Prokaryotic expression, purification and immunogenicity analysis of CpsD protein from *Streptococcus iniae*

Wang H.C.[1]; Wang K.Y.[1,2]*; Wang J.[1]; Xiao D.[3]; Huang J.L.[1]; Chen D.F.[4]

Received: February 2013 Accepted: September 2014

Abstract

Streptococcus iniae is a major cause of serious bacterial infections in both fish and human beings. Capsular polysaccharide (CPS) of *S. iniae* is vital to evade phagocytic clearance of the host and serves as an important protective antigen of *S. iniae* infection in aquatic animals. The CpsD gene was determined to be highly conservative in capsule polysaccharide operon. Prokaryotic expression of the CpsD gene of a clinical isolate of *S. iniae* from channel catfish and immunogenic examination of the recombinant protein were first described in this essay. The recombinant protein was expressed in the form of inclusion bodies (IBs). Induction conditions in *Escherichia coli* were optimized with 0.6mM Isopropyl β-D-1-Thiogalactopyranoside at 37°C for 5h after the culture mid-log phase in Luria Bertani (LB) medium. The recombinant protein CpsD was thus expressed and purified by immobilized metal affinity chromatography (IMAC), yielding approximate 582.47 mg the protein per liter culture. Western blot analysis showed that the purified CpsD had reactogenicity. It will possibly reveal more details of capsule synthesis and capsule regulation during various stages of the *S. iniae* infectious process.

Keywords: *Streptococcus iniae*, Capsular polysaccharide, Prokaryotic expression, Purification, Western blot analysis.

1-Department of Basic Veterinary, College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, Sichuan Province, 625014, China.

2-Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Ya'an , Sichuan Province, 625014, China.

3-Tongwei Co. Ltd., Chengdu, Sichuan Province, 610041, China.

4-Department of Aquaculture, College of Animal Science and Technology, Sichuan Agricultural University, Ya'an , Sichuan Province, 625014, China.

*Corresponding author's email: kywangsicau@126.com

Introduction

Streptococcus iniae is a β-hemolytic, Lancefield serogroup negative, gram positive streptococci (Yuasa *et al*, 1999). In the past few decades，the aquatic zoonotic pathogen has represented a threat to the worldwide aquaculture industry, causing economic losses adding up to a hundred million dollars annually (Agnew and Barnes, 2007). To date, *S. iniae* infections have been documented in over 30 species of fish (Shoemaker *et al*., 2001; Agnew and Barnes, 2007; El Aamri *et al*., 2010; Chen, *et al*., 2011), including salmon (Goh *et al*., 1998), tilapia (Shoemaker *et al*., 2001), channel catfish (Chen *et al*., 2011) and trout (Rajagopal *et al.*, 2003). Furthermore, it also causes human infection with septic arthritis complicating chronic gout and bacteremic- cellulitis, especially in immune-compromised individuals (Weinstein *et al*., 1997; Lau *et al.,* 2003; Koh *et al*., 2004). It is generally believed that *S. iniae* has been a zoonotic pathogen, holding an ability to cause severe systemic disease in both fish and human beings (Weinstein *et al*., 1997).

Like other systemic streptococcal pathogens, CPS of *S. iniae* is a significant virulence factor and is required for its pathogenesis and survival in the host (Buchanan *et al*., 2005; Miller and Neely, 2005). CPS helps the microorganisms to resist phagocytosis and delete innate host defenses during systemic infection. Multiple streptococcal capsule operons contain a conservative group of genes (CpsA ~E) that are collectively responsible for capsular chain length determination and export (Griffin *et al*., 1996; Cieslewicz *et al*., 2001; Morona *et al*., 2004). With regard to *cpsD* gene, it's predicted function is encoding an autophosphorylating protein tyrosine kinase，which is required for capsule polysaccharide chain length determination and export in *S. agalactiae* and *S. pneumonia* (Cieslewicz *et al*., 2001; Bender *et al*., 2003; Morona *et al*., 2003). Allelic exchange mutagenesis of *cpsD* gene of *S. iniae* showed that *cpsD* was likely required for expression level of *S. iniae* capsule (Locke *et al*., 2007). However, characterizations of CpsD product have not been studied yet.

