In vitro expression of enolase from *Streptococcus suis* serotype 2 and it's antigenicity

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Abstract:

***Streptococcus suis* serotype 2 (SS2) is one of the most important pathogens in the porcine industry and an important zoonotic agent. The absence of suitable vaccine or virulence markers makes SS2 infections more difficult to control. An immunoproteomics approach is used for identifying antigenic proteins in SS2 recognized enolase, which may represent strain-specific antigenic proteins and potential protective antigens. This study aims to clone, express enolase gene from SS2 and use western blotting to evaluate the antigenicity. Enolase gene from the SS2 strain was amplified with specific primers. The obtained PCR product was inserted into an expression vector, pGEX-4T1. The recombinant vector was then transformed into BL21 cells for protein expression. Subsequently, the immunological activity of the recombinant enolase was tested by western blotting with human sera in the convalescent phase. The glutathione S-transferase (GST)-tag fusion enolase was purified by glutathione sepharose affinity chromatography for further studies. The SS2 recombinant enolase was successfully expressed in *E. coli*. The western blotting analysis demonstrated that enolase has an antigenic property, which is recognized by patients naturally infected with SS2.**

***Keywords:* antigenicity, enolase, protein expression, Streptococcus suis serotype 2.**

***Classification numbers:* 3.1, 3.5**

Introduction

Streptococcus suis, commensal and opportunistic pathogens of swinea, α-hemolytic gram-positive cocci with 35 different serotypes. *Streptococcus suis* is an important pathogen in pigs and an emerging zoonotic agent in humans who are in contact with pigs or with their products [1]. *S. suis* infections in human are most often reported in countries where there is a high population of pigs. By the end of 2012, a total of 1584 cases had been reported in the literature (including 189 probable cases identified in 3 outbreaks), mainly from Thailand (36%), Vietnam (30%) and China (22%) [2]. Human infections with *S. suis* most frequently manifest as purulent meningitis, but septic shock with multiple organ failure, endocarditis, pneumonia, arthritis and peritonitis have also been reported [1]. In Thailand, China (including Hong Kong) and Vietnam, *S. suis* is an important cause of adult endocarditis, sepsis, and especially, meningitis [3]. Thirty-five serotypes (types 1-34 and 1/2) have been described based on capsular polysaccharides; serotype 2 is considered to be the most common pathogen in both humans and pigs [1, 4].

The lack of thorough knowledge about virulence markers and protective antigens can hinder the control of SS2 infections. Several approaches have been adopted to develop vaccines for *S. suis*. However, little success was achieved because the protection was either a serotype or strain dependent. More recently, interest has shifted towards the protein antigens of *S. suis* as vaccine candidates. Recently, immunoproteomics has become an effective approach for identifying immunoreactive proteins, which are essential antigens in the development of vaccine; they are also biomarkers for diagnostic and molecular therapy.

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Enolase, which is one of the remarkable antigenic proteins of SS2, was identified through immunoproteomics.

Enolase is a potential virulence factor of SS2; it was originally identified as a key glycolytic enzyme that catalyses the dehydration of 2-phosphoglycerate to phospho-enolpyruvate in the last steps of the catabolic glycolytic pathway [5, 6]. Recent studies have found that enolase plays an important role in many physiological and pathological processes, such as facilitating pathogen invasion into the host [7-9], cancer metastasis [10-14] and apoptosis [15, 16]. Recently, it was also recognized as an immunodominant antigen involved in the virulence of the Streptococcus species [6, 8, 17, 18]. It is noteworthy that enolase is an antigenic protein recognised by patient sera with SS2 infection based on the immunoproteomics approach [19]. In this study, the enolase gene from SS2 was cloned and expressed to confirm its antigenic property.

Methods and materials

Bacterial strain and patient sera collection

SS2 strains were isolated and identified in patients infected with *Streptococcus suis* serotype 2, as described in previous publication [19]. The patient sera were collected from the SS2 infected patients in convalescent phases who were hospitalized for treatment. Both the bacterial isolates and patient sera were stored in a deep freezer at -80°C [20]. One Shot™ TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific, Massachusetts, USA) and One Shot BL21 Star (DE3) Chemically Competent *E. Coli* (Thermo Fisher Scientific, Massachusetts, USA) were used as recipients of recombinant enolase, containing the vector pGEX- 4T1 (Amersham).

