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行政院衛生署疾病管制局九十年度自行研究計畫

百日咳疫苗抗原-pertactin 之免疫學研究與應用

The application and immunology studies of pertussis antigen-pertactin

自 行 研 究 成 果 報 告

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目　　錄

摘　　要

關鍵詞：百日咳、聚合酶鏈鎖反應、基因表現

百日咳外膜蛋白ｐｅｒｔａｃｔｉｎ雖然不是預防感染百日咳的疫苗必需抗原，但卻是多種市售疫苗中之輔助保護抗原。此蛋白不但可提升非細胞疫苗之保護效力，單獨使用時，亦可避免小鼠或小豬受百日咳之吸入攻擊感染。為了進一步了解此抗原免疫機制作為未來診斷及防疫應用參考，我們利用百日咳菌ｐｅｒｔａｃｔｉｎ基因進行聚合酶鏈鎖反應分段增殖，並將各段分別以大腸菌作基因表達，最後將以吸入攻擊實驗測定各段部分蛋白之保護效力。在獲得某一特定蛋白區段具有保護效力後，我們將進一步將此區段再細分成小段進行吸入攻擊實驗，期盼最後獲得具抗原決定位的ｐｅｐｔｉｄｅ以作為未來之應用研究。我們的結果發現，在將ｐｅｒｔａｃｔｉｎ基因分成三段互有重疊之核酸區段增殖時，C-端之蛋白對等核酸序列因大於 80%之ＧＣ高含量，只能在 15%甘油存在下由聚合酶鏈鎖反應增殖。而N-端之蛋白在ｐＥＴ系統表現時，也必需去除前兩個帶電價之氨基酸方可順利大量表現。然而所有表現之蛋白區段皆形成包涵體(ｉｎｃｌｕｓｉｏｎ　ｂｏｄｉｅｓ)。利用純化的包涵體作為抗原，我們並不能在吸入攻擊實驗中發現很明顯之保護作用。改變表現方法，以製造可溶性之蛋白將是我們未來努力的方向。

ABSTRACT

Key words: pertactin, PCR

Pertactin alone has been demonstrated to be effective in protecting mice and piglets against aerosol challenge with *Bordetella pertussis* strain 18323. In order to explore the immunological activity of pertactin, in this study, we intend to excise pertactin coding region to three regions (i.e. N-terminus, M-terminus, and C-terminus, approximately 1 kb each), separately amplified by PCR, and expressed the corresponding peptides in *E. coli*. The recombinant partial peptides are then subjected to aerosol challenge to isolate the protective region of pertactin. To the region that exhibits the protection activity, we will further cut it into three smaller regions and repeat the aerosol analysis until the small peptide epitope being isolated. Due to very high GC content (>80%), we found that the C-terminus of pertactin can be amplified by PCR only when 15 % glycerol is present. In addition, in the N-terminus amplification, the amino acid (i.e. Asp.Trp.) right next to initiation codon (i.e. AUG) were mutated to Ala. Ser. codons (i.e. GCAAGC) to be overexpressed by pET system. Unfortunately, all peptides are produced as inclusion bodies in *E. coli*. And, only a trace protection activity was observed when these inclusion bodies were used as antigens in the aerosol challenge assay. An improvement in the production of soluble recombinant peptides may be required to solve this problem.

INTRODUCTION

In order to prevent various adverse reactions [1-3] resulted from the vaccination of whole cell pertussis vaccines, several effective acellular vaccines which were demonstrated to be much safer have been developed sine 1980s [4]. The analysis of all commercialized vaccines revealed that the purified pertussis toxin [5] is an essential component. However, two other pertussis proteins, i.e. filamentous hemagglutinin (FHA) [6] and a 69 kDa outer membrane protein (named pertactin) [7-9], which were previously demonstrated to be associated with the cell adhesion in the early stage of infection, were found to be able to enhance the immunogenicity of vaccine. Because of this, in the receipt of at least three commercialized acellular vaccines (i.e. Biocine, Connaught Ltd, Leclele/Takeda), either or both these non-toxin antigens were added. Although the exact immunological mechanism is not yet clear, based upon their biological activity (i.e. cell adhesion), it is believed that FHA and pertactin may provide vaccine in the function of infection prevention. While the primary antigenic activity of pertussis toxin may be more related to disease prevention.