In the present study, CpsD gene of *S. iniae* isolated from channel catfish was massively expressed in BL21(DE3) /pLysS and purified by the extraction of inclusion bodies (IBs). We furthermore optimized the expression conditions including concentration of IPTG (Isopropyl b-D-1-thiogalactopyranoside), induction temperature and induction time. Anti-serum against the recombinant protein CpsD expressed in *E. coli* was also performed.

Materials and Methods

Bacterial strain and plasmid

The *S. iniae* strain DGX07 was isolated from infected channel catfish (Guangxi Province, China) and preserved in the authors' laboratory. *E. coli* DH5α strain and BL21(DE3) /pLysS strain were used as host strains for recombinant protein heterologous expression. Cloning vector pMD19-T was purchased from TaKaRa Company. Expression vector pET-32a (+) was conserved by the authors' laboratory.

Construction and identification of

recombinant expression vector

A complete open reading frame (ORF) of 720bp sequence (GenBank accession no.JF795257) was amplified by polymerase chain reaction (PCR) with the following primers:

forward primer

5'-CGGATCCATGTCACAATTAAATTTAGTA3';

reverse primer

5'AAGCTTTCACTTTCTGGAATGTTTTTTGGATGA-3',

containing the *Bam*H I and *Hind* III restriction sites (bold), respectively. The PCR parameters were 5 min at 94°C and 30 cycles of 1min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension time of 10 min at 72°C. The PCR products were purified by using Agarose Gel DNA Extraction Kit (TaKaRa) was cloned into the pMD19-T vector, followed by excising using *Bam*H and *Hind* Ⅲ. The purified gene was inserted into pET-32a (+), generating the recombinant expression vector pET-32a(+):CpsD, then transformed into *E. coli* DH5α. The positive recombinant clone was selected by Amp/IPTG/X-Gal agar plate and then verified by colony PCR, restriction enzyme analysis and DNA sequencing (Hajam *et al*., 2013).

Induction expression of the recombinant protein with IPTG

The recombinant expression plasmid was transformed into *E. coli* BL21 (DE3) /pLysS competent cell, followed by selection by Amp/IPTG/X-Gal agar plate and identified by colony PCR. A single positive bacterial colony was inoculated into 5 ml Luria-Bertani (LB) broth with ampicillin (100μg/ml) and incubated in a shaker overnight at 37°C. The overnight culture *E. coli* BL21 (DE3) /pLysS containing recombinant plasmid pET-32a(+): CpsD and expression plasmid pET-32a(+) (negative control) were diluted into 50ml of fresh LB medium with ampicillin (100mg/ml), then incubated by shaking at 37°C until reaching mid-log phase (OD_{600}nm=0.5~0.6). The positive culture was subsequently induced at 37°C by using 1mM IPTG. Each sample was collected at 4h post-induction, and both the *E. coli* BL21 (DE3) /pLysS with or without the positive plasmid were cultured without IPTG as blank control. Each bacterial culture was harvested by centrifugation at 8000r/min for 10min at 4°C. Broth culture precipitate was suspended in 20ml 20mM Tris-HCl buffer (pH8.0), 2 times. Then the broth culture lysed in 10ml 20Mm Tris-HCl buffer (pH8.0). The concentrated cells were then sonicated in an ice water bath. Cells were then harvested by centrifugation at 8000r/min for 15min at 4°C and lysed by 5× sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) loading buffer (1M Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, and 0.05% bromophenol blue, with 0.05% β-ME), followed by boiling for 10min. A 12.5% SDS-PAGE gel was performed to verify the expression of the recombinant protein. The negative control and the blank control were analyzed in parallel, as described above (Li *et al*., 2011).

Optimization of the expression conditions

To maximize the quantity of the recombinant protein, culture conditions were optimized with induction temperature, IPTG concentration and induction period. Bacterial growth conditions were as described above. For optimizing induction

temperature, the bacterial cultures were induced with 1.0 mM IPTG for 4 hours and grown at three different temperatures (28°C, 34°C and 37°C). For IPTG concentration, the bacterial cultures were induced with different final concentrations (0.2, 0.4, 0.6, 0.8, 1.0 1.2, 1.5 and 2.0 mM) and incubated for 4h at 37°C. For incubation time, the bacterial cultures were induced with 0.6 mM IPTG and cultured at 37°C for 2h, 3h, 4h, 5h, 6h, 8h, 10h and overnight. Total cells of each tube were analyzed by SDS-PAGE as described above (Liu and Yang, 2012).