Amplification of enolase gene and cloning enolase gene into expression vector

A PCR assay was performed by using forward primer (5'-GGC GGA TCC ATG TCA ATT ATT ACT GAT G-3') and reverse primer (5'-ACG CTC GAG TTA TTT TTT CAA GTT GTA GAA TGA G-3'), which specifically targets 1305-bp of the enolase gene of locus taq SSUBM407_1397 of *Streptococcus suis* BM407 complete genome data (NC_012926.1). The primes were design in the Geneious v8.1 software, which contained *BamH*I and *Xho*I sites at 5'and 3' ends, respectively [21]. The PCR amplification was profiled as follows: initial denaturation at 94°C for 2 minutes, followed by 36 cycles of 95°C for 15s, 55°C for 30s and 68°C for 30s in Veriti® Thermal Cycler (Applied Biosystems, CA, USA).

The PCR products were purified using the QIA quick PCR Purification Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Ten of the purified enolase PCR products and 0.32 µM of the primer were used for direct sequencing. To sequence both strands, two specific PCR primers were run for each enolase PCR product samples. The chromatograms were analysed by the Geneious software v8.1 and compared with the enolase sequence data available in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/), using the BLASTn plugin of the Geneious software [21]. All sequences were aligned using ClustalX [22].

The 1305 bp PCR product, which corresponded to the obtained enolase, was restricted, gel eluted and inserted into the expression vector pGEX-4T1 in frame with the GST-tag sequence. The recombinant plasmid was introduced into *E. coli* TOP10 by heat shock transformation, and the clones were selected by growing the cultures on Luria-Bertani (LB) agar in the presence of 100 µg/ml ampicillin. For identifying the positives clones, miniprep was performed, and then, the putative clones were checked by restriction digestion.

Expression of recombinant S. suis enolase

The recombinant vectors were transformed into *E. coli* BL21 for expression. The transformation procedure followed the instructions of TransformAid Bacterial Transformation Kit (Thermo Fisher Scientific, USA) [23]. A single recombinant colony of BL21 was grown on a SOB (super optimal broth) medium at 37°C overnight with shaking. The protein expression was induced by the addition of 0.1 mM isopropyl b-D-1-thiogalactopyranoside (IPTG). One hour later, the cells were collected by centrigugation, and the cell pellet at were stored at -20°C; this collection process continued for a period of 4 hours with 1 hour intervals.

SDS-PAGE and Western Blotting

The recombinant enolase protein was resolved in 2 separate acrylamide gels. After electrophoresis, one gel was alternatively stained with SimplyBlue™ SafeStain (Novex, Life Technologies), and then digitalized, with the Gel Doc™ XR+ System (Bio-rad, USA).

The second gel was subjected to western blot assay. The proteins were transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham, GE) with a Mini-Trans-Blot Cell (Bio-Rad, CA, USA) at 250 mA (100 V) for one hour at 4°C. Then, the membranes were blocked with PBS-T containing 5% (w/v) skim milk. Membranes were incubated for one hour with a patient serum in 1:1500 dilutions in PBS-T, containing 2% (w/v) skim milk in a Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad, CA, USA). The incubated membranes with the Goat Anti-Human IgG-HRP conjugated (Southern Biotech, Alabama, USA) as secondary antibodies diluted in the ration 1:50000 in PBS-T, containing 5% (w/v) skim milk, in one hour. The membranes were developed with Luminata™ Forte Western HRP substrate (Merck Millipore Corp., Darmstadt, Germany), and images were acquired with a ChemiDoc™ XRS+ System and were analysed by Image Lab Software (Bio-Rad, CA, USA).

Affinity purification of GST fusion enolase

The GST-tag added to the enolase allowed its isolation by affinity chromatography on glutathione column. Overnight cultures of *E. coli* BL21 cells harbouring recombinant enolase were sub-cultured in an LB medium with ampicillin 100 µg/ml and chloramphenicol 35 µg/ml and incubated at 37°C until an optical density of 0.6 at 600 nm (OD_{600}) was reached. Then, the *E. coli* were grown with 0.1 mM IPTG for 3 hours. The cells were centrifuged, and then, the pellet was re-suspended in ice-cold 1X PBS buffer with protease inhibitors. After sonication on ice, the lysates were centrifuged at 10,000×g for 10 minutes, and the recombinant protein was purified from the filtered supernatants by affinity chromatography. The procedure was performed as described in the Recombinant Protein Purification Handbook-GE. Healthcare [24].