In spite of the similarity in the cell adhesion properties of FHA and pertactin, unlike FHA, the immunization with pertactin alone has been proved to be effective in protection of mice [10,11] and piglet [12] against aerosol challenge with virulence *B. pertussis* strain 18323. In human, following infection, both antibodies against pertactin and the specific T cells [13,14] were significantly elicited. Although the nucleic acid sequence [15] and the three dimensional structure [16] of pertactin has been published, little of biological activity of pertactin (including the immunological mechanism) has been developed. In our previous studies, we have cloned the coding region of pertactin from *B. pertussis* ATCC 9340 strain, and successfully overexpress the recombinant protein (> 100

mg/L of culture) in *E. coli* [17]. In addition to the usage of recombinant pertactin in the formulation of acellular vaccine, it was also used in the biological activity assay. Recently, we have demonstrated, in another study, that the recombinant pertactin exhibit a remarkable adhesion activity and effectiveness in the protection activity in aerosol challenge. The activities in both aspects are at least equivalent to the authentic pertactin isolated from pertussis.

Based on the facts that no Cys. residue was found in pertactin and the effectiveness of recombinant pertactin produced in *E. coli* (lack post-tranlational modification), it was expected that pertactin possess a linear epitope (not comformational epitope as in pertussis toxin). By using the strategy of peptide screen, the antigenic epitopes should thus be able to be isolated. In the current study, we propose to generate partial peptide sequences from pertactin, rather than adopt the conventional and costly “peptide mapping”, to map the antigenic epitope of pertactin. By using the developed technologies in recombination protein expression, cell adhesion, and aerosol challenge, we would assay and narrow the possible region of pertactin that may represent as an antigenic epitope. Hopefully, the isolated peptide would be applied in the development of new diagnosis reagents for the future epidemiological studies.

MATERIALS AND METHODS

Pertussis strain and genomic DNA isolation

Similar to our early studies, *Bordetella pertussis* strain ATCC 9340 was cultivated as described in previous study [18]. The genomic DNA was prepared by using Qiagen Genomic-tip (c.a. 10223) by the procedure as described in the supplied instruction. Prior to polymerase chain reaction, the genomic DNA was re-centrifuged to pellet the insoluble substance.

Partial coding sequence amplification by PCR

Based on the coding sequence of pertactin (approximately 1.8 kb) [15], we have design three pair of primers to separately amplify three regions of pertactin (i.e. nucleotide sequence corresponding to the C-terminus (from nucleotide 1250 to 2052; 802-bp fragment), middle M-region (from nucleotide 750 to 1665; 915-bp fragment), and N-terminus (from nucleotide 250 to 1250; 1000-bp fragment)). The M-region was designed to encompass a 500 bp and 415 bp overlapped region with N-terminus and C-terminus regions respectively. The primers for N-terminus was upstream sense primer 5'-ATATCC**ATG**GC-AAGCAACCAGTCCATCGTCAAG-3' and downstream antisense primer 5'-ATATCTCGAGTTAGTGGTGGTGGTGGTGGTGCGCTTTCCCCTGGGCA-TG-3'. For M-region, the upstream sense primer was 5'-AAAACC**ATG**GT-GCTGCGCGACACCAAC-3', and the down stream antisense primer was 5'-GATCCTCGAGTTAGTGGTGGTGGTGGTGGTGGCCGCTGGCGTCCTG CATG-3'. For C-terminus, the upstream sense primer was 5'-AAAGC-**CCATGG**CCCAGGGGAAAGCGCTGC, and the downstream antisense primer was 5'-ATATACTCGAGATTGCTTTCGGCGTACCAGAG-3'. All sense primers incorporated a restriction recognition site of *Nco*I and translation