Purification of the recombinant protein

Expression host bacteria *E. coli* BL21 (DE3) /pLysS transformed with recombinant expression vector were cultured at 37°C in 1L LB medium with ampicillin (100 μg/ml). The bacteria were induced with optimized conditions and then harvested by centrifugation at 8000 r/min for 10min at 4°C. The broth culture was then lysed with 0.1 mg/ml lysozyme in 50ml 20 Mm Tris-HCl buffers (pH8.0) overnight at 4°C and lysed by sonication. The suspension was centrifuged at 8000 r/min for 20 min at 4°C, and the yield was then kept on ice. The pellet was re-suspended in 50 ml Wash buffer (2 M urea,50mM pH8.0 Tris-HCl buffer, 1mM EDTA, 150mM NaCl, and 0.1% Triton X-100), 5 times (each time lasted 10 minutes). After these procedures and centrifugation, the resulting subsidence was dissolved in 8M urea till the solution was clear. The solution was subsequently diluted in 2×25 mM Tris-HCl pH8.0. A Ni^{2+}-NTA agarose resin column (Bio-Rad) was employed for purifying the protein, performed as the manufacturer's standard protocol. The purified recombinant protein was then stored at 4°C for 3 days or at -70°C for a longer time (Liu and Yang, 2012; Zhu *et al*., 2012).

Immunization of rabbits with CpsD

Anti-serum was prepared by immunizing two rabbits. Normal rabbit blood serum (about 5 ml of blood sample) was collected as negative controls, then the rabbits were traperitoneally injected initially with 0.8 mg purified CpsD with complete Freund's adjuvant (Sigma) on the back and proximal limbs (0.1 ml /site). Subcutaneous booster injections of 0.7mg protein in incomplete Freund's adjuvant (Sigma) were given at intervals of 10 days, for two times. Then the animals were intravenously immunized with 0.2 mg of protein after 10 days. Finally, the animals were sacrificed and the blood was harvested. The anti-serum was harvested and purified by precipitation of ammonium sulfate, then purified by the DEAE-Sepharose column (Bio-Rad). The IgG fraction was purified by ion exchange column chromatography following the manufacturer's instructions. The purified IgG fraction was analyzed by 12.5% SDS-PAGE (Zhang *et al*., 2010).

Western Blotting

Protein samples were separated on a 12.5% SDS-PAGE gel and then electroblotted onto polyvinylidene fluoride (PVDF) membrane (TIANGEN) for 90min at 120V. The PVDF membrane was blocked with 3% bovine serum albumin (BSA) in TBST Buffer (20mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20, pH 8.0) for 90 min at 37°C. The membrane was washed 3 times

(each time lasted 10 minutes) and then incubated with prepared anti-serum (diluted 1:500 in TBST buffer with 0.5% BSA) overnight at 4°C, while applying the normal rabbit blood serum as negative control. The membranes were washed (as described above) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sangon，China), diluted into 1:2500 in TBST buffer containing 0.5% BSA for 1 h at 37°C. After that, the membranes were washed three times with TBST, and immuno-reactive protein was visualized by using diamino benzidine (DAB). The membrane was washed with distilled water till the band was clear enough (Zhang *et al*., 2010; Zhu *et al*., 2012).

Results

Construction and identification of recombinant expression vector

CpsD gene of *S. iniae* isolated from channel catfish was amplified and then revealed by agarose gel electrophoresis (Fig.1A, lane 3). The products were inserted into plasmid pMD19-T to form the recombinant plasmid, and was confirmed and recollected by double restriction enzymes digestion (Fig.1A, lane2). Subsequently the gene fragment was inserted into the expression plasmid pET-32a (+), forming the recombinant expression plasmid pET-32a (+)/CpsD. The transformation was performed with competent *E. coli* DH5α cells for screening. After that, the positive transformant was selected by the Amp/IPTG/X-Gal agar plate and identified by PCR and double restriction enzymes digestion (Fig.1B). The sequence of the confirmed clones was submitted to sequence analysis, indicating that there was no nucleotide error in the recombaint CpsD gene (data not shown).

