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CLONING, EXPRESSION AND PURIFICATION OF M-CELL SPECIFIC BINDING PEPTIDE (CO1) FUSED WITH GFP

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SUMMARY

Despite many advantages over injection vaccines such as cost effectiveness, safety and easy to use, and so on, oral vaccines are negligibly concerned. This is mostly because of the availability of vast surface in the gastro-intestinal tract, thereby requiring lot of antigens which could hamper their potential. To circumvent this issue, a novel strategy for targeting antigens to M cells (microfold cells), a minority of cells located in the small intestine for antigen transportation, is utilized by making a fusion protein comprised of an antigen with an M cell specific ligand. Discovered via biopanning, Co1 peptide is a potential ligand because of its small size (12 amino acids) and having an adjuvant capacity. To develop a monitoring model, we fused GFP (green fluorescent protein) as a monitoring marker with Co1 peptide. Initially, *co1-gfp* fusion gene was created by overlap extension PCR on GFP-encoded vector backbone, then it was incorporated into an expression vector pET22b before transforming into *E. coli* DH5α. The recombinant vector was screened by PCR method, sequenced and aligned with designed sequences. The in-frame vector was then introduced into *E. coli* BL21(DE3) for expression by inducing with 0.5mM IPTG. Fusion protein was purified using Ni-affinity chromatography. The results showed that the fused genes were in-frame cloned and completely matched with the designed sequences. SDS-PAGE and Western blot analyses showed Co1-GFP protein expressed in soluble form and could be purified at one-step elution of 500mM imidazole. The purified fusion protein could emit fluorescent light under UV excitation. Collectively, recombinant Co1-GFP fusion protein was successfully produced and its potential applications need to be warranted.

Keywords*: cell targeting, Co1 peptide, GFP, M cell, oral vaccine, overlap extension PCR*

INTRODUCTION

Gastro-intestinal tract, the widest surface area of human body (about 200 – 300 m^2) (Helander, Fandriks, 2014) is the ideal target for many exogenous pathogens such as Influenza virus, *Vibrio cholerae*, Type I reovirus, Rhinovirus, etc. (Plotkin, 2005). For the prevention of mucosal infections, the most effective way is to use oral vaccines. In fact, oral vaccines possess many advantages including easy to use and an ability to invoke both mucosal and systemic immune responses. However, the number of oral vaccines are quite humble because of the difficulty in delivering antigens into the gut lumen (Kim *et al.,* 2010).

Gut develops a defensive barrier called GALT (Gut-associated lymphoid tissue) such as Peyer's patches (PP) to fight pathogens (Neutra *et al*., 2001). Follicle associated epithelium (FAE) covers the PP comprising a kind of epithelial cell called microfold cells (M cells) which can actively transport luminal macromolecules via transcytosis process (Bockman, Cooper 1973; Owen, 1977). The unique function of M cells in transportation of intact antigens is provided by many specific characteristics. The apical surface of M cells lacks brush border as well as a thinner glycocalyx layer and the reduction of hydrolytic enzyme. Moreover, M cells have a "pocket shape" formed by basolateral membrane invagination which reduces travel distance of antigens to immune cells including dendritic cells (DCs) and lymphocytes. Antigens taken up by M cells can be immediately captured by immune cells and induces effective immune responses (Neutra *et al*., 2001).

Due to the transcytosis ability, M cells appear to act as a promising target for antigen delivery. However, targeting M cells are not simple because M cell population is very small in the FAE. Thus, a ligand targeting M cell is employed in order to overcome the hindrance. In fact, many microorganisms infiltrate body by targeting specific ligands on the apical surface of M cells, several molecules/ligands have been identified such as FimH component of *E. coli* binding to GP2 (Terahara *et al*., 2008, Hase *et al*., 2009), Hsp60 of *Brucella abortus* binding to cellular prion protein (PrPc) (Nakato *et al*., 2009). However, most pathogen-derived molecules are quite large hence they are not easily integrated with antigens, and the possibility of side-effects and safety measure need to be concerned (Kim *et al.*, 2010).

