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Original article

Cloning and Expression of recombinant *O*-acetylserine lyase from human *Streptococcus suis* serotype 2 in *Pichia pastoris*

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Abstract *Streptococcus suis* is recognized worldwide as an important swine pathogen, which occasionally infects humans and causes fatal illnesses. *O*-acetylserine lyase (OASL) is responsible for the final step of the cysteine biosynthesis, a key step in bacterial sulfur metabolism and offers a means by which inorganic sulfur maybe incorporated into cellular components. Recently, OASL was reported as a putative virulence-associated factor and immunogenic protein in *S. suis.* And this suggests that it may be involved in pathogenesis. In this study, the gene coding for OASL was cloned from a clinical *S. suis* serotype 2 strain. A full-length gene encoding OASL was amplified by the OASL gene-specific primer. The amplified fragments were cloned into pGAPZαA vector and expression as a His-tagged recombinant protein in *Pichia pastoris*. The expressed sample collected from the supernatant was first analyzed by SDS-PAGE. After that the recombinant proteins were detected by Ni2+-NTA enzyme conjugate. The Western immunoblotting showed that either antibodies present in patient serum suffered from *S. suis* infection or rabbit serum raised against whole cell lysate of homologous and heterologous strains can bind to recombinant OASL. These results verify that OASL had antigenicity and guide for evaluating its antigenic potential in further studies. **Chiang Mai Veterinary Journal 2014; 12(2): 121-129**

Keywords: human *Streptococcus suis*, recombinant *O*-acetylserine lyase, recombinant protein

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Introduction

Streptococcus suis is a major swine pathogen causing a wide range of diseases. It is also an important emerging zoonotic agent that causes similar diseases in humans who come into contact with swine or their products, resulting in fatal diseases such as meningitis, endocarditis and septic shock (Arends & Zanen, 1988; Gottschalk & Segura, 2000; Staats, Feder, Okwumabua, & Chengappa, 1997). *S. suis* serotype 2 is considered to be the most virulent and the most frequently found in patients. In Thailand, humans have the second high rate of *S. suis* infection after China but it presents the highest rate of mortality (Lun, Wang, Chen, Li, & Zhu, 2007). At present, *S. suis* infection is still frequently found among people in Northern region of Thailand, especially in Chiang Mai, Lam Phun and Payao provinces (Fongcom, Pruksakorn, Mongkol, Tharavichitkul, & Yoonim, 2001; Leelarasamee, Nilakul, Tien-Grim, Srifuengfung, & Susaengrat, 1997; Takeuchi D, 2012; Wangkaew, Chaiwarith, Tharavichitkul, & Supparatpinyo, 2006; Wangsomboonsiri, Luksananun, Saksornchai, Ketwong, & Sungkanuparph, 2008).

Until now, the pathogenesis of *S. suis* infection has not been completely understood, although several virulence factors have been proposed and, to date, the major diagnostics of the disease still depends on historical examination and specimen culture. This process takes a long time and becomes a leading cause of the delay of appropriate treatment; combined with rapid progress of the disease, it can lead to the death of patients. Thus, the rapid diagnostics is urgently required to rescue patients. Accordingly, the immunogenic protein which elicits a strong immune response in humans is beneficial for both diagnostics and vaccine purposes. *O*-acetylserine lyase (OASL) is the enzyme responsible for the final step of sulfur assimilation pathway, namely the cysteine biosynthesis, in bacteria and plants (Hell, 1997; Jeff D. Evans, 2002). Recently, the important role of genes involved in cysteine biosynthesis in gram-positive bacteria has been reported (Albanesi D, 2005; Fernández M, 2002; Lithgow JK, 2004; Osaki, Takamatsu, Tsuji, & Sekizaki, 2000). It is suggested that OASL, a key enzyme in cysteine biosynthesis, is also one of the putative virulence associated factors in many microorganisms including *S. suis* (Hill CE, 2003; Stancik LM, 2002 ; Wu, Zhang, & Lu, 2008). Interestingly, it is also immunogenic protein in *S. suis* (Osaki et al., 2000; Walker J, 2006; Zhang & Lu, 2007) and can be only recognized by sera from *S. suis* infected patients, but not sera from healthy humans, preimmune rabbits and patients who are infected with other microorganisms (Kanruethai Wongsawan, 2010). This suggests that OASL causes specific immune response to *S. suis*.

