Structure and Function Analysis of House Dust Mite Group 10 Allergen (Der p 10)

การศึกษาโครงสร้างและหน้าที่ของสารก่อภูมิแพ้ Der p 10 จากไรฝุ่นบ้าน

Nareerat Wongchum (นารีรัตน์ วงษ์ชุ่ม)* Dr.Sarin Chimnaronk (ดร.ศรินทร์ ฉิมณรงค์)**
Dr.Surapon Piboonpocanun (ดร.สุรพล พิบูลโภคานันท์)***

ABSTRACT

House dust mite allergens are one of the major causes of respiratory diseases such as asthma. Tropomyosin, the major muscle protein, is identified as one of HDM allergens and called group 10 or Der p 10. Importantly, antibody against tropomyosin of dust mite species could only react to tropomyosin of other invertebrate species such as cockroach and shrimp, but not that of vertebrate. To date, it is unknown why only invertebrate but not vertebrate tropomyosin causes allergic reactions. To answer this perplex event, structure analysis of HDM tropomyosin would be needed. This project aims to express recombinant Der p 10 in bacteria *Escherichia coli*. Two Der p 10 cDNA constructs with either N-terminal 6xHis-fused or C-terminal 6xHis-fused were constructed. Over expression and Nickel-affinity chromatography purification were performed. The yield and purity of purified rDer p 10 of N-terminal 6xHis-fused rDer p 10 more than C-terminal 6xHis-fused significantly. Crystallization and immunological assay of rDer p 10 will be further performed.

บทคัดย่อ

สารก่อภูมิแพ้จากไรฝุ่นเป็นสาเหตุที่สำคัญต่อการเกิดโรคระบบทางเดินหายใจ เช่น โรคหอบหืด สารก่อภูมิแพ้จากไรฝุ่นกลุ่มที่ 10 (Der p 10) หรือ Tropomyosin เป็นองค์ประกอบหลักของเซลล์กล้ามเนื้อ ผลของงานวิจัยพบว่า antibody ที่จำเพาะต่อ Tropomyosin ของไรฝุ่น สามารถปฏิสัมพันธ์กับ Tropomyosin ของสัตว์ไม่มีกระดูกสันหลังเท่านั้น เช่น แมลงสาป กุ้ง เป็นต้น เนื่องจากยังไม่มีการศึกษาสาเหตุของคนไข้โรคภูมิแพ้ ที่มีการตอบสนองเฉพาะต่อ Tropomyosin จากสัตว์ไม่มีกระดูกสันหลังเท่านั้น เพื่อที่จะตอบคำถามนี้ การศึกษาด้านโครงสร้างของ Tropomyosin ของไรฝุ่น จึงมีความจำเป็น ดังนั้น โครงงานวิจัยนี้มีวัตถุประสงค์เพื่อผลิต สารก่อภูมิแพ้ Der p 10 โดยอาศัยการแสดงออกใน *Escherichia coli* ผู้วิจัยได้ทำการสร้างพลาสมิดเวคเตอร์ที่สามารถผลิต rDer p 10 ซึ่งต่อกับ Histidine จำนวน 6 ตัว (6xHis) ที่ด้าน N-terminal (Der p 10-N His) หรือ C-terminal (Der p 10-C His) หลังจากทำการผลิตโปรตีนในปริมาณมาก และทำให้บริสุทธิ์โดยใช้เทคนิค Nickel-affinity chromatography ผลการทดลองพบว่า พลาสมิดเวคเตอร์ Der p 10-N His สามารถผลิตสารก่อภูมิแพ้ Der p 10 ได้มากกว่าพลาสมิดเวคเตอร์ Der p 10-C His นอกจากนี้โปรตีน N-His-Der p 10 ยังมีความบริสุทธิ์สูงกว่าอย่างมีนัยสำคัญ โดยโปรตีนที่บริสุทธิ์แล้วจะนำไปวิเคราะห์สมบัติทางด้านการเป็นสารก่อภูมิแพ้ และศึกษาโครงสร้างของโปรตีนโดยอาศัยเทคนิค X-ray crystallography ต่อไป

Key Words : House dust mite allergens, recombinant allergen, structure of protein
คำสำคัญ : สารก่อภูมิแพ้จากไรฝุ่น รีคอมบิแนนท์โปรตีน โครงสร้างของโปรตีน

* *Master Student, Master of Science Program, in Molecular Genetics and Genetic Engineering, Institute of Molecular Biosciences, Mahidol University*
** *Lecturer, Institute of Molecular Biosciences, Mahidol University*
*** *Assistant Professor, Institute of Molecular Biosciences, Mahidol University*

1. Introduction

House dust mite allergens (HDM) are one of major causes of allergic diseases such as allergic rhinitis, allergic dermatitis and asthma. In Thailand, two predominant species of house dust mite are *Dermatophagoides pteronyssinus (Dp),* a source of Der p allergens, and *Dermatophagoides farinae (Df)*, a source of Der f allergens (1, 2). To date, more than 20 groups of HDM allergens, based on the basis of immunological and biological functions, have been reported.