In a recent study employed biopanning technique in order to find suitable M cells' ligands, a Co1 peptide has been identified which can bind to C5a receptor (Kim *et al*., 2010). Briefly, a random dodecapeptide phage library was screened on M-like cells and analysed. The determined peptide was rather short (only 12 amino acids) compared to other ligands. Besides that, Co1 peptide has an adjuvant ability, hence this peptide is of a promising ligand for oral vaccines (Kim *et al.,,* 2010).

Therefore, in this study, Co1 peptide was fused with a well known marker, green fluorescent protein (GFP). The recombinant protein was identified with anti 6-Histidine and tested by size comparison with GFP. Lastly, the recombinant protein was assessed based on its fluorescence under UV excitation.

MATERIALS AND METHODS

Construction of pET22b-co1-gfp vector

A *co1-gfp* gene was created using extension PCR method (Fig. 1). Specifically, GFP was amplified from pTurboGFP plasmid [a gift of Prof. Dr. Gregor Bucher, Ernst Caspari Haus (GZMB)] using PCR with primer pairs, Co1F (5'-actCATATGtcttttcatcaattacctgctcgttctcctttccctggtggatccggtatggag-3') and GFPR (5'-cgcCTCGAGttcttcaccggcatctgc-3') predesigned to cover the complete *co1* gene and flanking restriction site sequences. Restriction enzymes were underlined. Co1-GFP encoding gene was digested with *Nde*I and *Xho*I (Thermo Scientific, USA) before being fused into respective restriction sites in pET22b plasmid by using T4 ligase (Thermo Scientific, USA). The ligation product was introduced into *E. coli* DH5α competent cells. Positive transformants were screened with T7 primers. A digestion with *Nde*I/*Xho*I and sequencing were performed to evaluate the plasmids obtained from the transformants. The recombinant plasmid was named pET22b-co1-gfp.

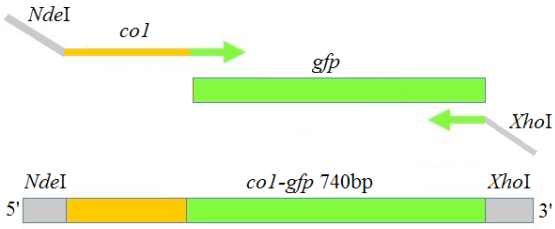

Figure 1. Schematic diagram of co1-gfp gene amplification.

Expression of Co1-GFP

pET22b-*co1-gfp* was introduced into *E. coli* BL21(DE3). Bacteria were grown in LB-Amp medium until OD_{600} reached 0.6-0.8 prior to inducing with IPTG 0.5mM in shaking condition for 4 hours. Total cells were sonicated under ice-cooled condition, centrifuged at 5000 rpm for 5 minutes to collect supernatant containing soluble proteins. Negative control was *E. coli*/pET22b, GFP was used as a size comparison. Total proteins were analyzed with SDS-PAGE and Western blot with anti 6xHis and visualized by using ECL kit (GE Healthcare, USA).

Co1-GFP purification

In this study, nickel-charged sepharose 6 (GE Healthcare, USA) was packed into column followed by adding protein solution. After 3 washing steps with binding buffer (0.5M NaCl, 0.02M NaH_2PO_4, 0.025M imidazole), the proteins were eluted from the column by adding elution buffer (binding buffer containing 0.5M imidazole). Eluted protein fractions were undergone SDS-PAGE and Western blot to evaluate their purity.

RESULTS AND DISCUSSION

Construction of pET22b-*co1-gfp* vector

The recombinant pET22b-*co1-gfp* was generated as described in Materials and Methods section. To verify cloning precision, the vector was sequenced and aligned with designed sequence. The alignment showed that the cloned genes were

matched with designed sequence and the *co1* gene was cloned in frame with *gfp* gene (Fig. 2).

