Journal of Animal and Veterinary Advances 11 (20): 3800-3805, 2012
ISSN: 1680-5593

Prokaryotic Expression of *p1* Gene of *Yersinia ruckeri* Isolated from Channel Catfish (*Ictalunes punctatus*) and Optimization of Expression Conditions

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Abstract: The *p1* gene of *Yersinia ruckeri* (*yrp1*) which was isolated from channel catfish was amplified by PCR with specific primers and inserted into pMD19-T vector. The positive recombinant plasmid was selected and sequenced. Then, the *yrp1* gene was subcloned into pET-32a (+) vector and transformed into BL21 (DE3) followed by induction with IPTG and detection with SDS-PAGE. Optimization of the induction conditions were conducted. The results showed that the recombinant protein with a molecular mass of about 72 kDa was mostly packaged into inclusion bodies. The optimization of induction process conditions led us to perform the fusion protein induction at 37°C for 4 h with 0.8 mM IPTG.

Key words: *Yersinia ruckeri*, *p1* gene, prokaryotic expression, optimization, China

INTRODUCTION

Yersinia ruckeri (*Y. ruckeri*) can cause an acute, contagious and highly lethal disease which named yersiniosis or Enteric Redmouth disease (ERM) in all ages of fish (Ross *et al*., 1966) and is recognized as one of the most prominent pathogenic bacteria. *Y. ruckeri* was initially isolated from rainbow trout (*Oncorhynchus mykiss*) in America in 1952 (Rucker, 1966) and it spread through Australia, North America, Europe and South Africa in the next a few decades and caused remarkable economic losses in the salmonid farming industry (Furones *et al*., 1993). Since, the first report of ERM in silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Aristichthys nobilis*) in China (Bo-Hai *et al*., 1991), it began to break out in China and the susceptible hosts expanded to some other aquatic animals such as carp, shrimp, channel catfish, etc. according to the reports in recent years (Xue-Feng *et al*., 1997; Fang-Ling *et al*., 2010; Bo-Hai *et al*., 1991).

Extracellular products of *Y. ruckeri* including haemolysins (Fernandez *et al*., 2007), proteases (Secades and Guijarro, 1999) and lipases (Romalde and Toranzo, 1993), reproduce some characteristic signs of ERM such as haemorrhage in the intestine and the mouth when injected into rainbow trout. A extracellular metalloprotease termed Yrp1 (*Yersinia ruckeri* protease 1) can digest a wide variety of extracellular matrix and muscle proteins (actin, myosin, gelatine, fibrinogen and laminin) and lead to membrane alterations and pores in the capillary vessels, causing typical haemorrhages especially around the intestine and mouth (Fernandez *et al*., 2003). Therefore, it seems clearly that the Yrp1 protein contributes importantly to the pathogenicity of *Y. ruckeri*. In addition to this as described by Fernandez *et al*. (2003), the Yrp1 protein could elicit a strong protection against the infection of *Y. ruckeri* when it was inactivated by heat and used as an immunogen. Consequently, the Yrp1 protein can be made into vaccine to prevent the outbreak of ERM due to its excellent immunogenicity. The Yrp1 protein used in that study was extracted from the extracellular products of *Y. ruckeri* by ammonium sulfate precipitation and ion-exchange chromatography (Secades and Guijarro, 1999). However, there are some problems for large-scale preparation of the Yrp1 protein vaccine, such as complex operation and high cost. The effective way to solve these problems is to obtain the Yrp1 protein through expression *in vitro* because the immunogenicity of the Yrp1 protein is almost unaffected owing to its complete primary structure and specific senior structure.

Researchers cloned the *yrp1* gene which encoding the Yrp1 protein and did codon usage bias analysis of it during the previous study. Based on these, researchers

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intend to construct the recombinant expression vector and optimize the expression conditions, thereby obtaining a large amount of Yrp1 protein. These researches might lay the foundation for large-scale preparation of the Yrp1 protein vaccine.

MATERIALS AND METHODS

Bacterial strain, plasmid, chemicals and kits: *Y. ruckeri* strain FF003 was isolated from channel catfish farm outbreaks in Sichuan province, China and preserved in the researchers' laboratory. *Escherichia coli* (*E. coli*) DH5α and BL21 (DE3) competent cells were purchased from Tian Gen Biotech company, China. Cloning vector pMD19-T was purchased from TaKaRa company, China as well as all chemicals and kits used in this study. Expression vector pET-32a (+) was provided by the researchers' laboratory.