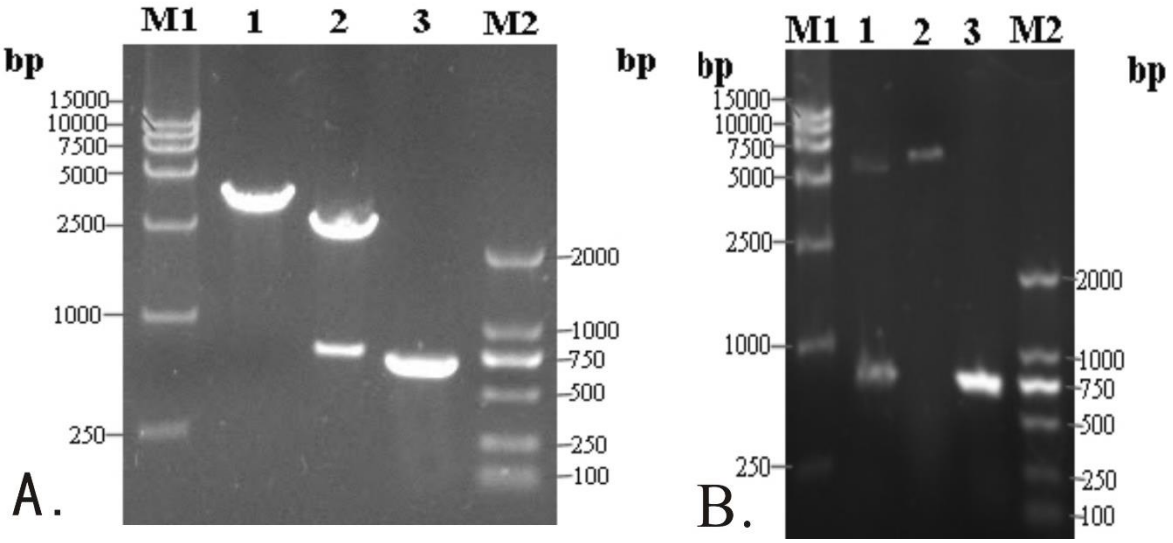

Figure 1: PCR amplification of *S. iniae* CpsD gene and identification and construction of the recombinant T-clone vector and expression vector.

(A) Lane M1: DNA Marker (DL15000); lane 1: pMD19-T/CpsD digested with Bam HI; lane 2: pMD19-T/CpsD digested with Bam H I and Hind Ⅲ; lane 3: PCR

amplification product of CpsD; lane 4: DNA Marker (DL2000) (B) Lane M1: DNA Marker (DL15000); lane 1: pET-32a(+) /CpsD digested with Bam HI and Hind III; lane 2: pET-32a(+) /CpsD digested with Bam HI Lane 3, product of the bacterial colony PCR; Lane M2. DNA Marker (DL2000).

Induction expression of the recombinant protein with IPTG

The confirmed recombinant expression vector pET-32a (+): CpsD was transformed into the expression host *E. coli* BL21(DE3) /pLysS competent cell. The verified transformants by colony PCR were selected for recombinant protein expression (data not shown). The expression of recombinant CpsD protein was induced by IPTG with 1mM IPTG induction at 37°C for 4h. SDS-PAGE showed a specific band of approximately 46 kDa (CpsD ≈26 kDa, His6-tag= 20kDa, His6-tag- CpsD ≈ 46 kDa，Fig. 2A, lane 9), corresponding to the expected molecular mass. Meanwhile, no distinct band was present for both the un-induced *E. coli* BL21(DE3) /pLysS carrying recombinant plasmid pET-32a(+):CpsD and the induced *E. coli* BL21(DE3)/pLysS carrying the pET-32a (+) empty vector (Fig. 2A). We examined the relative distribution of the expressed recombinant protein after sonication and the result showed that the target protein was almost expressed in the insoluble fraction, as forms of IBs (Fig. 2A).

Optimization of the expression conditions

We examined the CpsD expression level at three different conditions: induction temperature, IPTG concentrations and duration of induction. For optimization of CpsD expression level, as shown in Fig. 2, the optimal induction temperature was 37°C (Fig. 2A, lane 9), since the expression level was higher than that at 28°C (Fig. 2A, lane 5) or 34°C (Fig. 2A, lane 7). The optimal concentration for IPTG induction was 0.6 mM (Fig. 2B, lane 4), compared to other inductions with different IPTG concentrations. The optimal induction time was 5h (Fig. 2C, lane 4), compared with other induction time.

