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Expression of recombinant major surface protein 5 of *Anaplasma marginale* (*A. marginale*) at different temperatures

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Background : *Bovine anaplasmosis is an important tick-borne disease caused by Anaplasma marginale (A. marginale) and infected in ruminants, mostly in cattle. This disease occurs in tropical and subtropical regions including Thailand and causes a major problem to livestock productions. The major surface protein 5 (MSP5) is one of outer membrane protein of A. marginale which as an immunodominant protein encoded by a single gene and also highly conserved gene.*

Objective : *The aim of this study was to optimize the conditions for the expression of recombinant major surface protein 5 (rMSP5) of A. marginale.*

Methods : *The msp5 gene of A. marginale was cloned into the pET100/D-TOPO® vector to produce an pET100-msp5-6xHis fusion gene construct. The recombinant proteins were expressed by the plasmids in Escherichia coli host strain BL21 star™ (DE3) at different temperatures (16, 25 and 37 ^{o}C) for 6 h. The proteins were analyzed by SDS-PAGE and confirmed the target protein by Western blotting using antisera against His.*

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Results : *After induction with 0.1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) at different temperatures for protein expression, the protein was not produced at 6 h for 16 ^{o}C. On the other hand, the rMSP5 protein was produced at 25 and 37 ^{o}C for 2-6 h but the expressive protein at 25 ^{o}C showed lower yield than that at 37 ^{o}C.*

Conclusion : *In this study, the best condition for rMSP5 protein expression was cultured at 37 ^{o}C for 4 h. The protein was identified as the rMSP5 at the molecular weight of 26 kDa.*

Keywords : *Bovine anaplasmosis, Anaplasma marginale, MSP5, recombinant protein, protein expression.*

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เหตุผลของการทำวิจัย : *Anaplasma marginale (A. marginale) เป็นสาเหตุที่ทำให้เกิดโรคพยาธิในเม็ดเลือดโคและกระบือ (bovine anaplasmosis) ซึ่งก่อให้เกิดปัญหาสำคัญในอุตสาหกรรมการผลิตสัตว์ และส่งผลกระทบอย่างมากทางเศรษฐกิจ Major Surface Protein 5 (MSP5) เป็น immunodominant protein ที่อยู่บนเยื่อหุ้มเซลล์ของเชื้อ A. marginale จึงมีบทบาทสำคัญในการนำมาใช้พัฒนาการตรวจวินิจฉัยโรค bovine anaplasmosis ให้มีความแม่นยำและรวดเร็วมากขึ้น ดังนั้นการศึกษาการผลิต recombinant major surface protein 5 (rMSP5) จึงมีความจำเป็นเพื่อใช้ในการศึกษาและพัฒนาการตรวจวินิจฉัยทางภูมิคุ้มกันให้มีความจำเพาะต่อโรค ซึ่งจะเป็นประโยชน์ในการรักษาต่อไป*

วัตถุประสงค์ : *เพื่อศึกษาการแสดงออกของ rMSP5 ของเชื้อ A. marginale ในสภาวะอุณหภูมิที่แตกต่างกัน*

วิธีการทำวิจัย : *ในการศึกษานี้ยีน msp5 ได้ถูกเชื่อมต่อเข้ากับ pET100/D TOPO vector และทำการกระตุ้นให้เกิดการแสดงออกของโปรตีนใน E.coli BL21 star™ (DE3) โดยใช้ Isopropyl β-D-1-thiogalactopyranoside (IPTG) ที่อุณหภูมิ 16, 25 และ 37 องศาเซลเซียส เป็นเวลา 6 ชั่วโมง และทำการวิเคราะห์การผลิต rMSP5 ด้วยวิธี SDS-PAGE และ Western blot*

ผลการศึกษา : *การแสดงออกของ rMSP5 ในอุณหภูมิที่แตกต่างกันนั้น ไม่พบการแสดงออกของ rMSP5 ที่ 16 องศาเซลเซียส ในเวลา 6 ชั่วโมง ในขณะที่ 25 และ 37 องศาเซลเซียส พบการแสดงออกของ rMSP5 ในเวลา 2 - 6 ชั่วโมง แต่อย่างไรก็ตามที่ 25 องศาเซลเซียสพบการแสดงออกของ rMSP5 ต่ำกว่าที่ 37 องศาเซลเซียส*

สรุป : *หลังจากถูกกระตุ้นให้มีการแสดงออกของโปรตีนด้วย IPTG นั้น สภาวะอุณหภูมิที่เหมาะสมในการแสดงออกของโปรตีน rMSP5 คือที่ 37 องศาเซลเซียส เป็นเวลา 4 ชั่วโมง โดยโปรตีน rMSP5 มีขนาดประมาณ 26 kDa*