Tropomyosin, 36 kDa major muscle protein found in both vertebrate and invertebrate such as shrimp, cockroach, dust mite. Tropomyosin of *Dp* mites is one of mite allergens causing allergic reactions. Therefore, it is named as group 10 allergen (Der p 10). HDM tropomyosin is also identified as pan-allergen which demonstrated by monoclonal IgG antibody to Der p 10 could react to other invertebrate tropomyosin from shrimp and cockroach (2, 3, 4, 5). Interestingly, the same specific IgG against Der p 10 could not bind to any vertebrate tropomyosins such as pork, beef, and chicken. To date, it is still unknown how specific IgE from allergic patients react to only invertebrate tropomyosin but not vertebrate tropomyosin. To have better understanding of this interaction between specific IgE and invertebrate tropomyosin, structure analysis of dust mite tropomyosin was carried out. This project is aimed to produce a high yield of recombinant Der p 10 in *Escherichia coli* for crystallization. By comparison dust mite Der p 10 structure with known vertebrate tropomyosin, we might be able to answer how invertebrate tropomyosin is pan-allergen.

2. Materials and methods

2.1 Construction of chimeric His-Der p 10 cDNA

Total RNA was extracted from homogenized 20-30 purified *Dermatophagoides pteronyssinus* mites (a collaboration with Assist Prof. Nat Malainual and Assoc Prof Vanna Mahakittikun, Siriraj Hospital, Mahidol University) using QIAGEN total RNA extraction kit. The total RNA was used as a template for RT-PCR. Der p 10-specific primers were used: Der p10F: 5' GTCCTCTCGAGAAAAGAGAGGCTATG GAGGCCATCAAGAATAA 3', Der p10R: 5' GTGG TGCGGCCGCTTAATAACCAGTAAGTTCGGC 3'. Der p 10 cDNA was subcloned into plasmid pPICZ□ and transformed into *E.Coli* cells. Sequence of Der p 10 cDNA was confirmed by DNA sequencing. Since we would like to express recombinant Der p 10 in *E.Coli* cells, primers containing cloning sites of pET28b were used to amplify Der p 10 from pPICZ□-Der p 10.

To obtain a high yield of recombinant Der p 10 that suitable for X-ray crystallization, two plasmids containing chimeric 6xHis/Thrombin cleavage site at either N-terminal or C-terminal of Der p 10 cDNA were constructed (Figure 2).

Der p 10-specific primers for pET28b were used: Dp10pET28HisNF: 5' GCACGCCATATGGAG GCCATCAAGAATAAAATG 3', Dp10pET28HisNR: 5' GGGCTCGAGTTAATAACCAGTAAGTTCGGC AAATG 3', Dp10pET28HisCF: 5' GCACGCCCATG GAGGCCATCAAGAATAAAATG 3', Dp10pET28 HisCR: 5' GGGCTCGAGGCTGCCGCGCGGCACC AGATAACCAGTAAGTTCGGCAAATG 3'. PCR amplified N-terminal His/Thrombin-Der p 10 cDNA was digested with NdeI and XhoI and PCR amplified C-terminal His/Thrombin-Der p 10 cDNA was digested

with NcoI and XhoI. Digested cDNA was ligated with NdeI/XhoI or NcoI/XhoI digested pET28b.

Recombinant plasmids were transformed into JM109 cells (Figure 1). The positives clones were selected, extracted plasmids and sequenced (Macrogens). Sequencing results showed correctable Der p 10 cDNA sequences both N-terminal and C-terminal His.

The recombinant plasmids were transformed into BL21pLysS cells. The positive clones were screened (6).

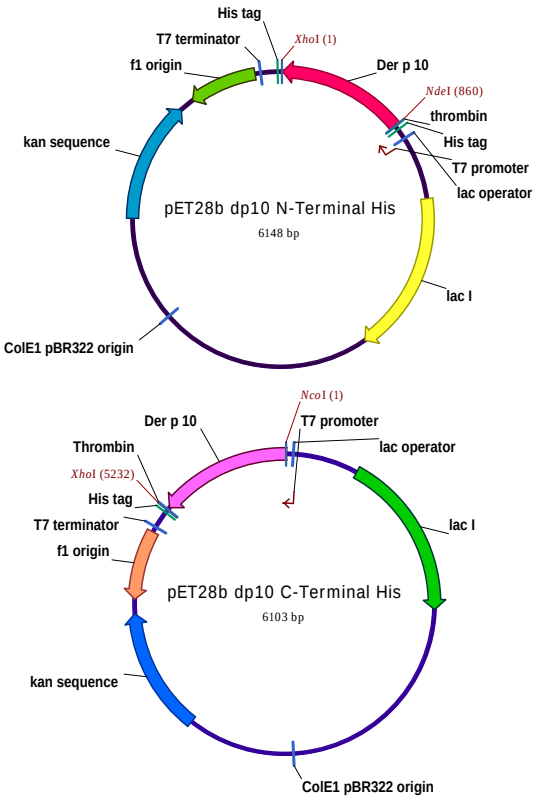

Figure 1 The recombinant vector for N-terminal 6xHis-fused and C-terminal 6xHis-fused.

