressed efficiently as high soluble protein with molecular weight 16 kDa in *E. coli*. To further improve FljB1 productivity, the conditions of cultivation and induction were also optimized systematically. The conditions for some flagella expression in our work were adopted for this FljB1 expression. These conditions were listed in the following: 1 mM IPTG, induction of the OD_{600}= 0.6 at 28°C for 5 h [7, 8, 9]. The protein was purified and assessed the ability to bind to natural antibody.

3.1. The effects of different expression conditions

3.1.1. Cultivation temperature

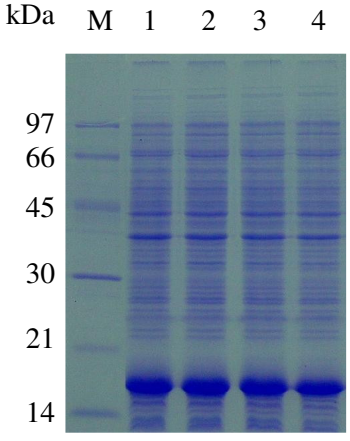

Fig 1. SDS-PAGE analysis of FljB1 protein expression under different temperatures

Lane 1-4: the soluble protein cultured at 28℃, 30℃, 32℃ and 37℃, respectively; M: molecular weight marker

The temperature affects recombinant protein expression significantly, especially for soluble protein expression [18]. The recombinant strain was cultivated in LB medium at different induction temperatures (37°C, 32°C, 30°C and 28°C). The expressed proteins due to the

temperature effect were determined by SDS-PAGE (Fig. 1). The results revealed that the target protein was achieved a high concentration in soluble proteins at 30°C; thus, the following cultivation was carried out at 30°C.

3.1.2. Induction timing

In the process of recombinant protein expression in *E. coli*, IPTG induction is the turning point between cell growth and recombinant protein synthesis. The addition of IPTG triggers the transcription of foreign gene in the plasmid and, consequently, brings great changes to the metabolism of host cells by initiating the translation of heterologous protein. The effect of induction timing was evaluated by adding IPTG at different stages of growth phase according to this growth profile. Comparison of expression level of soluble target protein indicated that the amount of soluble protein varied in a wide range of induction time. The maximum yield of recombinant protein was obtained when induced at an OD_{600} value of 0.6.

3.1.3. IPTG concentration

For the expression vector with a T7 Lac promoter, final IPTG concentration should be optimized because of its great contribution to recombinant protein expression and serious harm to cell growth [5]. The final concentration of IPTG was examined from 0, 0.1, 1, 10, 100, 1000, 50.000 µM. The result of IPTG concentration examination has shown that the recombinant protein was synthesised very effectively even though at the low concentration (0.1 µM IPTG) (Fig 2). Thus, this will be reduced the cost of product due to IPTG is an expensive substrate.

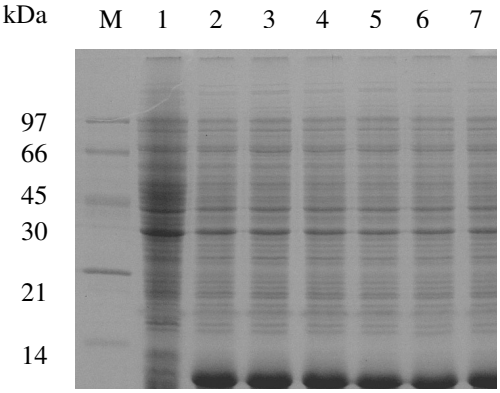

Figure 2. SDS-PAGE analysis of effect of IPTG concentration on the expression level
Lane 1: soluble protein without IPTG; 2-7: soluble protein induced with IPTG at final concentration is 0.1, 1, 10, 100, 1000, 50 000 µM, respectively;M: molecular weight marker

3.1.4. Post-induction time

After adding the IPTG into the medium, the target protein begins to synthesize. However, the accumulation of target protein is not proportional to the expression time. The optimal expression time was determined by analyzing samples taken at 1, 2, 3, 4, 5 and 18 h after induction at the optimized conditions. The result was analyzed by SDS-PAGE (Fig. 3). The product reached the highest concentration after a 4 h induction. Overtime expression influenced the

productivity and decreased the amount of recombinant protein in total soluble proteins.