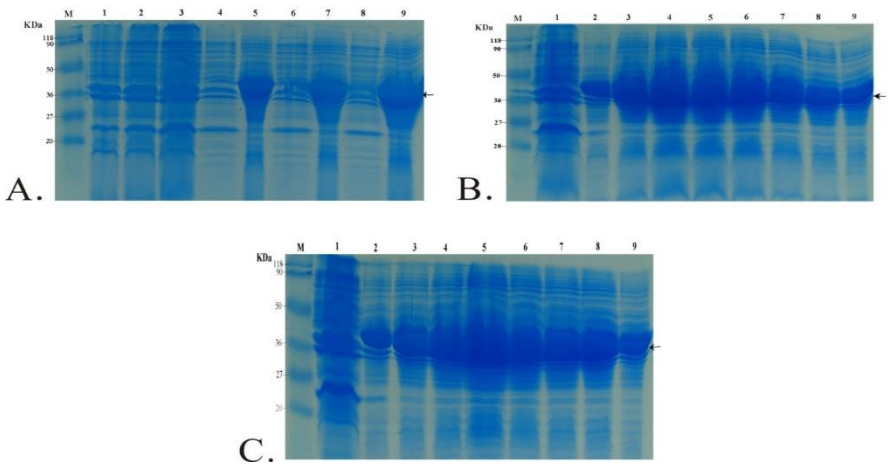

Figure 2: Analysis of expression and optimization of the expression conditions of recombinant protein on a 12.5% SDS-PAGE gel.

The direction of the arrow represented the target protein. (A) Expression and optimization of the temperature for CpsD. Lane M: prestained protein marker; Lane 1: the un-induced BL21 (DE3)/pLysS within pET-32a (+) plasmid; Lane 2: the induced BL21 (DE3)/pLysS within pET-32a (+) plasmid; Lane 3: the un-induced BL21(DE3) /pLysS within pET-32a(+)/CpsD plasmid. Lane4, 6, 8: Soluble fractions of the induced BL21(DE3) /pLysS within pET-32a(+)/CpsD plasmid at 28℃, 34℃ and 37℃, respectively; lane5, 7, 9: IBs of the induced BL21(DE3)/pLysS within pET-32a(+)/cpsD plasmid at 28℃, 34℃ and 37℃, respectively. (B) The determination of the optimal induced IPTG final concentration. Lane M: prestained protein marker; Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9: the induced IPTG final concentrations were 0Mm, 0.2mM, 0.4mM, 0.6mM, 0.8mM, 1.0mM, 1.2mM, 1.5mMand 2.0mM, respectively.(C) The determination of the optimal duration of induction. Lane M: prestained protein marker; Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9: the induced durations were 0h, 2h, 3h, 4h, 5h, 6h, 8h, 10h and overnight, respectively.

Purification of the recombinant protein

In the present study, the host bacteria *E. coli* BL21(DE3)/pLysS carrying pET-32a(+): CpsD plasmid were allowed to grow in 1L LB medium supplemented with ampicillin (100 µg/ml). Host bacteria was then collected and sonicated as described above, and the purification of the IBs was very effective. There were few other proteins examined by SDS-PAGE (Fig. 3, lane 2). Since the recombinant CpsD protein contains a His6-tag of 20 kDa, we used a single IMAC chromatographic step on Ni^{2+}-NTA agarose for purifying IBs, following the manufacturer's instructions. The purity of the eluted recombinant protein was examined by SDS-PAGE, and a single band was observed (Fig. 3, lane 1). According to the Modified Bradford Protein Assay Kit (Sangon, China), 582.47 mg of purified protein/1L culture was obtained.

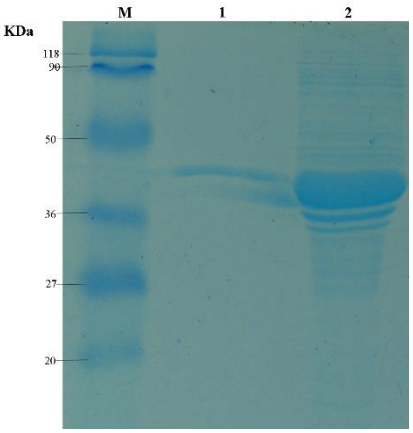

Figure 3: analysis of purified recombinant protein on 12.5% SDS-PAGE.