2.2 Determination of protein solubility

The conditions that suitable for soluble expressed protein were determined as described in QIAGEN protocol. Expression conditions that were tested with 0.2, 0.4, 0.6, 0.8 and 1.0 mM IPTG at 18°C, 25°C and 37°C. Western blot analysis by Ni-HRP was done to confirm the best condition of expression.

2.3 Large scale expression and purification

Large scale expression was performed at the predetermined condition that yields high amount of soluble rDer p 10. The His-rDer p 10 was purified through Nickel affinity chromatography.

3. Results and discussion

The size of His-Der p 10 cDNA from PCR reaction was ~1,000 bp. Moreover, the remaining template pPICZα-dp10 was also seen in the gel. His-Der p 10 cDNA was purified by gel extraction (Figure 2).

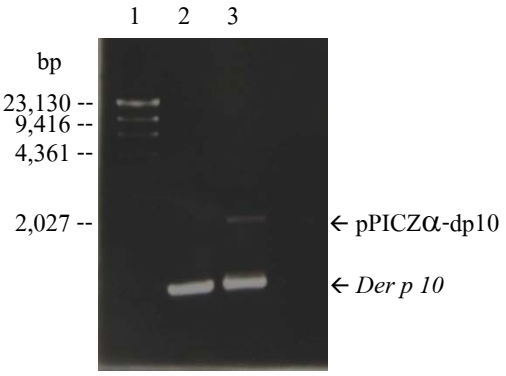

Figure 2 PCR products of Der p 10 cDNA (Lamda HindIII marker; lane 1, N-terminal His-Der p 10 cDNA; lane 2, C-terminal His-Der p 10 cDNA; lane 3).

The recombinant plasmids were transformed into BL21(DE3)pLysS cells to express recombinant Der p 10.

The suitable expression condition that yielded high amount of soluble rDer p 10 were:

For N-terminal His-Der p 10, induced with 1.0 mM IPTG and maintained cells at 25°C for 7 hours (Figure 3A).

For C-terminal His-Der p 10, induced with 0.6 mM IPTG and maintained cells at 18°C for overnight (Figure 3B).

Western blot result using Ni-HRP anti-His confirmed the expressed His-Der p 10 in the soluble fractions (Figure 4A, B).

Large scale expression (1.2 liters of bacterial culture) was performed for both N-terminal His- Der p 10 and C-terminal His-Der p 10 with optimal conditions as described above. The His-Der p 10 in soluble fractions was purified through Nickel affinity chromatography (HiTrap chelating HP column). SDS gel analysis showed a high yield of N-terminal His- Der p 10 (~8 milligrams per liter of bacterial culture) was obtained with a background from high MW proteins (Figure 5A). On the other hand, a small amount of C-terminal His-rDer p 10 (~0.5 milligrams per liter of bacterial culture) was obtained but with a high background from other proteins (Figure 5B).

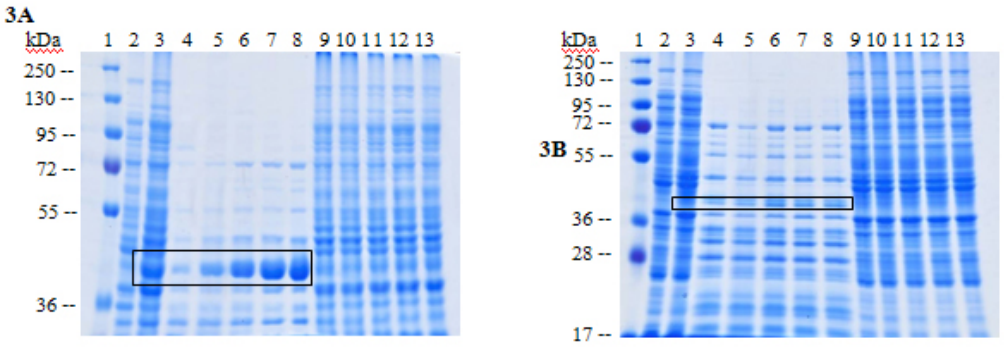

Figure 3 Determination of protein solubility *(A)* N-terminal His-Der p 10 from 25°C culture *(B)* C-terminal His-Der p 10 from 18°C culture (PageRuler plus prestained protein ladder; lane 1, Non-induce control; lane 2, Induce control; lane 3, Soluble fractions that induced with 0.2, 0.4, 0.6, 0.8 and 1.0 mM IPTG; lane 4-8, Insoluble fractions that induced with 0.2, 0.4, 0.6, 0.8 and 1.0 mM IPTG; lane 10-13, The boxes indicated rDer p 10 protein).

