CLONING AND EXPRESSION OF pigC GENE IN ESCHERICHIA COLI

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SUMMARY

Prodigiosin (Pg), which is particularly of interest because of anticancer and antimicrobial activities, can be produced through the pigC-catalyzed condensation reaction of 4-methoxy-2, 2′-bipyrole-5-carboxyaldehyde (MBC) and 2-methyl-3-amilpyrrole (MAP). Therefore, the PigC protein plays an important role in prodigiosin biosynthetic pathway. However, studies related to PigC protein have not been carried out in Vietnam yet. In this work, the pigC gene was cloned and expressed in Escherichia coli DH10B and BL21 (DE3), respectively. Using PCR and universal primers, we amplified a fragment of 3 kb covering entire coding region of the pigC gene from Serratia sp. strain M5. The pigC gene was inserted into pJET1.2 vector, and then transformed into E. coli DH10B. The sequence of a recombinant vector pJET1.2/pigC was evaluated by using whole colony PCR amplification. Sequence alignment results revealed that the obtained pigC gene possesses 71.5% and 75.4% of nucleotide identity in comparison with two strains, Serratia 39006 and Serratia sp. AS9 published in GenBank with their respective accession numbers of AJ833001 and CP002773. The recombinant vector pJET1.2/pigC was used to reamplify pigC, and the acquired amplicon was inserted into pET22b vector at the site of HindIII and Xhol. The clone E. coli BL21 (DE3) containing recombinant vector pET22b/pigC was expressed in the auto-induced medium. The presence of PigC protein in the lysate was identified as a 100 kDa band through Western Blot analysis using anti his-tag antibody. Afterward, the PigC protein was purified by Ni-NTA column, and its expression level was quantified through SDS-PAGE analysis. The results of our study provide a potential material for producing prodigiosin from recombinant protein in Vietnam.

Keywords: MAP, MBC, pigC, prodigiosin, Vietnam

INTRODUCTION

Prodigiosin (Pg), a red pigment belonging to the prodiginine group, is a tripyrrolic secondary metabolite isolated from Serratia marcescens. Recently, prodigiosin and its related compounds have been particularly of interest because of their biological activities (Darshan, Manonmani 2015). The selective anti-cancer property of prodigiosin and its analogues were well-demonstrated on many cancer cell lines (Diaz-Ruiz et al., 2001; Montaner et al., 2000; Tomás et al., 2003) as well as in vivo tests (Wang et al., 2016; Yamamoto et al., 1999; Zhang et al., 2005). In addition, prodigiosin and its derivatives also have antiparasitic and antimicrobial activities (Rahul et al., 2015; Suryawanshi et al., 2017). In Vietnam, there were a few studies interested in S. marcescens and prodigiosin. Nguyen (2015) extracted and evaluated antibiotic of prodigiosin against Bacillus subtilis and Staphylococcus aureus from S. marcescens strain M10. The anti-insect property against Spodoptera litura of S. marcescens and prodigiosin was also detected (Nguyen, Nguyen, 2015). However, the opportunistic human pathogen S. marcescens species is harmful to human health, making it unsuitable for drug production by fermentation process (Domröse et al., 2015; Liu et al., 2017). Therefore, genetic engineering could create promising strategy for Pg production.

A biosynthetic gene cluster consisting 14-15 genes was identified to be responsible for Pg production in Serratia (Harris et al., 2004). Among these, a membrane located enzyme named PigC, with an approximate molecular weight of mass 100 kDa, plays a crucial role in the final step to produce prodigiosin.
Materials and Methods

Materials

Serratia sp. M5 strain was provided by Enzyme Biotechnology laboratory, Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology (VAST). E. coli DH10B (Invitrogen) and E. coli BL21 (DE3) strains (Novagen) were used as cloning and expression hosts, respectively. Vector pJET1.2 was utilized as cloning one and pET22b (Novagen) as expression vector. E. coli and Serratia sp. strains were respectively grown in media containing 1% NaCl, 1% tryptone, 0.5% yeast extract (Merck, Germany) at 37°C and 30°C.