Expression of all lanes is accomplished at optimal conditions (at 37°C for 5 h with 0.6 mM IPTG induction). Lane M: prestained protein marker; Lane 1: the purified of recombinant protein by IMAC chromatographic step on Ni^{2+}-NTA agarose; Lanes 2: IBs of the induced within pET-32a(+))/CpsD plasmid, after washing with Wash Buffer.

Western blot analysis

The anti-serum was harvested and then purified (According to the method mentioned above). The purified IgG fraction was analyzed by SDS-PAGE (Fig. 4, lane 1).The purified anti-serum reacted with the recombinant protein by western

blot analysis. The results showed that a single specific band approximately 46 kDa (Fig. 5, lane 1) was visualized. Meanwhile, the negative serum did not show any band in western blots (Fig. 5, lane 2). The results indicated that the recombinant protein had good reactivity and specificity.

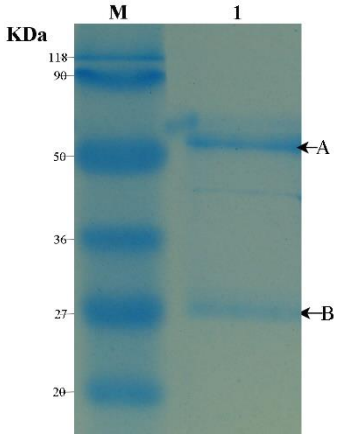

Figure 4. Analysis of purified rabbit anti-serum IgG on 12.5% SDS-PAGE. Lane M: prestained protein marker; Lane 1: purified rabbit anti-serum (the direction of A and B arrow represented heavy chain and light chain, respectively).

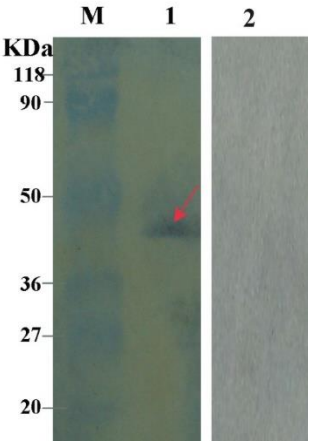

Figure 5. Western blot analysis of purified CpsD. The direction of red arrow represented the target protein. Lane M: prestained protein marker; Lane 1: recognition of antiserum with purified CpsD; lane 2: western blot analysis with negative rabbit serum.

Discussion

Polysaccharide capsule of expression have a key role on *S. iniae* infection of human and aquatic animals, that facilitates protecting from phagocytic clearance (Allen and Neely, 2011). CpsD gene of *S. iniae* is notably conservative in capsule operon of both virulent and commensal strains. Some studies have revealed that CpsD serves a crucial role in CPS formation. Deletion of CpsD gene of *S. iniae* could significantly attenuate *S. iniae* infection of hybrid striped bass, which is likely responsible for Chain length determination and export (Locke *et al*., 2007). However, the CpsD protein plays some role in capsule synthesis and capsule regulation during various stages of the *S. iniae* infectious process which remains unknown. Therefore, our work might lay the foundation for detecting the capsule regulation of the *S. iniae* infectious process.

In the present study, the CpsD gene was PCR amplified from *S. iniae* virulent strain that infected channel catfish. The CpsD gene was inserted into expression vector pET-32a (+) plasmid and the recombinant protein was expressed and purified. Generally, high expression levels of recombinant protein in *E. coli* often results in the formation of IBs, insoluble and inactive protein aggregates (Schein, 1990; De Bernardez Clark, 1998). In this study, the target protein was also expressed through the form of IBs. Expression of recombinant protein in prokaryotes such as *E. coli* is a common and inexpensive method to rapidly obtain large quantities of the expected protein (De Bernardez Clark, 1998). To date, there is no report in literature on the optimization expression conditions of this protein in *E. coli*. For the first time, we reported that the optimal growth condition for expression of CpsD was 37°C for 5h with 0.6 mM IPTG. The