คำสำคัญ : *โรคพยาธิในเม็ดเลือดโคและกระบือ, Anaplasma marginale, MSP5, recombinant protein, การแสดงออกของโปรตีน.*

Anaplasma marginale, the cause of bovine anaplasmosis in ruminants mostly cattle is endemic in the tropical and subtropical areas.[1] The transmission of *A. marginale* is affected by tick and blood contaminated fomites such as needles and ear-tagging devices. [2] The clinical symptoms of the disease include fever, severe anemia, jaundice, weight loss, abortion, decreased milk production and often death in animals over two years old. Hence, the anaplasmosis causes significant economic loss to livestock productions in many countries worldwide including Thailand. [3] The conventional method for diagnosis of anaplasmosis is based on giemsa-stained blood smears. However, this method is not reliable for detecting *A. marginale* in pre-symptomatic stage of the animals and PCR assay should be an alternative method for confirmation of the strain identity. [4]

Presently, the six major surface proteins (MSPs) of *A. marginale* have been identified from bovine erythrocyte, designated as MSP1a, MSP1b, MSP2, MSP3, MSP4 and MSP5. As for the MSP1a, MSP1b and MSP2 there have been reports about the function of these proteins which are adhesin for bovine erythrocytes. In addition, the function of MSP1a was also shown to be adhesin for tick gut cells [5], while the function of MSP3, MSP4 and MSP5 have not yet been reported.

The MSP5 is an immunodominant protein encoded by a single gene. [6] The *msp*5 gene is present in the bovine erythrocytes in all stages of *A. marginale.* [7] The MSP5 protein is a potentially useful as a diagnostic antigen in a competitive enzyme-linked immunosorbent assay (cELISA) for diagnosis of anaplasmosis. [8] As for rMSP5, it is important to have information regarding its structure and function in the future. In this study, we have optimized the conditions for expression of rMSP5 of *A. marginale* in order to provide information for development of diagnostic technique of anaplasmosis in the future.

Materials and Methods

Construction of msp5 gene

The TOPO® cloning reaction was performed by adding of the PCR products of *msp*5 gene into the pET100/D-TOPO® vector (Invitrogen Life Technologies). The plasmid DNA was transformed into *Escherichia coli* by using the heat-shock method. Briefly, the 10 ng of plasmid DNA was added into competent *Escherichia coli* host strain BL21 star™ (DE3) (Invitrogen). The cells were incubated on ice for 30 min and transferred to heat-shock for 1 min at 42 ^{o}C. Then the cells were placed back on ice immediately. The Super Optimal broth with Catabolite repression (S.O.C.) medium (Invitrogen) was added into the transformed cells and incubated for 30 min at 37 ^{o}C. Then, the transformation reaction was added into 10 ml of Luria-Bertani (LB) broth containing 100 μg/ml of ampicillin and incubated with shaking at 37 ^{o}C for overnight.

Protein expression of the msp5 gene

The protein expression was carried out by inoculating the cells in LB broth and incubated with shaking at 37 ^{o}C to an optical density of 600 nm (OD_{600}) was 0.5 - 0.8 (mid-log phase). For induction, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added in the culture to a final concentration of 0.1 mM and incubated with shaking at difference temperature (16,

25 and 37 ^{o}C) for 6 h. The cell culture was removed at every 2 h until 6 h to choose the best time for expression. The cells were centrifuged at 8,000 xg for 10 min at 4 ^{o}C as well as collected and frozen at -20 ^{o}C.

Protein analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The cell pellet was resuspended in buffer containing 20 mM HEPES and 50 mM NaCl, pH 7.4, mixed with electrophoresis sample buffer (200 mM Tris-HCl, 8% SDS, 40% Glycerol and 0.4% Bromophenol blue, pH 6.8) and boiled for 5 min. Protein samples were separated by 12% polyacrylamide gel in electrophoresis buffer (192 mM glycine, 25 mM Tris and 0.1% SDS) at constant 80 Voltages. The gels were stained with Coomassie Brilliant Blue staining solution and destained with destain buffer (10% (v/v) of EtOH and 10% (v/v) of glacial acetic acid in distilled water).

Western Blot analysis

After the proteins were separated by SDS-PAGE, the gel was soaked in transfer buffer (25 mM Tris, 150 mM Glycine and 10% (v/v) ethanol). Subsequently, the proteins were transferred onto nitrocellulose membrane by wet transfer technique with constant current for 1 h. The membrane was blocked in 5% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) buffer at room temperature for 45 min. Then, the membrane was incubated with Anti-His as a primary antibody at dilution of 1:3000 for 45 min. The membrane was washed with 0.1% tween 20 in PBS for 3 times. The secondary antibody (anti-mouse IgG antibody) conjugated with the alkaline phosphatase (ALP) in 5% BSA at dilution of 1:5000 was added on to the membrane and incubated for 45 min. After washing, the color reaction was developed by using ALP substrate (75 mg/ml of NBT and 50 mg/ml of BCIP) and the reaction was stopped by adding of distilled water.