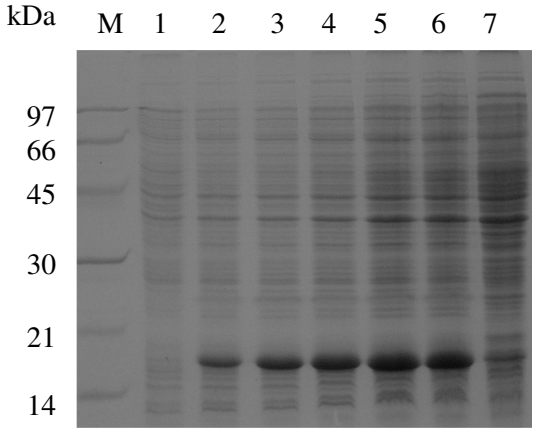

Figure 3. SDS-PAGE analyses the expression level of FljB1 in *E. coli* at the different post-induction time.
Lane 1-7: soluble protein collected at the 0, 1, 2, 3, 4, 5 and 18 post-induction, respectively; M: molecular weight marker

3.2. FljB1 Purification

As above present, *fljB1* was inserted into the pET22b(+) vector, thus *E. coli* synthesized recombinant protein FljB1 has 6 C-terminal histidine residues. FljB1 protein from cellular extract was purified by affinity chromatography on Hitrap chelating resin. Effectiveness of purification was determined by SDS-PAGE (Fig. 4). The result revealed that the fusion protein with a high purity was achieved after a one-step affinity purification process. In the downstream purification, nickel-affinity chromatographic operation increased the percentage of fusion protein with $(His)_6$ tag from 64 to 85% [18].

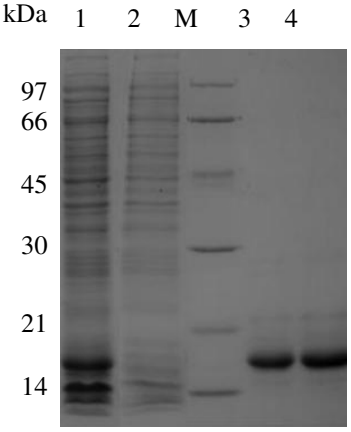

Figure 4. SDS-PAGE analyses the FljB1 purification
Lane 1: Crude cellular lysate of the soluble protein of recombinant strain harboring fljB1; 2: non-binding protein; 3, 4: Elution fraction after affinity chromatography (FljB1 protein); M: molecular weight marker

3.3. FljB1 binding ability with natural antibody

Protein FljB1 purified by affinity chromatography was checked the ability of the binding to natural antibody by western blot. Serum collected from 2 infected experimentally with *S. typhimurium* and *S. enteritidis* respectively, 2 healthy chickens were used to assess the specific binding with FljB1. The specific antibody response to FljB1 was detected only in sera from *S. typhimurium*-infected chickens (Fig. 5) but not in sera from *S. enteritidis*-infected and healthy ones.

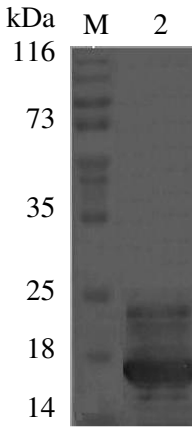

Figure 5. Western blot analysis the antigenicity of purified recombinant FljB1

Lane 1: reactivity of the purified FljB1 with antibodies in serum from *S. typhimurium*-infected chicken;M: molecular weight marker

The gold standard for one diagnosis of immunology should be a test that is sensitive enough for the early detection of acute infection and the detection of latent infections, specific for the differentiation between the species, and economical with regards to materials and time [1]. So far, no immunological assays with native antigens can meet the standards due to the nonspecific reaction and complicated preparation of the antigen [1, 19]. The researchers usually make capture ELISAs kit (ELISA kit detecting antigen) to reduce the difficult of preparation enough native antigen. But these methods take a long time to run a test, from 36 to 48 h normally. However, they were still cross-reacted with some species in *Enterobacteriaceae*, which are not suitable for some other immunodiagnostic assays.

A bacterial expression system has an advantage over others because the procedures of expression and purification are much easier. This is more relevant when producing a protein on a large scale if its antigenicity will not be impaired by incorrect folding, which may sometimes happen [**Error! Reference source not found.**]. The present study takes advantage of the bacterial expression system. Moreover, the smaller molecular mass of recombinant FljB1 might be an outstanding advantage in development of rapid immunochromatographic assay for diagnosis of *S.* Typhimurium infection.