level of purification of CpsD was determined to a single band by SDS-PAGE. In *S. pneumoniae*, Weiser *et al*. (2001) also revealed that the CpsD protein was recognized by rabbit anti-CpsD IgG by western blot analysis. The antiserum against the recombinant protein CpsD was raised to detect the levels of expression in the transparent (T) and opaque (O) variants of *S. pneumoniae,* showing amounts of CPS correlated with expression of CpsD: the higher expression level of CpsD, the more amounts of CPS, and vice versa (Weiser *et al*., 2001). It further revealed that CpsD is vitally important in the process of capsule synthesis. In the present study, the prepared anti-serum against CpsD protein of *S. iniae* is raised to detect reactogenicity with CpsD protein by western blot analysis, showing that CpsD protein is a favorable antigenicity of the recombinant protein. Hence, in the next step of the study, we will analyze the possible relationship between multiple virulent isolates of *S. iniae* and the CpsD protein by the prepared antibody, and further clarify that amounts of CPS correlated with expression level of CpsD in the *S. iniae*. Meanwhile, we can also explore dynamic relationships among amounts of capsule synthesis, capsule regulation and expression level of CpsD of *S. iniae* during various stages of the *S. iniae* infections.

Acknowledgements

This study was supported by grants from the Chang jiang Scholars and Innovative Team Development Plans of Ministry of Education, China (IRT0848).

References

Agnew, W. and Barnes, A.C., 2007. *Streptococcus iniae*: an aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. *Veterinary Microbiology*, 122, 1-15.

Allen, J.P. and Neely, M.N., 2011. The *Streptococcus iniae* Transcriptional Regulator CpsY is required for protection from neutrophil-mediated killing and proper growth in vitro. *Infection and Immunity*, 79, 4638-4648.

Bender, M.H., Cartee, R.T. and Yother, J., 2003. Positive correlation between tyrosine phosphorylation of CpsD and capsular polysaccharide production in *Streptococcus pneumoniae*. *Journal of Bacteriology*, 185, 6057-6066.

Buchanan, J.T., Stannard, J.A., Lauth, X., Ostland, V.E., Powell, H.C., Westerman, M.E. and Nizet, V., 2005. *Streptococcus iniae* phosphoglucomutase is a virulence factor and a target for vaccine development. *Infection and Immunity*, 73, 6935-6944.

Chen, D.F., Wang, K.Y., Geng, Y., Wang, J., Huang, X.L. and He, M., 2011. Pathological changes in cultured channel catfish Ictalurus punctatus spontaneously infected with *Streptococcus iniae*. *Diseases of Aquatic Organisms*, 95, 203-208.

C**ieslewicz, M.J., Kasper, D.L., Wang, Y. and Wessels, M.R., 2001**. Functional analysis in type ia group B *Streptococcus* of a cluster of genes involved in extracellular polysaccharide production by diverse species of Streptococci. *Journal of*

Biological Chemistry, 276, 139-146.

De Bernardez Clark, E., 1998. Refolding of recombinant proteins. *Current Opinion in Biotechnology*, 9, 157-163.

El Aamri, F., Padilla, D., Acosta, F., Caballero, M., Roo, J., Bravo, J., Vivas, J. and Real, F., 2010. First report of *Streptococcus iniae* in red porgy (*Pagrus pagrus*, L.). *Journal of Fish Diseases*, 901-905.

Goh, S.H., Driedger, D., Gillett, S., Low, D.E., Hemmingsen, S.M., Amos, M., Chan, D., Lovgren, M., Willey, B.M. and Shaw, C., 1998. *Streptococcus iniae*, a human and animal pathogen: specific identification by the chaperonin 60 gene identification method. *Journal of Clinical Microbiology*, 36, 2164.

Griffin, A.M., Morris, V.J. and Gasson, M.J., 1996. The cpsABCDEgenes involved in polysaccharide production in *Streptococcus salivarius* ssp. thermophilus strain NCBF 2393. *Gene*, 183, 23-27.

Hajam, I.A., Dar, P.A., Chandra Sekar, S., Nanda, R.K., Kishore, S., Bhanuprakash, V. and Ganesh, K., 2013. Co-administration of flagellin augments immune responses to inactivated foot-and-mouth disease virus (FMDV) antigen. *Research in Veterinary Science*, 95, 936-941.

Koh, T., Kurup, A. and Chen, J., 2004. *Streptococcus iniae* discitis in Singapore. *Emerging Infectious Diseases*, 10, 1694.