Table 1. Primer pairs used in this study.

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>PigC_universal_F</td>
<td>5’TATTCCAYTTCGCCAATCAGGACA 3’</td>
</tr>
<tr>
<td>PigC_universal_R</td>
<td>5’ AAATTCCGYCACKAYAMAGCC 3’</td>
</tr>
<tr>
<td>PigC_HindIII_F</td>
<td>5’AAAAAAAGCTTTAATCCTACCTGTTGGA 3’</td>
</tr>
<tr>
<td>PigC_XhoI_R</td>
<td>5’AAAAAATCGAGGCATCGGACGTTCTC 3’</td>
</tr>
<tr>
<td>pJET1.2F</td>
<td>5’ CGACTCACTATAGGAGACGGCC 3’</td>
</tr>
<tr>
<td>pJET1.2R</td>
<td>5’ AAGAACATCGATTTCGATGCG 3’</td>
</tr>
<tr>
<td>T7 Promoter</td>
<td>5’TATACGACTCATATAGG 3’</td>
</tr>
<tr>
<td>T7 Terminator</td>
<td>5’ GTAGTTATTGCCTAGCGG 3’</td>
</tr>
</tbody>
</table>

Methods

Primer design for amplification of pigC gene

Because of highly variable sequences of pigC among strains available in Genbank, complete sequences of Pg clusters from different strains available in Genbank were downloaded and aligned. The consensus sequences being 300 nucleotides away from the pigC gene were chosen to design primers. After cloning, the obtained sequence of the region covering the pigC gene was used to design expression primers. The primer sequences were listed in Table 1.

Total DNA extraction and sequence amplification

Total DNA was extracted from S. macescens using GeneJET Genomic purification kit according to the manufacturer’s instruction. The pigC gene was amplified with pigC universal pair of primers (Table 1) using the following program: 98°C for 30 sec; 40 cycles of 98°C 10 sec, 58°C for 15 sec, 72°C for 1 min 30 sec, and a final cycle of 72°C for 5 min.

The targeted band was purified via GeneJET gel extraction kit according to the vendor’s manual. This candidate sequence was then ligated into pJET1.2 blunt vector and transformed into E. coli DH10B chemically competent cells as described before. Several colonies were selected for further examination using plasmid extraction and sequencing by pJET1.2 specific primers (Table 1).

PigC encoded gene was reamplified from colonies containing recombinant vector using the primers pigC_HindIII_F and pigC_XhoI_R (Table 1). The amplified product was digested with HindIII and XhoI (ThermoFisher Scientific, USA) and ligated into the vector pET22b. The ligated product was transformed into E. coli DH10B strain (Sambrook et al., 2001). Some clones were further examined using PCR, plasmid extraction, enzyme digestion and sequencing by T7 primers (Table 1).
Expression of pigC in E. coli BL21 (DE3) and protein purification

The recombinant vector with the correct sequence was transformed into BL21 (DE3) and expressed using auto-induced medium. In brief, overnight culture was inoculated into auto-induced medium containing tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, KH₂PO₄ 6.8 g/l, Na₂HPO₄ 7.1 g/l, (NH₄)₂SO₄ 3.3 g/l, MgSO₄ 2 mM, glucose 0.05%, glycerol 0.5%, and lactose 0.2% w/v). Firstly, the culture was incubated at 37°C/200 rpm for 3 hours. The temperature was then decreased to 16°C and the culture was incubated for 16 hours for protein expression. Afterwards, the bacteria were pelleted by centrifugation at 6,000 g for 10 min. The pellet was resuspended in phosphate buffered saline (PBS, pH 7.4) plus 8M urea (Sigma-Aldrich, USA) and sonicated for 1 min to release proteins from lysate. Subsequently, the soluble extract was centrifuged at 13,000 g for 30 min at 4°C and the supernatant was transferred to a new tube.