The aim of this study was to produce recombinant OASL in *Pichia pastoris* and determine its antigenicity. An advantage of yeast system is the ability to produce a large amount of recombinant protein in large-scale fermentation which is required in the next experiment for immunogenic investigation of protein in the future.

Materials and Methods

Bacterial strains, plasmids, and reagents

S. suis serotype 2 LPH02 strain isolated from a patient from the Lam Phun hospital, Lam Phun, Thailand, was grown in Todd-Hewitt broth (Difco, Detroit, MI) supplemented with 0.6% yeast extract, at 37°C under 5% CO_2 for 18 h. DNA was extracted using the GeneAid Kit (Geneaid Biotech, Taiwan). *Escherichia coli* XL-1 blue competent cells (Invitrogen, USA) were used as the host strain for the vector construction. The pGAPZαA plasmid, *P. pastoris* X-33 strain (Invitrogen, USA) and T4 DNA ligase (Fermentas, Lithuania) were used in cloning and expression. Zeocin™ (Invitrogen, USA) was use as drug resistance marker for selection in *E. coli* and *P. pastoris*. The sequences of primers used in this study were listed in Table 1.

Construction of expression plasmids

The gene encoding OASL protein containing an *Eco*RI and *Not*I recognition sites was amplified by PCR using the specific primers designed based on complete coding sequence of *S. suis CysM* for OASL (GeneBank: AB028865.1). The forward primer contained ATG start codon and *Eco*RI restriction site while the reverse primer incorporated *Not*I restriction site (Table 1). Vector pGAPZαA used for cloning and expression in *P. pastoris* contained zeocin™ resistance gene (*Sh*ble ORF) for selection in both *E. coli* and *P. pastoris*. Transcription of the gene of interest is controlled by the *GAP* promoter (glyceroldehyde-3-phosphate dehydrogenase promoter) and terminator (*AOX1* TT) from *P. pastoris*. This *GAP* promoter region allows integration of the vector into the *P. pastoris* genome. The amplified fragments was digested with *Eco*RI and *Not*I and ligated into *Eco*RI/*Not*I predigested pGAPZαA to construct the recombinant vector pGAPZαA-OASL where OASL gene was led by the signal peptide sequence *S. cerevisiae* α-mating factor (α-factor) under the transcriptional control of the *GAP* promoter. pGAPZαA-OASL construct was transformed into *E.coli* XL-1 blue cells by heat-shock method (Joseph Sambrook, 2001). Positive clones were selected on LB low salt agar containing 25 μg/ml of Zeocin™. Integration of the gene encoding OASL into vector pGAPZαA was verified by restriction enzymes digest, colony-PCR (Joseph Sambrook, 2001) and DNA sequencing.

Table 1 Primers used for OASL cloning.

Primers	Sequences
F-*suis*_E	5'-AGTCGAATTCATGGCTATTTATCAAAACAT-3'
R-*suis*_N	5'-ATATGCGGCCGCATCATTGAACTCATAAAG-3'
pGAP Forward	5′-GTCCCTATTTCAATCAATTGAA-3′
3′*AOX1*	5′-GCAAATGGCATTCTGACATCC-3′

[#] *Eco*RI and *Not*I restriction site are underlined.