Analysis the expression of Co1-GFP

An *E. coli* BL21(DE3) colony containing pET22b-*co1-gfp* plasmid was induced protein expression with IPTG. Three fractions were prepared from induced cells including total protein, soluble protein, and insoluble protein fractions. *E. coli* BL21(DE3)/pET22b-GFP was also induced in order to obtain GFP alone to compare size with the Co1-GFP fusion protein. Negative control was *E. coli* BL21(DE3)/pET22b. All protein samples were analyzed with SDS-PAGE and Western blot.

On the SDS-PAGE gel, the *E. coli* BL21(DE3)/pET22b-*co1-gfp* created an accumulated band (Fig. 3; well 2) about 30 kDa while negative control had no similar band (Fig. 3; well 1). This band was likely the Co1-GFP fusion protein, since its size was slightly bigger than GFP (Fig. 3; well 5) due to the addition of Co1 peptide. The fusion Co1-GFP was not visibly detected in the insoluble well (Fig. 3; well 4).

In order to assure the protein bands on the SDS-PAGE were Co1-GFP, a Western blot with anti 6xHis antibody was carried out. Because the Co1-GFP recombinant protein was designed with 6xHis at C terminal, therefore, it gives a signal on Western blot. GFP consisted of 6xHis was used for comparing size with Co1-GFP. In Western-blot film, the total and the soluble samples had one band for each (Fig. 3; well 2', 3'), with sizes correlated to bands on the SDS-PAGE gel, suggested that the proteins were successfully blotted into the membrane. The insoluble sample showed a slight band compared to the total and the soluble samples, similar with SDS-PAGE result. Collectively, the over-expressed protein bands were recombinant proteins, mostly soluble, with size larger than GFP. In conclusion, the protein expressed by *E. coli* BL21(DE3)/pET22b-*co1-gfp* was Co1-GFP.

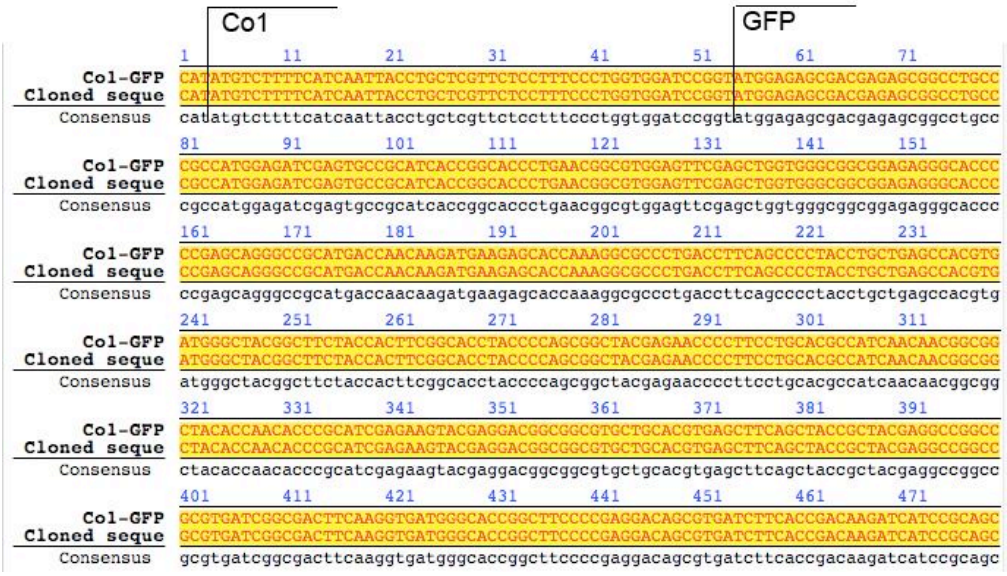

Figure 2. Sequence alignment between designed gene and a positive clone (Only 5' partial alignment is shown). Name of each gene is marked at their first codons.