Cloning and sequencing of the *yrp1* gene: *Y. ruckeri* strain FF003 was routinely cultured on nutrient broth at 28°C for 24 h. Then, the genomic DNA of it was extracted by using Bacterial Genomic DNA Extraction kit according to the manufacturer's instruction. The coding region of the *yrp1* gene was amplified with one pair of primers. Forward Primer (P1) 5'-GGATCCATGAAAGCAAGTAG TAATAAA-3' and the reverse Primer (P2) 5'-AAGCTTCG AATACATCCAAACAATA-3' with the BamH I and Hind III restriction sites (underlined), respectively. The two primers were synthesized in TaKaRa company and PCR was conducted in a 25 μL reaction mixture containing 0.5 μL of each primer (20 pmol each), 1.0 μL DNA template (5 ng), 12.5 μL MasterMix and 10.5 μL water. The PCR conditions were: 95°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min and then a final extension at 72°C for 10 min. The PCR products were fractionated on 1.0% agarose gel electrophoresis and stained with Gold View. After that the PCR products were purified by using Agarose Gel DNA Extraction kit according to the manufacturer's instruction. The purified PCR products were cloned into pMD19-T vector followed by transformation into *E. coli* DH5α competent cell. Then, the positive recombinant clone was selected by the Amp/IPTG/X-Gal agar plate. Recombinant plasmid was identified by bacterial colony PCR with aforementioned conditions, digested with restriction enzymes BamH I and Hind III and fractionated in 1% agarose gels. DNA sequencing was also carried out in TaKaRa company followed by alignment with BLAST N Software (Gotea *et al*., 2003).

Subcloning of the *yrp1* gene: The recombinant plasmid pMD19-T-yrp1 and expression vector pET-32a (+) were digested with restriction enzymes BamH I and Hind III followed by fractionation in 1% agarose gels. The intended products (pET-32a (+) vector and the *yrp1* gene) were purified by using DNA Fragment Purification kit according to the manufacturer's instructions. The purified fragment of the *yrp1* gene was ligated with the counterparts of expression vector pET-32a (+) at 16°C for 16 h by using T_4 DNA ligase to generate a recombinant expression plasmid named pET-32a (+) -yrp1. Then, the ligation mixture was transformed into *E. coli* DH5α competent cell by heat shock method and the positive recombinant clone was selected by Amp/IPTG/X-Gal agar plate. Recombinant plasmid was identified by bacterial colony PCR with a pair of specific primers. Afterwards, the recombinant plasmid was extracted by using Plasmid Purification kit according to the manufacturer's instructions and identified by digestion with restriction enzymes BamH I and Hind III. Then, the recombinant expression plasmid was transformed into *E. coli* BL21 (DE3) competent cell followed by selection with Amp/IPTG/X-Gal agar plate and identification with bacterial colony PCR.

Induction expression of the recombinant protein with IPTG: *E. coli* BL21 (DE3) which containing recombinant plasmid pET-32a (+) -yrp1 was inoculated into 10 mL of Luria-Bertani (LB) medium containing ampicillin (100 mg mL^{-1}) to grow overnight at 37°C with constant agitation (120 r min^{-1}). The culture was used to inoculate into 100 mL LB containing ampicillin (100 mg mL^{-1}) with vigorous shaking in a fermenter until the OD_{600} value reached at 0.5-0.6. The recombinant protein was induced by the addition of Isopropyl-β-D-Thiogalactopyranoside (IPTG) with final concentration of 1.0 mM. *E. coli* BL21 (DE3) which containing plasmid pET-32a (+) was dealt as described above as negative control and both the two kinds of *E. coli* BL21 (DE3) were cultured without induction of IPTG as blank control.