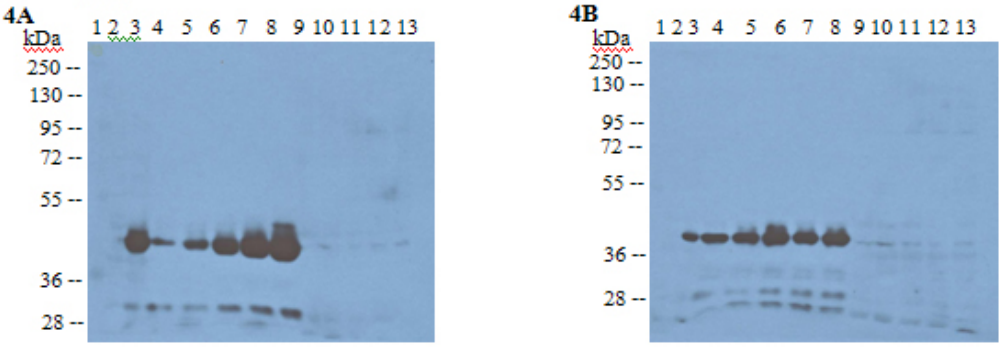

Figure 4 Western blot analysis using HRP-Ni anti-His *(A)* N-terminal His-Der p 10 from 25°C culture *(B)* C-terminal His-Der p 10 from 18°C culture (PageRuler plus prestained protein ladder; lane 1, Non-induce control; lane 2, Induce control; lane 3, Soluble fractions that induced with 0.2, 0.4, 0.6, 0.8 and 1.0 mM IPTG; lane 4-8, Insoluble fractions that induced with 0.2, 0.4, 0.6, 0.8 and 1.0 mM IPTG; lane 10-13).

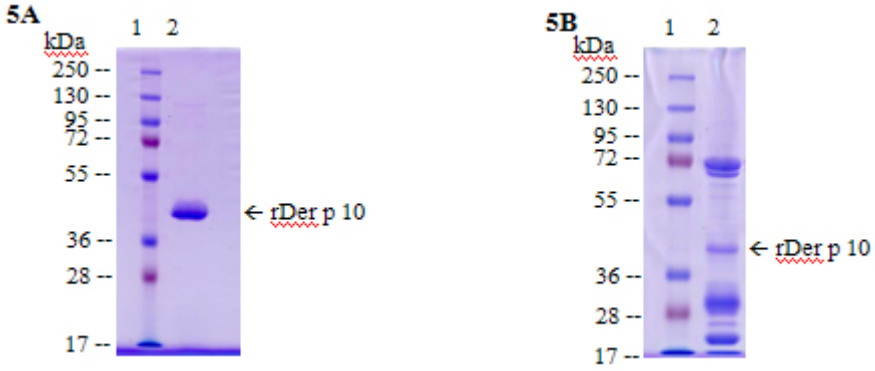

Figure 5 Nickel affinity chromatography purification of *(A)* N-terminal His-Der p 10 and *(B)* C-terminal His-Der p 10 (PageRuler plus prestained protein ladder; lane 1, Nickel affinity chromatography purified rDer p 10; lane 2).

Naturally, tropomyosin forms helical structure. Fusion of 6x-Histidine to C-terminal of rDer p 10 may misform the helical structure of rDer p 10. This might affect solubility of the protein. Thus, the yield of C-terminal His-Der p 10 was lower than that of N-terminal His-Der p 10 by. In addition, after purified through Nickel affinity column, other proteins were also observed with purified C-terminal His-Der p 10. It might was affected from internal folding of C-terminal 6xHis-tag that difficultly to bind in column. Thus, only Nickel affinity column purified N-terminal His-Der p 10 will be further purified through Size-exclusion Gel filtration column before used in X-ray crystallization and immune reactivity test.

4. Conclusions

The plasmids containing both N-terminal and C-terminal His-Der p 10 were successfully constructed. Suitable conditions that yielded high amount of soluble rDer p 10 protein were 1.0 mM IPTG at 25°C for 7 hours for N-terminal His-Der p 10 and 0.6 mM IPTG at 18°C for overnight for C-terminal His-Der p 10. However, only N-terminal His-Der p 10 yielded in high amount after Nickel affinity chromatography purification.

Acknowledgements

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