Results and discussion

Enolase sequences analysis

Amplification of the full length coding sequences (CDS) of the enolase gene was confirmed by gel electrophoresis of the amplified fragments. The expected size of about 1305 bp was obtained, which corresponded to the SS2 enolase gene (Fig. 1).

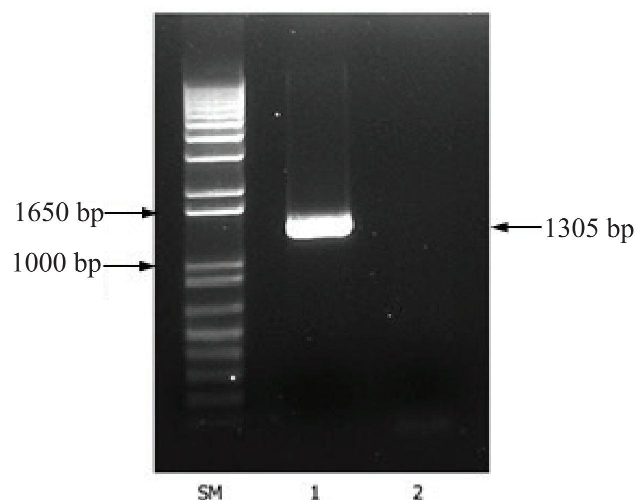

Fig. 1. PCR amplification of enolase gene from *S. suis* serotype 2. The PCR products were separated on 1.0% agarose gel. SM: 1 kb DNA Ladder (D0428, SIGMA DNA); lane 1: PCR product; lane 2: Negative control.

The raw data of enolase sequences were analysed with a multiple alignment algorithm in Geneious v8.1 with the enolase sequence from the reference genome of *S. suis* BM407 (NC_012926) in the Genbank (https://www.ncbi.nlm.nih.gov/nuccore/NC_012926.1). The sequence assembly data showed that our sequences matched with the *eno* CDS at locus_tag SSUBM407_1397 of the reference genome sequence (Fig. 2).

Cloning and expression of enolase in pGEX-4T1

The PCR products and pGEX-4T1 vector were digested by the *BamH*I/*Xho*I and purified by the ZymoClean™ Gel DNA Recovery Kit (Zymo Research Corp., USA). The enolase was cloned into the TOP10 *E. coli*. Double digestion of the enolase recombinant vector by *BamH*I/*Xho*I showed that the expected size of 1300 bp fragment on agarose gel electrophoresis corresponded to the enolase gene (Fig. 3).

Expression of recombinant enolase

The enolase gene was 1305 bp in size and encoded into a 435 amino acid protein with a predicted molecular mass of 52 kDa. The result of expression from the pGEX-4T1 is a GST-tagged fusion enolase in which the functional GST protein (26 kDa) is fused with the N-terminus of the recombinant enolase. By comparing the SDS-PAGE results of the non-induced and IPTG-induced BL21 strains (Fig. 4), it can be seen that induced BL21- pGEX 4T1-enolase lands