4. CONCLUSSION

In summary, a truncated *fljB* gene (*fliB1*) was cloned and expressed in *E. coli*. The recombinant protein was over-expressed at 30^{o}C, 0.1 µM IPTG and after 4 h induction. This protein was purified with the high purity under native conditions by affinity chromatography

using Hitrap chelating column. The FljB1 could be allowed to clearly differentiate *S. typhimurium*-infected chicken sera from *S. enteritidis*-infected or normal chicken sera. This is enough specific for the differentiation between the infection of these Salmonella species causing food poisoning. This is a good basis to build the ELISA kit detecting *S. typhimurium*-infected in chickens and develop some rapid immunochromatographic assay.

*Acknowledgement. T*he work was partially supported by the Program SIDA of Sweden Government through the project "Production of recombinant proteins for medical and agricultural use".

REFERENCE

1. Bruning A. - Equine piroplasmosis: an update on diagnosis, treatment, and prevention, Br. Vet. J., **152** (1996) 139-151.
2. De Almeida M. E., S. M. Newton, and L. C. Ferreira - Antibody responses against flagellin in mice orally immunized with attenuated *Salmonella* vaccine strains, Arch. Microbiol. **172** (1999) 102-108.
3. Hackett J., S. Attridge, and D. Rowley - Oral immunization with live, avirulent *fla_* strains of *Salmonella* protects mice against subsequent oral challenge with *Salmonella typhimurium,* J. Infect. Dis. **157** (1988) 78-84.
4. Kingsley R. A. and Baumler A. J. - *Salmonella interactions with professional phagocytes,* In Bacterial Invasion into Eukaryotic Cells, 321-342. Edited by T. A. Oelschlaeger and J. Hacker, New York: Kluwer Academic/Plenum, 2000.
5. Li P., Zhinan X., Xiangming F., Fang W., Peilin C. - High-level expression of soluble human Beta-Defensin-2 in E. coli, Process Biochem **39 (**2004) 2199-2205.
6. McSorley, S. J., B. T. Cookson, and M. K. Jenkins - Characterization of CD4_ T cell responses during natural infection with *Salmonella typhimurium,* J. Immunol. **164** (2000) 986-993.
7. Nguyen Thi Trung, Do Thi Huyen, Pham Thuy Hong, Truong Van Dung, Truong Nam Hai - Cloning and expression *fljB1* gene encoding for epitope of H:1,2 flagella antigen of *S.* Typhimurium in *E. coli* BL21, Proceeding of Vietnam national conference of Biotechnology, 2003a, pp. 1224-1227.
8. Nguyen Thi Trung, Do Thi Huyen, Pham Thuy Hong, Truong Van Dung, Truong Nam Hai - Cloning and expression *fljB2* gene encoding for epitope of H:1,2 flagella antigen of *S.* Typhimurium in *E. coli* BL21, Vietnam Biotechnology Journal **1** (1) (2003b) 25-31.
9. Nguyen Thi Trung, Do Thi Huyen, Pham Thuy Hong, Truong Van Dung, Truong Nam Hai - Cloning and expression *gm2* gene encoding for epitope of H:g,m flagella antigen of *S.* Enteritidis in *E. coli* BL21, Vietnam Biotechnology Journal **1** (2) (2003c) 144-148.
10. Noel H. Smith and Robert K. Selander - Sequence Invariance of the Antigen-Coding Central Region of the Phase 1 Flagellar Filament Gene (*fliC*) among Strains of *Salmonella typhimurium*, J. Bacteriol. **172** (2) (1990) 603-609.
11. Sambrook J, Russell D. W. - *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory, New York, 2001.
12. Stephen J. McSorley, Brad T. Cookson, Marc K. Jenkins - Characterization of CD_{4+} T cell responses during natural infection with *Salmonella typhimurium*, J. Immunol. **164** (2002)

986-993.