Results

Expression of recombinant MSP5 in Escherichia coli BL21 star^{TM} (DE3)

The recombinant plasmid pET100-*msp*5 was transformed into the *E. coli* BL21 star^{TM} (DE3) expression host and cultured in LB broth at different temperatures (16, 25 and 37 ^{o}C). After induction with 0.1 mM IPTG for 6 h, the induced culture at 16, 25 and 37 ^{o}C were analyzed by SDS-PAGE and compared with uninduced culture. The rMSP5 protein expressed at 16 ^{o}C did not show the target band on the gel (Figure 1). As for protein expression at 25 ^{o}C, the rMSP5 protein was shown on estimated molecular weight of 26 kDa at after induction 4-6 h (Figure 2), and the induced culture at 37 ^{o}C exhibited the rMSP5 protein on the target band on the size of 26 kDa at after induction 4 - 6 h (Figure 3).

Western blot analysis

The target band of rMSP5 protein at 37 ^{o}C was confirmed by Western blot using commercial anti-His. The results showed clearly band of expressed protein at 37 ^{o}C for 2 - 6 h (Figure 4).

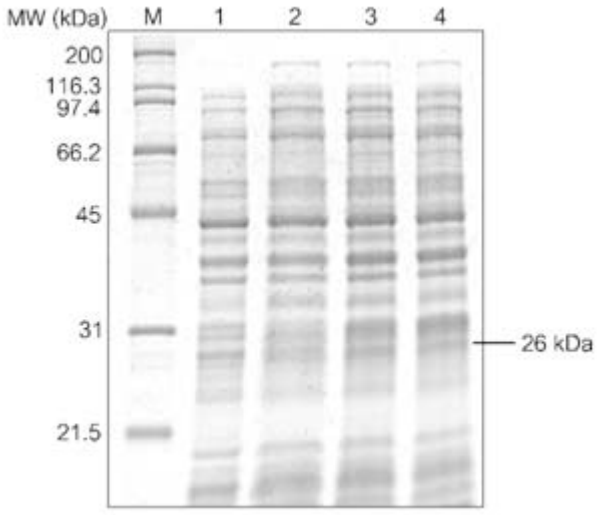

Figure 1. SDS-PAGE stained with Coomassie Brilliant Blue showed protein expression of BL21/pET100-*msp*5. The protein expression was induced by 0.1 mM IPTG at OD 0.5 - 0.8 and cultured at 16 ^{o}C. (M) Marker; (1) BL21/pET100-*msp*5 are not induced; (2) BL21/pET100-*msp*5 2 h after induction with IPTG; (3) BL21/pET100-*msp*5 4 h after induction with IPTG; (4) BL21/pET100-*msp*5 6 h after induction with IPTG.

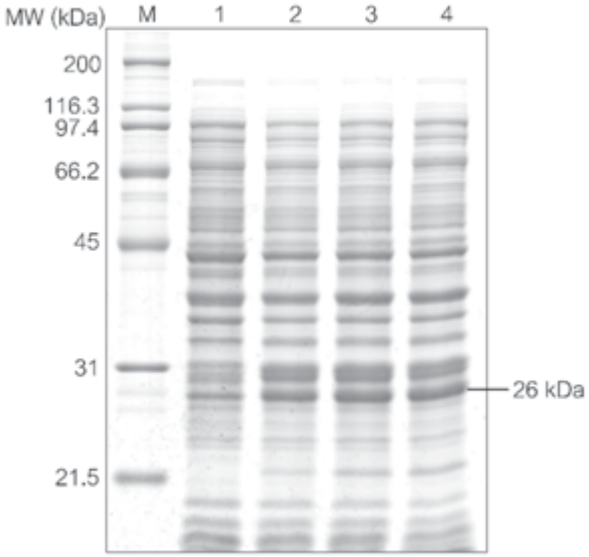

Figure 2. SDS-PAGE stained with Coomassie Brilliant Blue showed protein expression of BL21/pET100-*msp*5. The protein expression was induced by 0.1 mM IPTG at OD 0.5 - 0.8 and cultured at 25 ^{o}C. (M) Marker; (1) BL21/pET100-*msp*5 are not induced; (2) BL21/pET100-*msp*5 2 h after induction with IPTG; (3) BL21/pET100-*msp*5 4 h after induction with IPTG; (4) BL21/pET100-*msp*5 6 h after induction with IPTG.