Transformation into *P. pastoris* and selection of transformants

The pGAPZαA-OASL construct was linearized by restriction enzyme *Avr*II and transformed into *P. pastoris* X-33 by electroporation with 2000 V of charging voltage, 25 μF of capacitance and 200 Ω of resistance. The pGAPZαA vector was used as a negative control. Positive transformants were selected on yeast-peptone-dextrose-sorbitol (YPDS) plates containing 100 μg/ml of Zeocin™. The presence of OASL gene in the transformants was confirmed by colony-PCR using the F-*suis*_E and R-*suis*_N primers. Each colony was picked and suspended in 50 μl sterile water then heated at 80°C for 10 min. Supernatant (1 μl) was used as a template in PCR.

Expression of recombinant OASL in *P. pastoris* X-33

Positive transformants were cultured in a shaking flask containing 10 ml of YPD medium (1 % yeast extract, 2 % peptone, supplemented with 2 % glucose) at 30°C overnight. A 100 μl overnight culture was inoculated into a shaking flask containing 50 ml of YPD and incubated at 30°C. At each of the times indicated following: 0, 1 day, 2 days, 3 days, and 4 days, 1 ml of culture was transferred into separate tube and used to analyze the expression levels of recombinant protein and determine the optimal time to harvest. The supernatant was collected by centrifugation at maximum speed for 5 min and kept at -20°C until use.

Detection of recombinant OASL and its antigenicity

The supernatant was precipitated with trichloroacetic acid (TCA). The protein pellet was resuspended with 15 μl of 1M Tris-HCl pH 9 and applied to SDS-PAGE. The recombinant protein was visualized by Coomassie staining. To confirm the expression of recombinant OASL, the protein on gel was transferred to nitrocellulose membrane and detected with HisDetector™ Nikel-HRP (KPL, USA) according to the manufacturer's recommendations. The capacity of antibody to bind with recombinant OASL was preliminarily tested. After transfer onto nitrocellulose membrane, the presence of OASL antigen was detected with either rabbit antisera raised against whole cell lysate of homologous *S. suis* LPH02 strain (dilute 1: 200) or pooled *S. suis* infected patient sera (dilute 1: 100). Both rabbit antisera and patient sera were available and kept in freezer stock at -20°C in our laboratory. The blots then were probed with specific AP-conjugate goat anti-rabbit IgG or HRP-conjugate goat anti-human IgG (Bio-Rad, USA) and developed with BCIP/NBT (Amresco, USA) or TMB blotting substrate (KPL, USA), respectively.

Results and Discussion

Amplification of the gene encoding OASL and construction of recombinant vector pPICZA-OASL

Synthesis of the gene encoding OASL was carried out by PCR using OASL gene-specific primers. The products of expected size (927 bp) are shown in Figure 1A. To express OASL with

the C-terminal peptide containing in frame *c-myc* epitope and polyhistidine tag in *P. pastoris* X-33, we constructed the recombinant vector pGAPZαA-OASL, incorporating OASL open reading frame without a stop codon. It was done by ligation of the 927 bp fragment of *Eco*RI/*Not*I digested product to *Eco*RI/*Not*I predigested pGAPZαA in the end of 5′*GAP* promoter region. The pGAPZαA -OASL was propagated in *E. coli* XL-1 blue cells. A number of positive *E.coli* clones were observed in LB low salt agar containing 25 µg/ml of Zeocin. More than twenty colonies of positive clones were screened with colony-PCR using the OASL gene-specific primers. As shown in Figure 1B, all clones contained recombinant vector with a 927 bp of gene encoding OASL. Similar results were observed after restriction analysis was performed (data not shown). The pGAPZαA-OASL was further subjected to nucleotide sequencing using pGAP primer. The resulting sequence showed 100% identity to *O*-acetylserinelyase sequence of *S. suis* strain D9 (GenBank Accession no. CP002641.1) and ST3 (GenBank Accession no. CP002633.1) databases using Basic Local Alignment Search Tool (BLAST) program. The theoretical molecular weight (MW) of this recombinant protein was 43 kDa (http://web.expasy.org/compute_pi/).