initiation codon ATG. All antisense primers introduced an *Xho*I site and a termination codon TAA into the PCR products. However, only the antisense primers that were used for M-region and N-terminal incorporated six His. The PCR reaction condition for N-terminus fragment amplification was performed as: initial denaturation for 5 min at 94 ^{o}C, followed by 25 cycles of 94^{o}C for 20 sec, 50 ^{o}C for 20 sec, and 72^{o}C for 1 min 30 sec. For M-region, the initial denaturation was followed by 25 cycles of 94^{o}C for 20 sec, 55^{o}C for 20 sec, and 72^{o}C for 1 min 20 sec. And for C-terminus, the cycling parameters were 94^{o}C for 30 sec, 55^{o}C for 30 sec, and 72^{o}C for 1 min 30 sec. The PCR products were electrophoresis on a 0.8 % agarose gel and the desired fragments were eluted from the gel. Following the extraction with phenol/chloroform/isoamyl alcohol and chloroform, the DNA fragments were precipitated by 2.5 vol. of ethanol.

Recombinant plasmid construction and expression

The three PCR amplified DNA fragments (corresponding to three regions of pertactin coding regions) were digested with restriction enzymes *Nco*I and *Xho*I in a total volume of 30 μL solution and the resulted fragments were ligated to pET-22b that were digested with the same enzymes. The ligation mixtures were used to transform the competent bacteria DH5α. The resulted recombinant plasmids were analyzed by electrophoresis on a 0.8% agarose gel.

Recombinant constructs were expressed in LB media containing 50 μg/mL ampicillin. A single colony from each construct was cultivated in a 10 mL culture media and grown at 37 ^{o}C overnight. The culture was diluted with 100 mL culture media and continuously grown at the same temperature until OD_{600} reach 0.6-1.0. To the culture, 1 mM IPTG (final concentration) was added, and the induced expression was conducted for anther 3 h under the same conditions. Bacteria cells were collected by centrifugation and resuspended in a 2 mL

solution containing 20 mM Tis-HCl, pH 8.0 and 2 mM EDTA. Cells were then lysed by sonication with 30% output level for 15 sec each cycle and totally for three cycles. The lysed total lysate was analyzed by a 12% SDS-PAGE. In order to determine the solubility of recombinant protein, the total lysate was also centrifuged (12,000 rpm for 10 min) to separate the soluble and insoluble fractions of lysate, and separately analyzed by SDS-PAGE.

Aerosol challenge analysis by using recombinant proteins

In order to analyze the protection activity exerted by each recombinant peptide, the inclusion bodies of each peptide was assayed by aerosol challenge. Groups of six mice were immunized with 0.5 μg of recombinant N-terminus, M-region, and C-terminus peptide. A reference group of mice without vaccination was used as control. The procedure for aerosol challenge was similar to that has been developed in our laboratory. Seventeen days after immunization, mice were aerosol challenges by nebulizing with 2 mL of 1.5 OD_{650} (approximately 1 x 10^9 cfu for one OD_{650}) of virulence *B. pertussis* strain 18323, using Inhalation Exposure System, Model 099C A4212, Glas Col, USA). Mice were sacrificed at day 12 after challenge, lungs were collected and homogenized in 1% casamino acid, and appropriated amount of cell suspensions were plated on blood agar plates. The *B. pertussis* colonization was observed after 2 to 3 days incubation at 37 ^oC. The colony formation unit from each mouse was counted, and averaged in the group of six mice.

RESULTS AND DISCUSSION

Immunological studies of pertactin, compared with that of pertussis toxin, is quite limited. Due to the lack of cysteine residues and previous studies by others, It is believed that pertactin possess a linear epitope. Because of this, certain recombinant peptide derived from intact pertactin was expected to exhibit immunogenicity. In this study we attempt to divide the coding region of pertactin to small peptides and use aerosol challenge to analyze their protective activity against pertussis infection. As the period of study has been shorten to one year (originally, two years were proposed), we can actually carried out a preliminary study by a rough division into three fragments. Pertactin coding region was divided into three coding regions and separately amplified by PCR. As shown in Fig. 1, pertactin could be amplified as C-terminus, M-region, and N-terminus with the sizes of 802, 915, and 1000 bp, respectively. It was noted that the coding region of C-terminal region of pertactin was rather difficult to be amplified in spite of a great effort has been made. The similar difficulty has been encountered in our early studies “ genetic variation of local pertussis strains”. Among the PCR effectors that commonly used to enhance the sensitivity and specificity of PCR, we found only in the presence of 15% glycerol C-terminus could be amplified.