The solution containing the targeted protein was supplemented with lysis and column equilibration buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole and 8M urea) at a ratio of 1:1 and then incubated with HisPur™ Ni-NTA Resin (Thermo Fisher Scientific, USA) at 4°C. After 2 hours, the flow through was removed and the protein-bound resin in Ni-NTA column was washed thoroughly three times with wash buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, 50 mM imidazole (Sigma-Aldrich, USA) and 8M urea. The recombinant proteins were achieved by eluting with elution buffer including the same ingredients with wash buffer but high concentration of imidazole (500 mM) from the Ni-NTA column and stored at -20°C. The expression and purification level were quantified by SDS-PAGE and Western blot analyses.

RESULTS AND DISCUSSION

Cloning pigC region from Serratia sp. strain M5

Electrophoresis result showed a shaped band with approximately expected size of 3000 bps representing pigC region sequences (Figure 1A). After purification, this obtained band was inserted into pJET1.2 vector and transformed into E. coli DH10B strain. Five clones were randomly selected for plasmid extraction. Result indicated that two clones contained the recombinant pJET1.2/insert (Figure 1B). The clones were further confirmed the presence of the insert by PCR method using a pair of pJET1.2 primers (Table 1), and an obviously single band of around 3 kb corresponding to pigC was observed in these clones (Figure 1C). Additionally, Sanger sequencing these PCR products indicated that pigC region was successfully amplified and inserted into vector pJET1.2.

Sequence alignment indicated that the pigC sequence of Serratia sp. strain M5 isolated in Vietnam has over 98% identity at the nucleotide level when compared with pigC of other strain S. marcescens WW4 from GenBank. However, it should be noted that the difference of pigC gene between Serratia sp. M5 strain and the well-known strains S. marcescens 39006 as well as Serratia sp. AS9 was up to 25 – 30% (Table 2). Previous reports observed that the proportion of similarity between Serratia strain 39006 and S. marcescens Sma was 75.6% (Harris et al., 2004). The author’s hypothesis was that Serratia 39006 strain was atypical S. marcescens strain with significant differences in genome characteristics. The similar reason could be used to explain the difference between pigC of M5 strain and that of strains AS9, AS12 or AS13 of S. plymuthica (Neupane et al., 2012a; Neupane et al., 2012b; Neupane et al., 2012c). In addition, pigC clusters like Sma strain were observed in 6 pigmented strains belonging to S. marcescens (Harris et al., 2004).
Table 2. Nucleotide (lower triangle) and amino acid (upper triangle) identification matrix of pigC from Serratia sp. M5 strain in comparison to reference database from GenBank.

<table>
<thead>
<tr>
<th>No</th>
<th>Strain</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<td>HQ833702S. marcescens jx1</td>
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</table>

Detected in this construction, and an amino acid substitution at the position K265R was revealed when aligned with the pigC of S. marcescens WW4 (Figure 2). On the other hand, deduced amino acid sequence comparison showed some differences scattered throughout the protein sequence when compared to those of other strains Serratia sp. 39006 and AS9 (Table 2).
Recombinant vector pET22b/pigC was initially expressed in *E. coli* BL21 (DE3) in LB medium supplemented 100 μg/ml ampicillin and 1 mM IPTG at 37°C/6 hours or at 16°C/16 hours. However, the expression of pigC under this condition was really weak (data not shown). Previous reports indicated that maximum production of PigC was achieved using optimized auto-induced medium (You et al., 2018b), which was therefore used for expressing the targeted pigC protein. As a result, a
sharp band of approximately 100 kDa was observed in Western blot using anti-his-tag antibody (Figure 3A).