Bacterial culture was incubated for 4 h with vigorous shaking at 37°C and harvested by centrifugation at 8000 r min^{-1} for 10 min at 4°C. The pellet was suspended in 5 mL 20×mM Tris-HCl buffer and then lysed by sonication in an ice water bath. The supernatant was transferred into another tube and the pellet was resuspended in 3 mL 20×mM Tris-HCl buffer. Then, all the mixtures were added with 4 times volume of 5×SDS-PAGE loading buffer (0.313 M Tris-HCl, pH 6.8; 50% glycerol; 10% SDS and 0.05% bromophenol blue with 100 mM DTT). After that the cell lysates were boiled for 10 min, centrifuged at 12000 r min^{-1} for 10 min submitted to 12.5% sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then analyzed by

Coomassie brilliant blue R-250 staining. Briefly, the gel was stained with Coomassie brilliant blue R-250 overnight and destained in 6% acetic acid until a clear background was observed. The negative control and the blank control as described were analyzed in parallel.

Optimization of the expression conditions: To increase the production of the fusion protein, culture conditions for expression were optimized. For optimizing IPTG dose, the bacterial cultures were induced with different final concentrations (0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mM) and incubated at 37°C for 4 h. For incubation time optimization, the bacterial cultures were induced with 0.8 mM IPTG and allowed to grow at 37°C for 1, 2, 3, 4, 5, 6 and 7 h, respectively. For induction temperature optimization, the bacterial cultures were induced with 0.8 mM IPTG and allowed to grow at four different temperatures (18, 25, 30 and 37°C) for 4 h. Total tropina harvested from each test were assessed by SDS-PAGE as described.

RESULTS AND DISCUSSION

Cloning and sequencing of the *yrp1* gene: To isolate the *yrp1* gene, PCR was conducted on the genomic DNA of *Y. ruckeri* by using primers P1 and P2 which were specific to the *yrp1* gene. A band which was about 1400 bp was observed upon electrophoresis of the PCR products on agarose gel (Fig. 1). The approximate 1400 bp PCR product was purified and cloned into pMD19-T vector followed by identification through bacterial colony PCR and digestion with restriction enzymes BamH I and Hind III (Fig. 2) thus the positive recombinant plasmid was constructed, designated as pMD19-T-yrp1. After cloning and sequencing, the identical nucleotide sequence was analyzed by BLAST N Software. Sequence alignment indicated that the DNA sequence obtained by PCR displayed similarity of 100% to the *p1* gene of *Y. ruckeri* ATCC standard strain (GeneBank Accession No: AJ 318052), suggesting that there was no nucleotide error in the synthetic *yrp1* gene fragment which laid the foundation for expression of recombinant protein.

Subcloning of the *yrp1* gene: The prokaryotic expression vector pET-32a (+) which possesses a high stringency T7 lac promoter, T7 terminator and 6× His tag has been recognized as one of the most powerful tools for expression of recombinant proteins in *E. coli* (Brown *et al*., 1990). To construct the recombinant expression vector, recombinant plasmid pMD19-T-yrp1 and expression vector pET-32a (+) were digested with restriction enzymes Bam HI and Hind III. Then, the

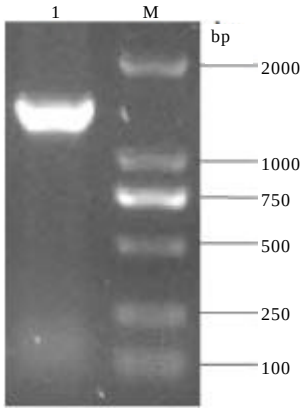

Fig. 1: PCR amplification result of the *yrp1* gene. M: DNA marker (DL2000); Lane 1: PCR product of the *yrp1* gene

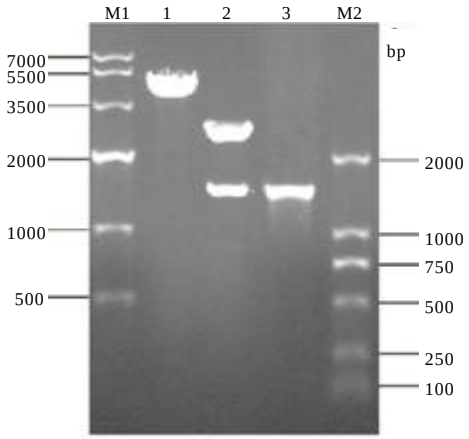

Fig. 2: Identification of the positive recombination plasmid through bacterial colony PCR and digestion with restriction enzymes BamH I and Hind III. M1: DNA marker (DL7000); Lane 1: digestion of the recombinant plasmid with BamH I; Lane 2: digestion of the recombinant plasmid with BamH I and Hind III; Lane 3: product of the bacterial colony PCR; M2: DNA marker (DL2000)