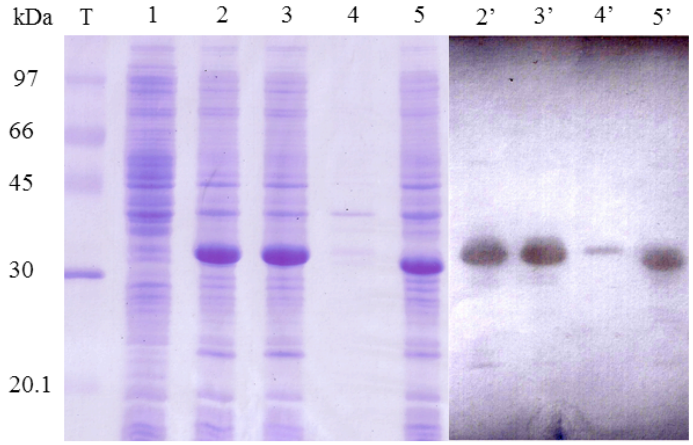

Figure 3. Analysis of fusion Co1-GFP by SDS-PAGE and Western blot. T: ladder; 1: negative control; 2 and 2': total protein samples; 3 and 3': soluble samples; 4 and 4': insoluble samples; 5 and 5': GFP products.

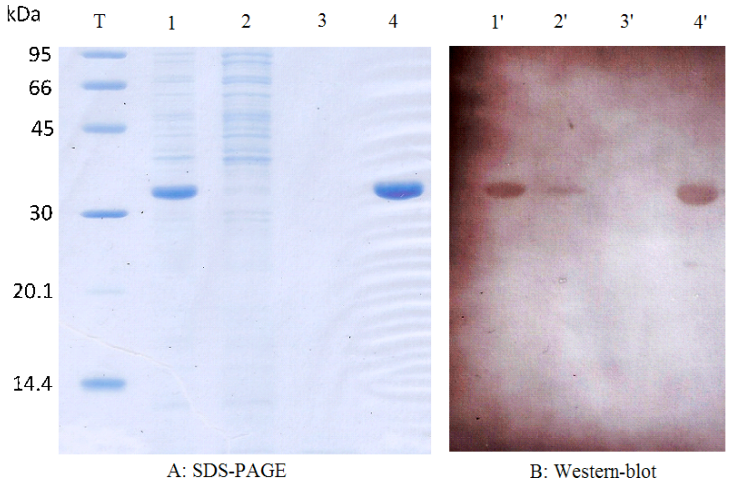

Figure 4. Purification of Co1-GFP analyzed by SDS-PAGE (A) and Western blot (B), T: ladder; 1 and 1': total protein samples; 2 and 2': flow-through samples; 3 and 3': washing samples; 4 and 4': elution samples.

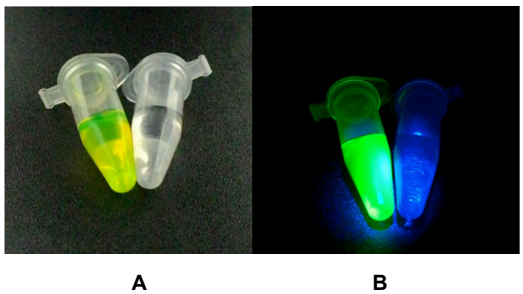

Figure 5. Fluorescent light of purified Co1-GFP. Purified Co1-GFP (right tube) and elution buffer (left tube) under normal light (A) and UV light (B).

Co1-GFP purification

After purification, four fractions including original protein samples, flow-through samples of loading step, washing step, and elution samples were evaluated with SDS-PAGE and Western blot. The results, including SDS-PAGE and Western blot film (derived from a duplicate of SDS-PAGE gel) demonstrated that the Co1-GFP protein was successfully purified. There was only a single band in the elution sample above 30 kDa which correlated with a wide band of the original sample (Fig. 4; wells 1, 1' and wells 4, 4'), while the loading and washing steps had no such bands (Fig. 4; wells 3 and 3'). A small band appeared in the flow-through well was due to overloading of the column (Fig. 4; wells 2 and 2').