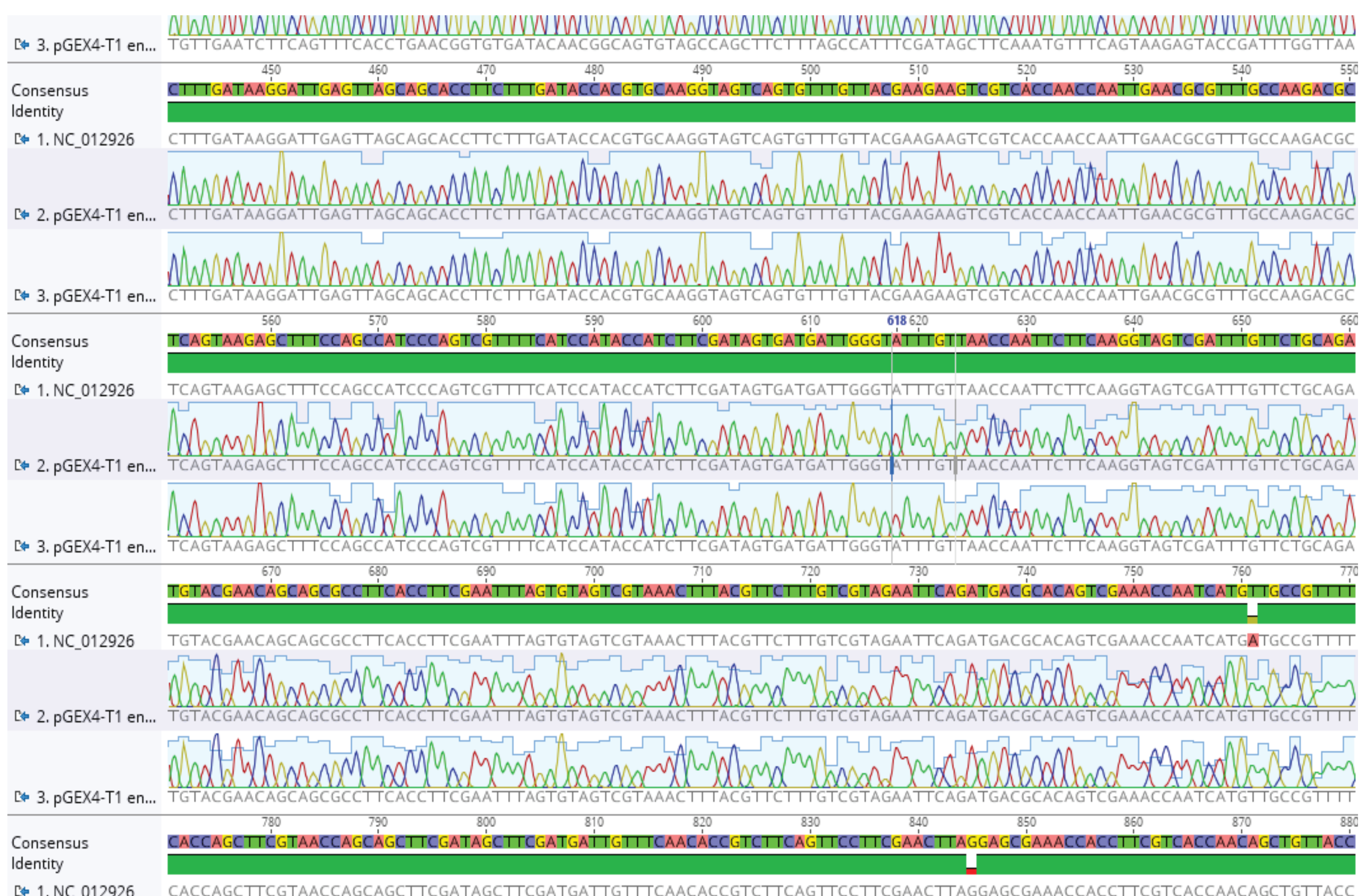

Fig.2. Analyse the chromatograms of *S. suis* enolase sequences by Align/Assembly plugin of Geneious v8.1.

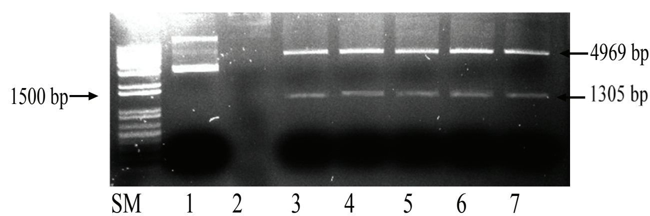

Fig. 3. Agarose gel electrophoresis of pGEX-4T1 recombinant vector digested *BamH*I/*Xho*I. SM: 1 kb DNA ladder; lane 1: pGEX-4T1-enolase undigested; lane 3-7: pGEX-4T1-enolase digested with *BamHI/XhoI*.