intended products were purified and ligated followed by transformation into *E. coli* DH5α competent cell. After that the positive transformant was selected by the Amp/IPTG/X-Gal agar plate and identified through bacterial colony PCR and digestion with restriction enzymes BamHI and Hind III (Fig. 3) thus the positive recombinant expression vector was constructed, designated as pET-32a (+)-yrp1. Afterwards, the recombinant plasmid pMD19-T-yrp1 was extracted from *E. coli* DH5α and transformed into *E. coli* BL21 (DE3) competent cell followed by selection with Amp/IPTG/X-

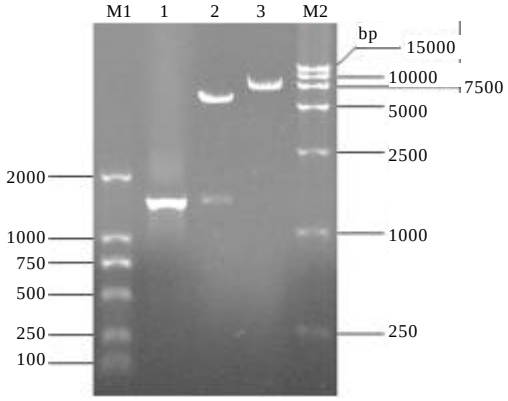

Fig. 3: Identification of the recombinant expression plasmid through bacterial colony PCR and digestion with restriction enzymes BamH I and Hind III. M1: DNA marker (DL2000); Lane 1: product of the bacterial colony PCR; Lane 2: digestion of the recombinant expression plasmid with BamH I and Hind II; Lane 3: digestion of the recombinant expression plasmid with BamH I; M2: DNA marker (DL15000)

Gal agar plate and identification with bacterial colony PCR. Here, *E. coli* DH5α strain was used as the cloning host for the *yrp1* gene due to its high transformation efficiency. For the expression of recombinant protein, the *E. coli* BL21 (DE3) strain was used. This strain harbors the T7 bacteriophage RNA polymerase gene which permits the specific expression of heterologous genes driven by the T7 promoter and has the advantage of being deficient in both the ompT and lon proteases (Mierendorf *et al*., 1994; Studier and Moffatt, 1986; Studier *et al*., 1990).

Induction expression of the recombinant protein: The *E. coli* BL21 (DE3) containing recombinant plasmid pET-32a (+)-yrp1 was induced with IPTG (final concentration 1.0 mM) at 37°C for 4 h. A distinct band of approximately 72 kDa of molecular weight, corresponding to the expected size of the 6× His-tagged Yrp1 fusion protein was observed (Fig. 4, Lane 1).

The recombinant protein was not detected in the uninduced *E. coli* BL21 (DE3) carrying recombinant plasmid pET-32a (+)-yrp1 (Fig. 4, Lane 2 and 6) nor was it found in the induced and uninduced bacteria containing empty pET32b (+) vector (Fig. 4, Lane 3, 4, 7 and 8). The relative distribution of the fusion protein in the supernatant or pellet of the cell lysate was examined after sonication. The results revealed that the recombinant protein was mostly expressed in the insoluble fractions with the form of inclusion bodies (Fig. 4, Lane 1).

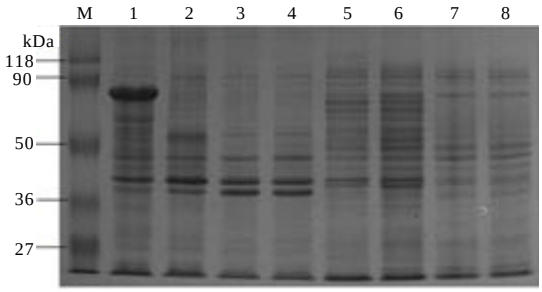

Fig. 4: Expression analysis of the Yrp1 fusion protein. M: protein marker; Lane 1: the induced BL21 (DE3) within pET-32a (+)-yrp1 plasmid (inclusion bodies); Lane 2: the uninduced BL21 (DE3) within pET-32a (+)-yrp1 plasmid (inclusion bodies); Lane 3: the induced BL21 (DE3) within pET-32a (+) plasmid (inclusion bodies); Lane 4: the uninduced BL21 (DE3) within pET-32a (+) plasmid (inclusion bodies); Lane 5: the induced BL21 (DE3) within pET-32a (+)-yrp1 plasmid (soluble fractions); Lane 6: the uninduced BL21 (DE3) within pET-32a (+)-yrp1 plasmid (soluble fractions); Lane 7: the induced BL21 (DE3) within pET-32a (+) plasmid (soluble fractions); Lane 8: the uninduced BL21 (DE3) within pET-32a (+) plasmid (soluble fractions)