Following the construction of recombinant plasmid containing three partial coding regions, the putative constructs were confirmed by restriction analysis with the same enzymes that were used for subcloning, i.e. *Nco*I and *Xho*I. As indicated (Fig. 2), the corresponding fragments (i.e. 1000 bp, 915 bp, and 802 bp) were obtained. Instead of subjecting to DNA sequencing, we directly used these constructs in the expression in *E. coli*.

The obtained constructs were expressed in bacteria as described in “Materials and Methods”. As shown in Fig. 3, all the recombinant peptides could be over-produced in *E. coli* with molecular weight about 44 kDa, 40 kDa, and 37 kDa corresponding to N-terminus, M-region, and C-terminus recombinant peptide respectively. The expression level all reached approximately 20 to 30 percent of total cellular protein content. In fact, the expression of N-terminus was initially rather unsuccessful. As the first two codons are either charged or bulky amino acids (Asp. and Trp.), these may result in a very low gene expression with pET system. We therefore substituted these two codons with GCA and AGC (corresponding to Ala. and Ser.). This modification in fact greatly improved the production of N-terminal recombinant peptide expression. Similar to our early studies (including pertactin and pertussis S1 subunit expression), unfortunately, all these recombinants were produced as inclusion bodies. As inclusion bodies were reported to be effective in eliciting the protective antibodies, we thus used them directly for aerosol challenge assay.

In aerosol challenge, similar to the results of our previous studies, the observed colony formation unit (cfu) was greatly varied among individual mouse in the same group. The non-immuized control mice was approximately 2.1 $x10^6$ per lung, while the average cfu of mice immunized with C-terminus, M-region, and N-terminus were 2.3, 1.4, and 1.6 $x10^6$ per lung. Only the mice immunized with M-region and N-terminal revealed a slightly decrease in colonization of virulence strain. However, the deviation of cfu (data not shown) in each mouse is too big to make data very persuasive. We currently not clear if the low protective activity of recombinant peptide possibly resulted from the use of inclusion bodies as antigen. To address this issue, we may need to modify the expression conditions in order to obtain soluble proteins that may confer a more reliable analysis. In spite the results were not as good as we expect, the two

regions (M-region and N-terminus) that exhibited a slightly protection activity also encompassed the RGD domain, which has recently been reported to be the essential region for the cell adhesion as well as the pertactin immunological activity [19]. This however is in consistent with our results.

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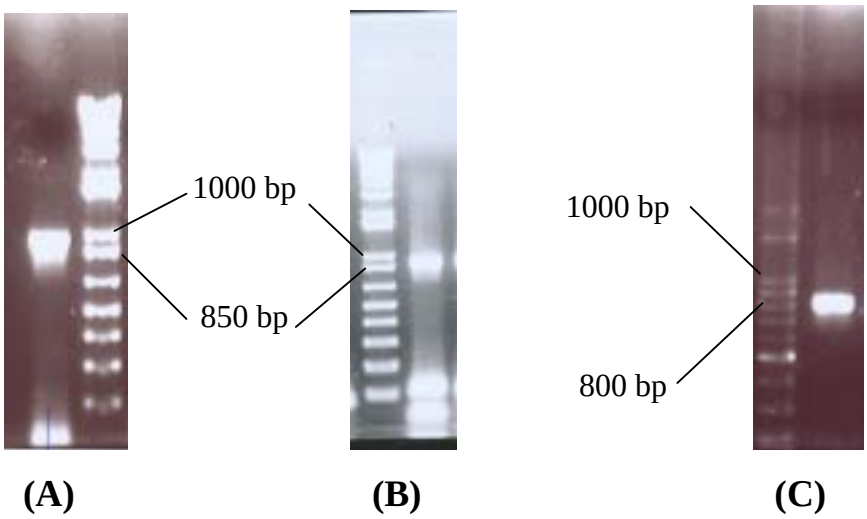