Transformation of recombinant vector pGAPZαA-OASL into *P. pastoris* and screening of transformants

To integrate pGAPZαA-OASL into *P. pastoris* genome, it was linearized by restriction digest with *Avr*II and transformed into *P. pastoris* X-33 competent cells by electroporation. A number of colonies were observed on YPDS plates containing 200 µg/ml of Zeocin™. Ten positive transformants grown on YPDS-Zeocin™ plates were screened by colony-PCR. A 927 bp of gene encoding OASL and transformant with the empty pGAPZαA vector were used as positive and negative controls, respectively. The results show that all ten transformants contained the gene encoding OASL (data not shown), suggesting the success of construction of recombinant

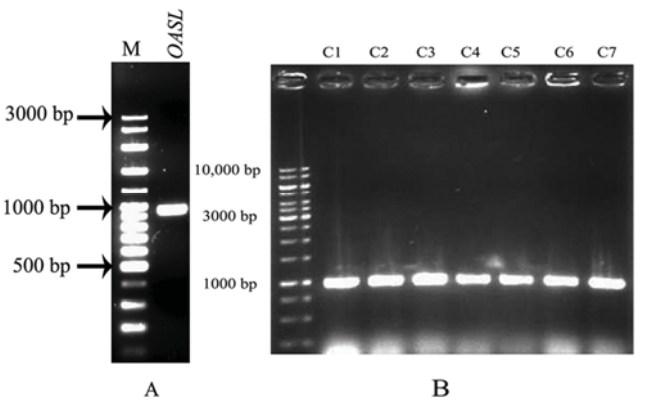

Figure 1. Amplification and cloning of the gene encoding OASL. (A) A 927 bp of PCR product was run in 1% agarose gel, 100 bp Plus DNA ladder marker (Vivantis, Malasia) was used to estimate the fragment size. (B) Cloning of A 927 bp of PCR product into pGAPZαA. The presence of the gene encoding OASL was checked by PCR in *E. coli* positive clones C1-C7.

Expression and detection of recombinant OASL

The highest OASL-producing *P. pastoris* was selected for subsequent large-scale protein expression. At different times, samples were taken from the culture and aliquots of the supernatant were resolved by SDS-PAGE analysis. The result showed a target recombinant OASL protein migrating at approximately 43 kDa (Figure 2A). The protein yields observed during the period of four days were equal in amount over the course of culture. None of the protein bands were detected from empty pGAPZαA transformants (data not shown).

Recombinant OASL expression was confirmed by western blot analysis with HisDetector™ Nikel-HRP (KPL, USA) (Figure 2B). The results showed that a band with molecular weight of about 43 kDa was positively stained and its lower protein band was also observed. However, immunoblot analysis with *S. suis* infected patient sera and rabbit antisera showed specific binding of sera to the 43 kDa protein only (Figure 2C), indicating that the positively lower band was non-specific binding. The result showed that recombinant OASL was antigenicity.

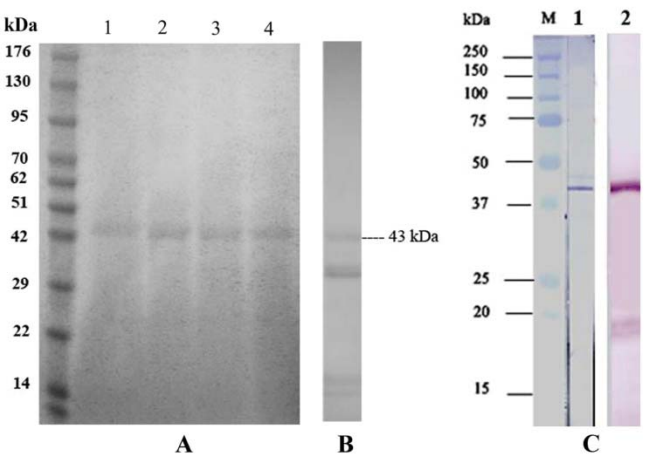

Figure 2. Analysis of recombinant OASL expression. (A) SDS-PAGE analysis of recombinant OASL in day1-4 (lane 1-4). (B) Immunoblot with Nikel-HRP. (C) Specific binding of recombinant OASL to S. suis infected patient sera (lane 1) and rabbit antisera (lane 2).