Lau, S.K.P., Woo, P.C.Y., Tse, H., Leung, K.W., Wong, S.S.Y. and Yuen, K.Y., 2003. Invasive *Streptococcus iniae* infections outside North America. *Journal of Clinical Microbiology*, 41, 1004-1009.

Li, J.F., Zhang, J., Zhang, Z., Kang, C.T. and Zhang, S.Q., 2011. SUMO mediating fusion expression of antimicrobial peptide CM4 from two joined genes in *Escherichia coli*. *Current Microbiology*, 62, 296-300.

Liu, Z.Q. and Yang, P.C., 2012. Construction of pET-32 α (+) vector for protein expression and purification. *North American Journal of Medical Sciences*, 4, 651.

Locke, J.B., Colvin, K.M., Datta, A.K., Patel, S.K., Naidu, N.N., Neely, M. N., Nizet, V. and Buchanan, J.T., 2007. Streptococcus iniae capsule impairs phagocytic clearance and contributes to virulence in fish. *Journal of Bacteriology*, 189, 1279-1287.

Miller, J.D. and Neely, M.N., 2005. Large-scale screen highlights the importance of capsule for virulence in the zoonotic pathogen *Streptococcus iniae*. *Infection and Immunity*, 73, 921-934.

Morona, J.K., Miller, D.C., Morona, R. and Paton, J.C., 2004. The effect that mutations in the conserved capsular polysaccharide biosynthesis genes cpsA, cpsB, and cpsD have on virulence of *Streptococcus pneumoniae*. *Journal of Infectious Diseases*, 189, 1905.

Morona, J.K., Morona, R., Miller, D.C. and Paton, J.C., 2003. Mutational analysis of the carboxy-terminal (YGX) 4 repeat domain of CpsD, an autophosphorylating tyrosine kinase required for capsule biosynthesis in *Streptococcus pneumoniae*. *Journal of Bacteriology*, 185, 3009-3019.

Rajagopal, L., Clancy, A. and Rubens, C. E., 2003. A eukaryotic type serine/threonine kinase and phosphatase in *Streptococcus agalactiae* reversibly phosphorylate an inorganic pyrophosphatase and affect growth, cell segregation, and virulence. *Journal of Biological Chemistry*, 278, 14429-14441.

Schein, C.H., 1990. Solubility as a function of protein structure and solvent components. *Nature Biotechnology*, 8, 308-317.

Shoemaker, C.A., Klesius, P.H. and Evans, J.J., 2001. Prevalence of *Streptococcus iniae* in tilapia, hybrid striped bass, and channel catfish on commercial fish farms in the United States. *American Journal of Veterinary Research*, 62, 174-177.

Weinstein, M.R., Litt, M., Kertesz, D.A., Wyper, P., Rose, D., Coulter, M., McGeer, A., Facklam, R., Ostach, C. and Willey, B.M., 1997. Invasive infections due to a fish pathogen, *Streptococcus iniae*. *New England Journal of Medicine*, 337, 589-594.

Weiser, J.N., Bae, D., Epino, H., Gordon, S.B., Kapoor, M., Zenewicz, L.A. and Shchepetov, M., 2001. Changes in availability of oxygen accentuate differences in capsular polysaccharide expression by phenotypic variants and clinical isolates of *Streptococcus pneumoniae*. *Infection and Immunity*, 69, 5430-5439.

Yuasa, K., Kitancharoen, N., Kataoka, Y. and Al-Murbaty, F.A., 1999. *Streptococcus iniae*, the causative agent of mass mortality in rabbitfish *Siganus canaliculatus* in Bahrain. *Journal of Aquatic Animal Health*, 11, 87-93.

Zhang, S., Ma, G., Xiang, J., Cheng, A., Wang, M., Zhu, D., Jia, R., Luo, Q., Chen, Z. and Chen, X., 2010. Expressing gK gene of duck enteritis virus guided by bioinformatics and its applied prospect in diagnosis. *Virology Journal*, 7, 168.

Zhu, X., Wang, D., Li, S., Xiao, Q., Tao, K., Hu, J., Cao, W. and Feng, W., 2012. Expression of a humanized single-chain variable fragment antibody targeting chronic myeloid leukemia cells in *Escherichia coli* and its characterization. *International Journal of Molecular Medicine*, 29, 939.