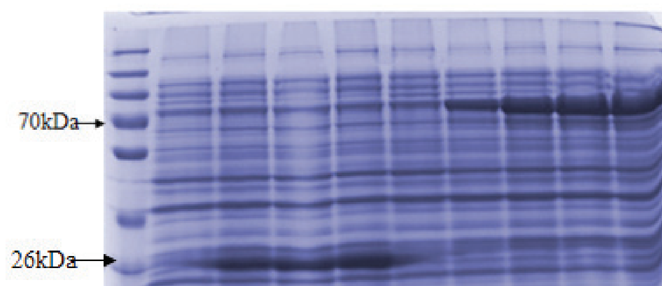

Fig. 4. SDS-PAGE analysis of recombinant enolase. Lane 1: PageRuler™ Prestained Protein Ladder Plus (Thermo Fisher Scientific Inc.); Lane 2: Non-induced BL21; Lane 3-5: Induced BL21-pGEX 4T1 empty; Lane 6-10: Induced BL21-pGEX 4T1-enolase at time zero, 1h, 2h, 3h and 4h, respectively.

have a strong band with an approximate size of 78 kDA. The other lanes (2-5) also have a light band with an approximate size of 78 kDA. The antigenic property of the recombinant enolase in the total protein of the induced BL21-pGEX 4T1-enolase was evaluated in the western blot assay.

Testing antigenic property of recombinant enolase

Western blotting was used to test the antigenic property of the recombinant enolase with sera from naturally infected patients. The primary antibody was diluted in a 1:1000 ratio, and the secondary antibody was diluted in a 1:50000 ratio. The protein reacted to the convalescent sera with a strong band with a predicted size of 78 kDa (Fig. 5).

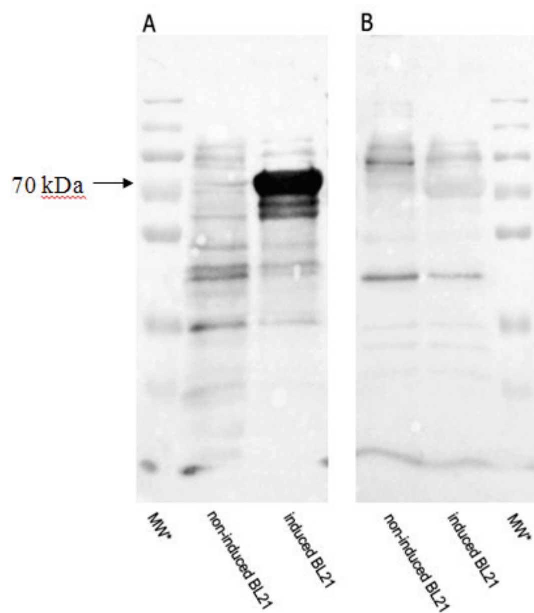

Fig. 5. Western blotting analysis of recombinant enolase. Convalescent serum **(A)** and negative serum **(B)**. MW: PageRuler™ Prestained Protein Ladder Plus (Thermo Fisher Scientific Inc.).

Purification of recombinant enolase

Protein purification was confirmed through a SDS-PAGE analysis. From the SDS-PAGE gel, it can be seen that there is a pure recombinant enolase in the elution fractions, and the fourth elution fraction contains the highest intensity band (Fig. 6).

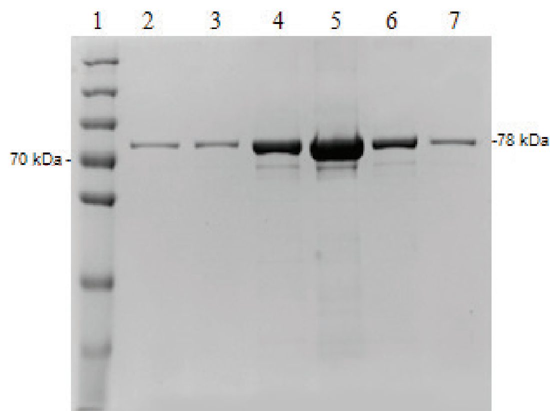

Fig. 6. SDS-PAGE analysis of affinity purification. Lane 1: Protein ladder; Lane 2-7: Elution fractions of purified enolase.

This evidence demonstrates that the recombinant enolase with antigenicity can be recognized by patient serum. It could be a candidate for developing a vaccine against SS2, which is present at the surface of all 35 *S. suis* serotypes. It contributes to *S. suis* adhesion to and invasion of host cells [8], and it is a highly conserved protein [25].

Conclusions

We have successfully cloned the SS2 enolase gene into the pGEX 4T1 vector and the recombinant enolase into BL21 cells. The recombinant enolase has antigenicity and can be recognized by patient sera infected SS2 in western blot assay.

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