Figure 1. Polymerase chain reaction amplification of three regions of pertactin. The coding region of pertactin were separately amplified by PCR as described in “Materials and Methods” (A) 1020 bp N-terminal region, (B) 915 bp M-region, (C) 800 bp of C-terminal region

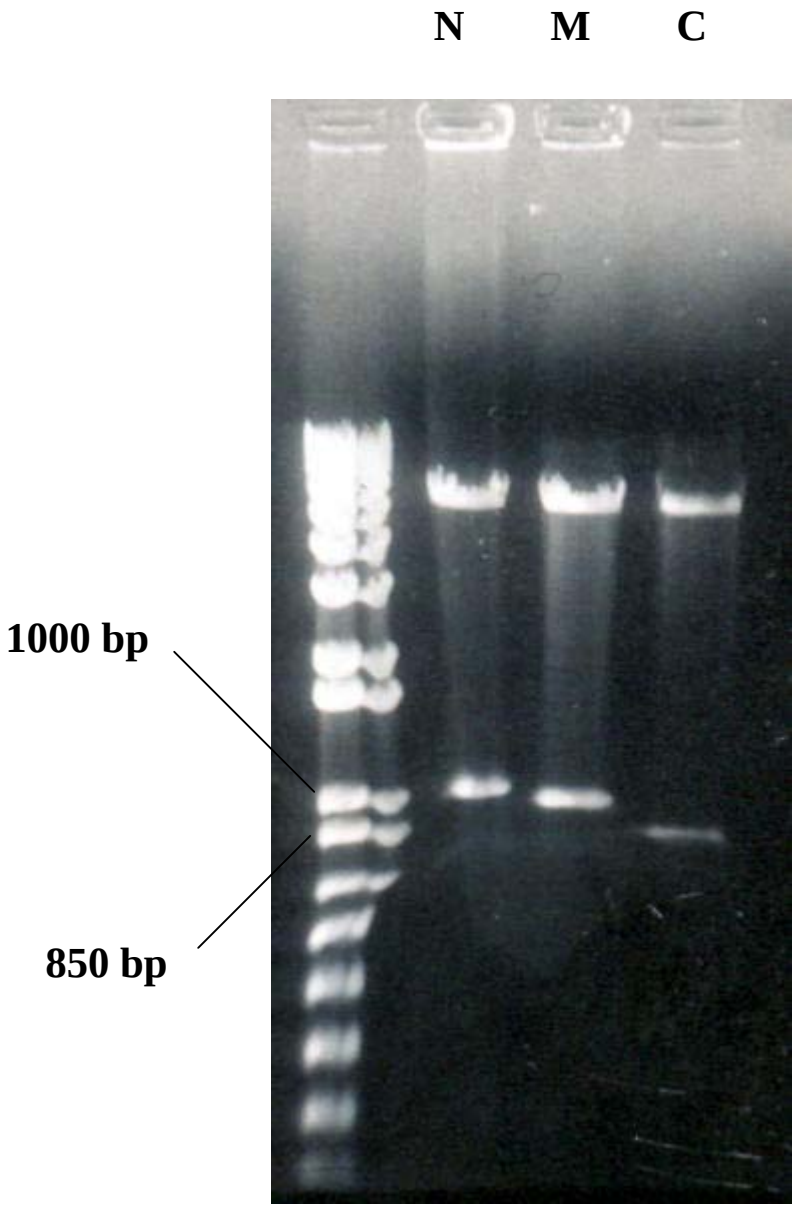

Figure 2. Agarose gel analysis of recombinant constructs of three peptides of pertactin. The recombinant partial protein constructs were prepared by subcloning of PCR products into pET-22b vector. The isolated recombinants were confirmed by digestion with *Nco*I/*Xho*I. Lanes N, M, and C indicate the results from constructs N-terminus, M-region, and C- terminus respectively.