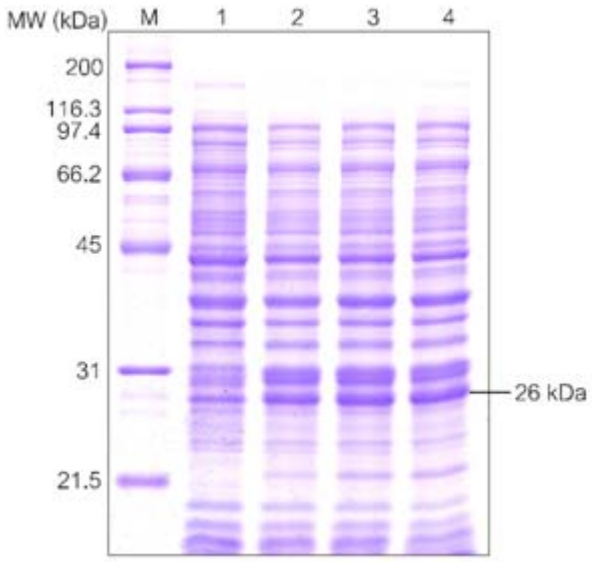

Figure 3. SDS-PAGE stained with Coomassie Brilliant Blue showed protein expression of BL21/pET100-*msp*5. The protein expression was induced by 0.1 mM IPTG at OD 0.5 - 0.8 and cultured at 37 ^{o}C. (M) Marker; (1) BL21/pET100-*msp*5 not induced; (2) BL21/pET100-*msp*5 2 h after induction with IPTG; (3) BL21/pET100-*msp*5 4 h after induction with IPTG; (4) BL21/pET100-*msp*5 6 h after induction with IPTG.

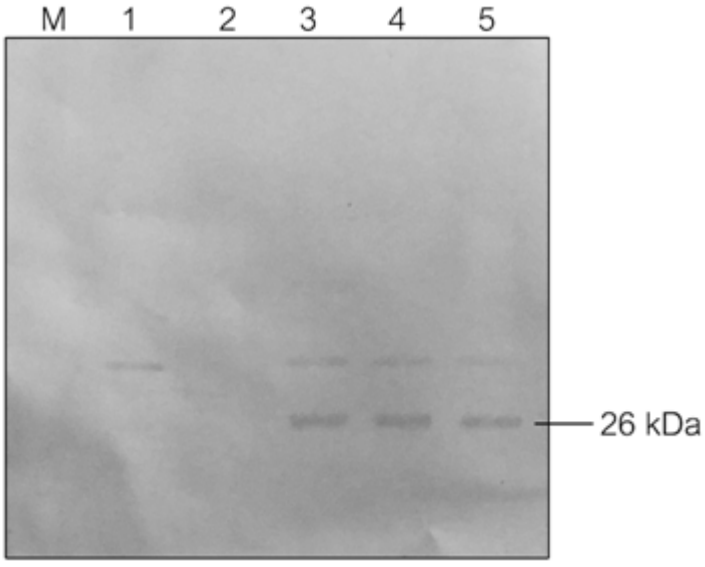

Figure 4. Western blot analysis of rMSP5 protein using anti-His antibodies. (M) Marker; (1) rMSP4 induced with IPTG (control); (2) rMSP5 not IPTG induced (control); (3) rMSP5 at 37^{o}C for 2 h after induction; (4) rMSP5 at 37 ^{o}C for 4 h after induction; (5) rMSP5 at 37 ^{o}C for 6 h after induction.

Discussion

This study, the *msp*5 gene isolated in Thailand and showed the molecular weight of rMSP5 protein at 26 kDa, being higher than other studied isolates. In the Florida isolate has been reported the molecular weight of rMSP5 is 19 kDa, which is constructed *msp*5 gene by using lambda ZAP and expressed protein in *E. coli* Y1090. [9] The rMSP5 from Havana isolate reported at 22 kDa. The gene of *msp*5 was cloned into pRSETB vector and expressed in *E. coli* BL21(DE3) pLysS strain. [10] This different size is occurred from different expression system. In the present study, we have used the pET100/D-TOPO® vector with histidine hexapeptide in N-terminal region for production of the recombinant fusion protein. The histidine tag residues fused to protein allow to increase the size of the recombinant protein.

The temperature of induction is one of the crucial factor affecting protein expression. In this study, the effect of different temperatures (16, 25 and 37 ^{o}C) on the rMSP5 expression levels showed the highest protein yields at 37 ^{o}C (2-6 h) compared with 25 ^{o}C. At 16 ^{o}C, rMSP5 has not been produced for 6 h because of the swift period of expression. In addition, the temperature at 25 ^{o}C showed the target band for 2 - 6 h after induction but the protein yields were lower than those at 37 ^{o}C. The duration time of expression at 37 ^{o}C for 4 h were sufficient to produce significant quantities of active protein because this time is an optimized condition for high yield of protein production. However, the higher temperature can cause a higher possibility of plasmid loss because of rapid growth of E. coli. Beside the study of temperature, for the further work, we will also concentrate on the duration time of protein expression for selecting the quality and activity of rMSP5.

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