13. Stocker B. A. and S. M. Newton - Immune responses to epitopes inserted in *Salmonella* flagellin, Int. Rev. Immunol. **11** (1994) 167-178.
14. Tenter A. M. and K. T. Friedhoff - Serodiagnosis of experimental and natural *Babesia equi* and *B. caballi* infections, Vet. Parasitol. **20** (1986) 49-61.
15. Toksoy E, Onsan ZI, Kirdar B. - High-level production of TaqI restriction endonuclease by three different expression systems in Escherichia coli cells using the T7 phage promoter, Appl. Microbiol. Biotechnol. **59** (2002) 239-245.
16. Tsolis R. M., Kingsley R. A., Townsend S. M., Ficht T. A., Adams L. G., and Baumler A. J. - Of mice, calves, and men: Comparison of the mouse typhoid model with other Salmonella infections, Adv. Exp. Med. Biol. **473** (1999) 261-274.
17. Vries N. D., Zwaagstra K. A., Huis in't VeLD J. H. J., Knapen F. V., Zijderveld F. G. V., Kusters H. G. - Production monoclonal antibodies specific for the i and 1,2 flagellar antigens of *Salmonella typhimurium* and characterization of their respective epitopes, Appl. Environ. Microbiol. **64** (12) (1998) 5033-5038.
18. Weickert M. J., Doherty D. H., Best E. A., Olins P. O. - Optimization of heterologous protein production in Escherichia coli, Curr. Opin. Biotechnol. **7** (1996) 494-499.
19. Weiland G. - Species-specific serodiagnosis of equine piroplasma infections by means of complement fixation test, immunofluorescence, and enzyme-linked immunosorbent assay, Vet. Parasitol. **20** (1986) 43-48.
20. Xuan X., A. Nagai, B. Battsetseg, S. Fukumoto, L. H. Makala, N. Inoue, I. Igarashi, T. Mikami, and K. Fujisaki - Diagnosis of equine piroplasmosis in Brazil by serodiagnostic methods with recombinant antigens, J. Vet. Med. Sci. **63** (2001) 1159-1160.
21. Zhinan Xu, Zhixia Zhong, Lei Huang, Li Peng, Fang Wang, Peilin Cen - High level production of bioactive human beta-defensin-4 in *Escherichia coli* by solution fusion expression, Appl. Mirobiol. Biotecnol., Biotechnological products and process engeneering **72** (2006) 471–479.
22. http://www.ifst.org/hottop20.html
23. http://www.who.int/medicacenter/factsheets/
24. http://www.Salmonella/org/info.html

TÓM TẮT

BIỂU HIỆN VÀ TINH SẠCH EPITOPE KHÁNG NGUYÊN ROI H:1,2 (FljB1) ĐỂ TẠO KIT CHẨN ĐOÁN *SALMONELLA*

Protein FljB là một trong hai kháng nguyên bề mặt của *S. typhimurium* đã được chứng minh là có khả năng sinh đáp ứng miễn dịch tương đương với tế bào vi khuẩn *S. typhimurium*. Do đó, FljB là một ứng cử viên sáng giá cho việc tổng hợp bằng công nghệ DNA tái tổ hợp để làm nguyên liệu tao kit phát hiện kháng thể kháng lại *S. typhimurium* trong huyết thanh của một số động vật. Nghiên cứu này trình bày sự biểu hiện của protein FljB1 là một vùng gen mã hóa epitope kháng nguyên trong *Escherichia coli* BL21. Protein tái tổ này được tinh chế bằng phương pháp sắc kí ái lực sử dụng cột Hitrap chelating (Pharmacia Biotech, Sweden). Bằng việc

sử dụng vector biểu hiện pET-22b(+) để thiết kế plasmid biểu hiện pETFljB1 chúng tôi có thể nhận được chủng *E. coli* tổng hợp nhiều protein FljB1 ở dạng tan. Năng suất cao nhất đối với protein FljB1 đã đạt được khi chủng *E. coli* mang plasmid pETFljB1 được nuôi trong môi trường LB ở 30^0C; cảm ứng bằng isopropyl β-D-galactopyranoside (IPTG) với nồng độ cuối cùng là 0,1 µM khi OD_{600}= 0,6; và mẫu được thu sau 4 giờ biểu hiện. Sau đó protein này được tinh sạch và kiểm tra khả năng liên kiết với kháng thể tự nhiên bằng phương pháp Western blot. Các kết quả nhận được cho thấy protein FljB1 tái tổ hợp có thể phù hợp để phát triển một thử nghiệm chẩn đoán miễn dịch hiệu quả vì tính đặc hiệu của nó.

Từ khoá: Salmonella typhimurium, FljB, kháng nguyên tái tổ hợp, western blot, tinh chế protein.