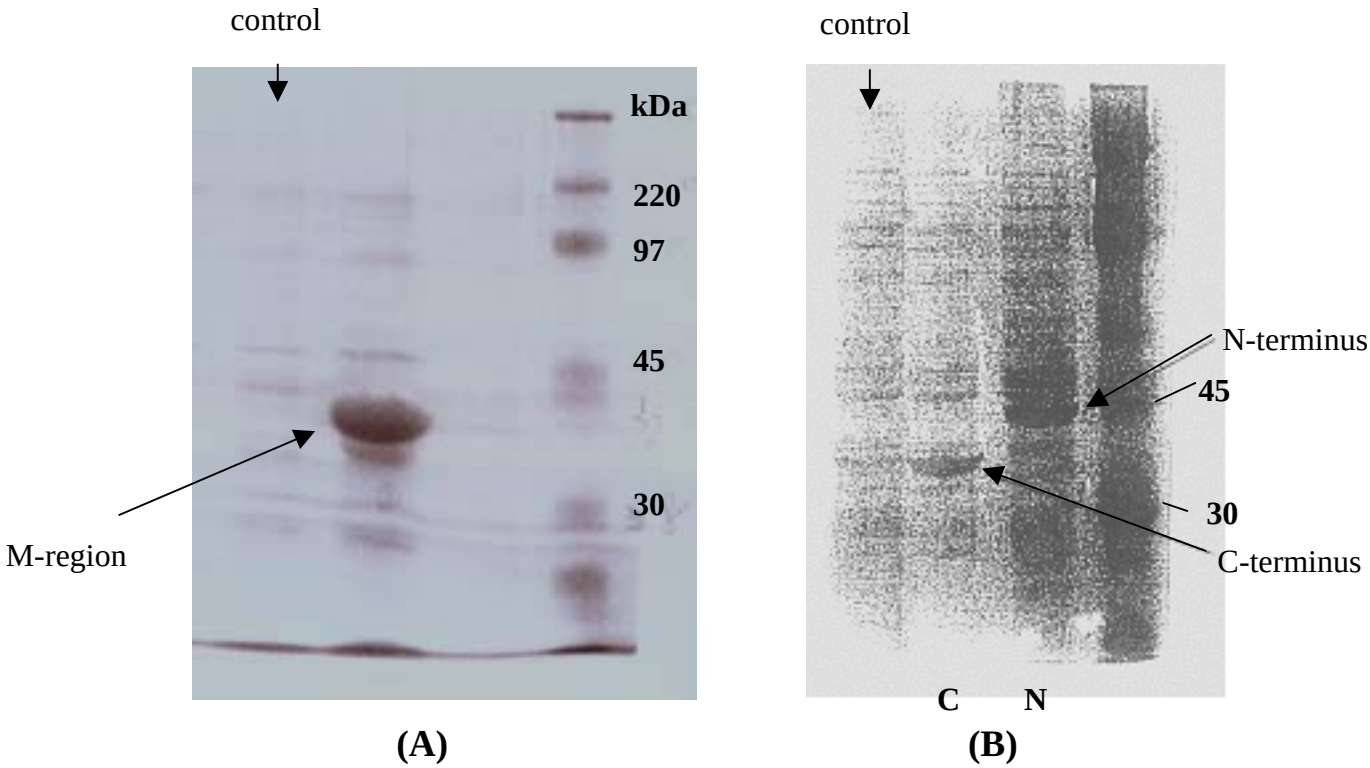

Figure 3. SDS-PAGE analysis for the expression of recombinant peptides of pertactin. The recombinant constructs were expressed in *E. coli* DH5α as described in "Materials and Methods" (A) recombinant M-region and (B) recombinant C- and N- terminal proteins. Arrows indicate the overproduction of these polypeptides.