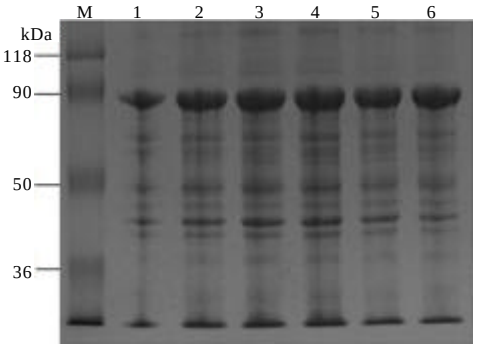

Fig. 5: Optimization of the final IPTG concentration. M: protein marker; Lanes 1, 2, 3, 4, 5 and 6 representing the induced IPTG final concentrations were 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mM, separately

Optimization of the expression conditions: The optimization of expression conditions as described in was conducted with different final IPTG concentrations, temperature for induction and duration of induction. Compared with other IPTG concentrations, the optimal concentration for IPTG induction was 0.8 mM (Fig. 5, Lane 4). As shown in Fig. 6, the optimal induction

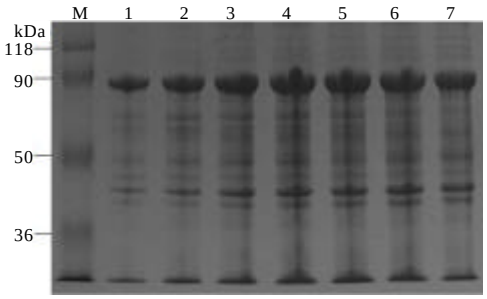

Fig. 6: Optimization of the culture time. M: protein marker; Lanes 1-7 representing the induced durations were 1-7 h, separately

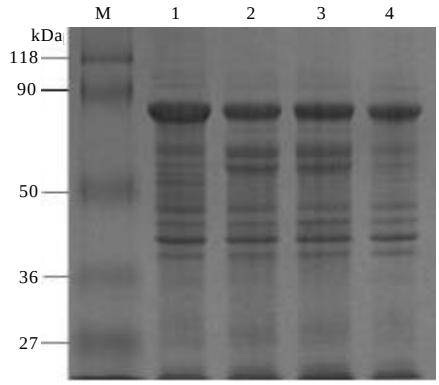

Fig. 7: Optimization of the culture temperature. M: protein marker; Lanes 1, 2, 3 and 4 representing the induced temperatures were 37, 30, 25 and 18°C

time was 4 h because the quantity of recombinant protein was higher than others. The optimal induction temperature was 37°C (Fig. 7, Lane 1) since, the expression level was higher than that at 18, 25 and 30°C. Therefore, the optimal expression conditions for Yrp1 were growth at 37°C for 4 h with 0.8 mM IPTG.

CONCLUSION

This study shows that the *yrp1* gene which encodes Yrp1 protein was amplified by PCR from genomic DNA of the *Y. ruckeri* strain FF003. After cloning and subcloning, the recombinant expression plasmid was constructed, designated as pET-32a (+)-yrp1. Then, the recombinant plasmid was transformed into BL21 (DE3) followed by induction with IPTG. Optimization of the induction conditions was carried out. The results indicated that the Yrp1 recombinant protein with a molecular mass of about 72 kDa was mostly packaged into inclusion bodies. The optimization of induction process conditions led us to perform the recombinant protein induction at 37°C for 4 h with 0.8 mM IPTG. Based on these, researchers can study the pathogenicity and immunogenicity of Yrp1 protein in further research and obtain a large amount of Yrp1 for large-scale preparation of the protein vaccine.

ACKNOWLEDGEMENTS

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

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