Conclusion

OASL is an indispensable enzyme that plays an important role in metabolic pathway; it is also immunogenic protein in *S. suis*. Thus, it is an attractive protein for the investigation of the possible use in diagnostics (in combination with other immunogenic proteins). In this study, OASL was successfully expressed in a secretory form in *P. pastoris*. Its antigenicity was observed after immunoblot analysis. To our knowledge, this was the first recombinant clones of *P. pastoris* harboring gene encoding OASL protein from human *S. suis*. Further studies will be focusing on the large-scale expression of OASL in *P. pastoris* and evaluate its antigenicity in animal model.

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โคลนนิ่งและการแสดงออกของโปรตีนลูกผสม o-acetylserine lyase จากเชื้อสเตรปโตคอคคัส ซูอิส ซีโรไทป์สอง สายพันธุ์ที่แยกได้จากผู้ป่วยในเชื้อ พิเชีย พาสทอริส

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บทคัดย่อ เชื้อเสตรปโตค็อกคัสซูอิส เป็นเชื้อก่อโรคที่สำคัญในสุกรทั่วโลกซึ่งสามารถติดต่อสู่คนและก่อให้เกิดโรคที่รุนแรงถึงแก่ชีวิต *o*-acetylserine lyase (OASL) เป็นโปรตีนที่ทำหน้าที่ในขั้นตอนสุดท้ายของกระบวนการสังเคราะห์กรดอะมิโนซิสเตอีน โปรตีนนี้มีรายงานว่าน่าจะเป็นปัจจัยที่เกี่ยวข้องในการก่อโรคของเชื้อรวมทั้งยังสามารถกระตุ้นให้มีการตอบสนองของระบบภูมิคุ้มกันได้ ในการศึกษานี้ได้ทำการโคลนยีนที่กำหนดการสร้างโปรตีน OASL จากเชื้อเสตรปโตค็อกคัสซูอิส ซีโรไทป์ 2 ซึ่งแยกได้จากผู้ป่วย โดยการสังเคราะห์และเพิ่มจำนวนยีนด้วยไพรเมอร์ที่มีความจำเพาะต่อยีนที่กำหนดการสร้าง OASL ทำการโคลนท่อนยีนเข้าสู่เวคเตอร์ pGAPZαA และให้มีการแสดงออกของโปรตีนลูกผสม OASL-His ในเชื้อ *Pichia pastoris* ตรวจวัดการแสดงออกของโปรตีนลูกผสม OASL-His โดยเก็บอาหารเลี้ยงเชื้อไปวิเคราะห์ด้วยเทคนิค Tricine-SDS-PAGE และตรวจยืนยันโดยใช้ Ni^{2+}-NTA enzyme conjugate ผลการทดสอบความสามารถของโปรตีนลูกผสม OASL-His ในการจับกับแอนติบอดีโดยทำ Western immunoblotting ด้วยซีรัมจากผู้ป่วยติดเชื้อเสตรปโตค็อกคัสซูอิส ซีโรไทป์ 2 และซีรัมกระต่ายที่ได้จากการฉีดกระตุ้นด้วยเชื้อสายพันธุ์เดียวกันกับที่ใช้ในการศึกษานี้และต่างสายพันธุ์ พบว่าซีรัมทั้งสองชนิดที่ใช้ทดสอบสามารถจับได้กับโปรตีนลูกผสม OASL-His ผลที่ได้แสดงให้เห็นว่า OASL-His มีคุณสมบัติในการจับได้กับแอนติบอดี และสามารถนำไปทำการประเมินหาศักยภาพในการจับกับแอนติบอดีในลำดับขั้นต่อไปได้

คำสำคัญ : สเตรปโตค็อคคัสซูอิสจากคน, โปรตีนลูกผสมโอออะซีติลเซอรีนไลเอส, โปรตีนลูกผสม