In order to purify the PigC protein for further study, the phase of recombinant PigC under expression conditions was investigated. Results indicated that recombinant PigC mostly accumulated in the pellet fraction (data not shown). Furthermore, it should be mentioned that PigC protein was demonstrated as a membrane binding protein in vivo and in vitro with full activity observed in pellets (Chawrai et al., 2012; You et al., 2018a). In these studies, therefore, a majority of recombinant PigC was in the insoluble fraction. In addition, enzymatic reaction indicated that enzyme activity was maintained in this fraction whereas no activity was identified in the supernatant. For all above the reasons, we further purified PigC protein under the denaturing condition using Ni-NTA resin. As expected, we achieved a purified precise band with the size corresponding to PigC protein (Figure 3B). This purified protein can be refolded and applied for examining the characteristics of recombinant PigC protein as well as producing prodigiosin.

Figure 3. (A) Western Blot result of expression of recombinant PigC protein in E. coli. M: marker gangnam stain (Intron), 1: E. coli BL21 (DE3) strain containing vector pET22b, 2: E. coli BL21 (DE3) strain containing recombinant vector pET22b_LacP_pigC; (B) Purification of PigC protein. M: marker Gangnam stain (Intron), 1: Purified PigC.

CONCLUSION

In the present study, the pigC sequence from Serratia sp. M5 isolated in Vietnam was successfully cloned into pJET1.2 vector. This gene, 2667 bps in length, encoded a protein of 888 amino acids. Sequence comparison indicated the obtained sequence of the pigC gene was highly similar to that of S. marcescens WW4, sharing over 98% of their DNA sequence. Meanwhile, it was 25 – 30% different from those of Serratia sp. 39006 and AS9 strains. The pigC gene was then inserted into pET22b and expressed in E. coli BL21 (DE3) using auto-induced medium. The targeted PigC, being a 100 kDa protein in SDS-PAGE as well as in Western blot analyses, was purified by Ni-NTA column. The prodigiosin production using the purified recombinant pigC is being examined in the further study.

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REFERENCES


potential applications. *J Food Sci Technol* 52(9): 5393-5407.


TÁCH ĐỒNG VÀ BIỂU HIỆN GEN pigC TRONG ESCHERICHIA COLI

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Tóm tắt

Prodigiosin (Pg) có hoạt tính kháng ung thư hoặc kháng vi sinh vật được tổng hợp từ phân ứng ngưng kết 4-methoxy-2, 2′-bipyrrrole-5-carboxyaldehyde (MBC) và 2-methyl-3-amylpyrrole (MAP) dưới sự xúc tác của enzyme PigC. Mác từ PigC động vai trò quan trọng trong quá trình tổng hợp probidiosins thấy nhiều chưa có nghiên cứu nào về protein này được thực hiện ở Việt Nam. Trong nghiên cứu này, chúng tôi đã tách đông và biểu hiện protein PigC ở Escherichia coli. Sử dụng cặp mới chủng cho vùng gen pigC, đoạn gen khoảng 3kb chứa pigC được khử hiệu đặc thành công từ chủng Serratia sp. chủng M5. Kết quả so sánh trình tự cho thấy, trình tự pigC có kích thước 2667 bp của chủng M5 phân lập tại Việt Nam tương đồng 98% với trình tự của các chủng S. marcescens khác, tuy nhiên khác biệt tối 30% khi so với chủng S. marcescens 39006 và AS9 với mã số tương ứng AJ833001 và CP002773. Trình tự gen mà họ protein PigC với kích thước 2664 bp sau khi được nhân lên sử dụng cặp mới có chứa vị trí nhánh biệt enzyme cắt HindIII và XhoI được đưa vào vector pET22b tạo thành vector tái tổ hợp có gắn đầu His và biểu hiện ở E. coli sử dụng mini trường tử cảm ứng. Kết quả di di cho thấy protein PigC đã được biểu hiện thành công với kích thước ~100 kDa và được kiểm tra bằng kit phát Western Blot sử dụng kháng thể kháng 6 histidine. Kết quả này tạo nguồn nguyên liệu khó giữa đầu cho việc nghiên cứu tạo prodigiosin từ protein tái tổ hợp ở Việt Nam.

Từ khóa: MAP, MBC, pigC, prodigiosin, Việt Nam