Fluorescent verification of purified Co1-GFP

Although the purified Co1-GFP shown a yellowish solution, UV excitation needed to be ascertained. The purified Co1-GFP was UV-excited at 480nm wavelength. In comparison to elution solution alone, the purified Co1-GFP emitted a well-known fluorescent light under UV (Fig. 5). Taken together, the Co1-GFP fusion protein had been successfully purified and retained its fluorescent ability.

CONCLUSIONS

In order to evaluate the M cell adhesion ability of the Co1 peptide, the present study conducted to fuse the M cell-targeting ligand with GFP protein. In this research, the fusion Co1-GFP was obtained in soluble fraction of recombinant *E. coli* cells expressed at 37^{o}C for 4 hours. Purified Co1-GFP retained its fluorescent ability. This prompts us to evaluate the binding capacity of the fusion protein *in vitro* and *in vivo*.

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TẠO DÒNG, BIỂU HIỆN VÀ TINH SẠCH PEPTIDE GẮN ĐỊNH HƯỚNG TẾ BÀO M (CO1) DUNG HỢP VỚI GFP

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TÓM TẮT

Mặc dù có nhiều ưu điểm so với vaccine tiêm như: giá rẻ, dễ dàng sử dụng và an toàn… vaccine uống vẫn chưa được quan tâm đầy đủ. Nguyên nhân chủ yếu vì bề mặt rộng lớn của ruột đòi hỏi một lượng lớn kháng nguyên cần để tạo nên đáp ứng miễn dịch hiệu quả gây khó khăn trong việc phát triển vaccine uống. Để giải quyết vấn đề, có một chiến lược mới nhắm trúng đích kháng nguyên vào tế bào M, một loại tế bào thiểu số trong ruột non có chức năng vận chuyển kháng nguyên, bằng cách tạo ra một protein dung hợp bao gồm một kháng nguyên và một ligand đặc hiệu tế bào M. Được phát hiện bằng kỹ thuật bio panning, peptide Co1 là một trong các ligand tiềm năng nhờ kích thước nhỏ (12 amino acid) và có đặc tính làm chất bổ trợ cho vaccine. Để phát triển một mô hình kiểm tra, chúng tôi sử dụng marker GFP để dung hợp với peptide Co1. Trước tiên gene dung hợp *co1-gfp* được thu nhận bằng phương pháp PCR nối dài khuôn (overlap extension PCR), sử dụng

DNA làm khuôn là vector mang gen mã hóa cho GFP, được nối vào vector pET22b trước khi biến nạp vào vi khuẩn *E. coli* DH5α. Những vector tái tổ hợp được chọn lọc bằng phương pháp PCR, giải trình tự và so sánh đối chiếu với trình tự *co1-gfp* thiết kế. Sau đó vector được biến nạp vào chủng *E. coli* BL21(DE3) để cảm ứng biểu hiện với IPTG 0.5 mM. Protein đích được tinh chế bằng kỹ thuật sắc ký ái lực ion kim loại (nickel). Điện di biến tính trên gel polyacrylamide (SDS-PAGE) và phân tích Western blot cho thấy protein Co1-GFP biểu hiện trong pha tan và được tinh chế thành công ở nồng độ imidazole dung ly là 500 mM. Đồng thời protein dung hợp đã được tinh chế vẫn mang khả năng phát huỳnh quang dưới kích thích UV. Như vậy, protein Co1-GFP dung hợp tái tổ hợp đã được tạo ra thành công, tuy nhiên tiềm năng ứng dụng vẫn cần được xác định.

Từ khóa: *GFP, nhắm trúng đích tế bào, PCR chồng nối, peptide Co1, tế bào M, vaccine uống*