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（NSC93-2313-B-002-086）

以植物細胞培養技術生產重組塵？過敏原蛋白 Der p2 之研究

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摘要

重組過敏原蛋白已在許多不同系統中表現， 而植物系統被認為是具有競爭力的外源蛋白表現系統。本研究中，從已轉入 CaMV 35S 啟動子驅動塵？過敏原蛋白 *Dermatophagoides pteronyssinus* 2 (Der p 2) 基因的轉殖煙草植株，來誘導癒傷組織與毛狀根，之後分別進行懸浮細胞培養與毛狀根培養，並利用自行建立之 Der p *2* 三明治酵素免疫分析法(sandwich-ELISA)進行定量。從 8 株懸浮細胞株中挑選出 Der p 2 產量最高的細胞株 S3，搖瓶培養 16 天，產量為 788.3 μg/ L；以生物反應器進行批次培養，產量在第 8 天即達 515.1 μg/ L。在轉植植株誘導毛狀根方面，共得 24 個細胞株，經 30 天三角搖瓶培養後，細胞株 R19 產量為 475.2 ng/mL。在重組 Der p 2 蛋白的抽出與純化方面，重組蛋白粗抽液經硫酸銨沉澱、陽離子交換樹酯 SP Sephadex、與自行建立之 Der p 2 單株抗體親合管柱處理後，可以獲得以 Der p 2 為主要蛋白之溶液，收率為 19.7 %。

Abstract

Recombinant allergenic proteins have been produced in a variety of different expression systems. Plants and plant cells are now considered as viable and competitive expression systems for large-scale protein production. In this research, the plantlet of transgenic tobacco contained CaMV 35S promoter chimeric with *Dermatophagides pteronyssinus* 2 (Der p 2) gene was used to induce formation of callus and hairy roots, and were studied for recombinant Der p 2 production. By Der p 2 sandwich enzyme-linked immunosorbent assay (sandwich-ELISA), cell line S3 expressed the highest Der p 2 productivity in 8 suspension cell lines. The maximum productivity in flasks was 788.3 μg/L; batch culture in bioreactor reached the highest productivity, 515.1 μg/L. Among 24 cell lines of hairy roots from transgenic tobacco, maximum productivity was 475.2 ng/mL. After the treatment of ammonia sulfate precipitation, cation exchange chromatography, and monoclonal anti-Der p 2 affinity column, recombinant Der p 2 protein was purified into homologous, the recovery rate was 19.7%.

Introduction

The Group 2 allergens are considered to be major allergens because 80-90% of mite-allergic individuals produce humoral and cellular response to this allergen (Heymann et al., 1989). The group 1 and 2 allergens of *Dermatophagoides* sp. induce high titers of IgE (van der Zee et al., 1988; Trombone et al., 2002) and Th2 cytokines in 80% of allergic patients (Hales et al., 2000; Hales et al., 2002). The relationship between the allergy and Der p 2 is significant, so our study focused on the group 2 allergen in *D. pteronyssinus*.

The Der p 2 is a protein consisting of 129 amino acid residues with a molecular mass of 14 kDa and is devoid of *N*-glycosylation sites. Large scale production of Der p 2 protein from *E. coli* have been reported (Mueller et al., 1997), but the Der p 2 protein are prone to form aggregated inclusion bodies in the *E. coli* protein production system. Besides this, the contamination of endotoxin for animal model test and the incorrect conformation of protein folding were the problems. Der p 2 was also expressed as a recombinant mature protein in the baker's yeast *S. cerevisiae* and possessed a good Der p 2 reactivity (Hakkrt et al., 1998), but the foreign protein that produced in yeast system are often hyperglycosylated and are commonly degraded. The Der p 2 had also been expressed in tobacco plantlet, suspension cell and potato plantlet. (Wang, 2002; Ho, 2002)

The term "plant cell culture" refers to the propagation of any plant-derived cell tissue in gently agitated liquid media (James and Lee, 2001). The advantages of plant cell culture systems include: (1) lower production costs; (2) plant contains no known human pathogens that could contaminate the final products; (3) higher plants generally synthesize proteins from eukaryotes with correctly folding, glycosylation, activity, protein assemble multimeric ability, low downstream processing requirement (Horn et al., 2004) (4) more controllable, reproducible environment, shorter growth cycle, and simple synthetic medium than whole transgenic plant (5) easy to scale-up culture for large scale production and storage cost is low (Ma et al., 2003). The plants that infected by *Agrobacterium rhizogenes* formed the hairy roots. "Hairy root" cultures have several properties: capable of unlimited propagation in culture media, fast growth, genetic and biochemical stability (Shanks and Morgan, 1999).

The most commonly used promoter for expressing foreign protein in plant system is cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985), which could achieve high-level expression of foreign proteins in plants. We used the plant cell culture system to express recombinant foreign proteins. Production of Der p 2 in plant cell cultures is the major aim of this work. We induced suspension cells and hairy roots from transgenic tobacco which Der p 2 gene was driven by a 35S promoter, and developed the cell cultures in flasks and bioreactor. We established the sandwich enzyme-linked immunosorbent assay (sandwich-ELISA) to quantify the concentration of Der p 2 in plant cell cultures. Finally, we also developed a process for purification of Der p 2.

Material & methods

CaMV 35S promoter/Der p 2 gene suspension cell and hairy root cultures

Der p 2-transgenic tobacco plantlets were kindly provided by Professor Chiang (Medicinal College, NTU), and we induced callus and hairy root from the transgenic tobacco. The construction of T-DNA region was shown in Fig. 1. We induced the calli from Der p 2-transgenic tobacco plantlets. Eight cell lines were selected to be cultured in flasks. The suspension cells were cultivated on MS liquid medium with 1 ppm 2,4-D , 1 ppm kinetin , 3% sucrose, and 0.1% bacto peptone, pH 5.6~5.8. The cultivation conditions were 27 °C, 100 rpm, in the dark, and cells were sub-cultured at every 20 d. The culture medium was the same as the medium described above in flask culture. The conditions for bioreactor culture were at 27 °C, 120 rpm, with 0.15 vvm aeration in the dark. Hairy roots were induced from transgenic tobacco plantlet by transformed with *A. rhizogenes* 1610. Finally, 24 sterilized cell lines were obtained. The medium of hairy root cultures was liquid ½ MS medium or solid ½ MS medium with 0.8% agar, and hairy roots were sub-cultured in 30 d.

Fig. 1 The construction of T-DNA region. T-DNA of CaMV 35S promoter/Der p 2 gene suspension cells and hairy roots fromtransgenic-Der p 2 plantlet. The construction of T-DNA in transgenic-Der p 2 plantlet was provided by Professor Chiang. The promoter combined with the fragment of enhancer (E) for expressing Der p 2. The NPT II is a selection marker by using kanamycin.

Residual sugar determination

We determined the residual sugar by HPLC (Chromatocorder 21, SYSTEM INSTRUMENTS Co., Tokyo, Japan) with RI detector (Model 1770, Bio-Rad, CA, USA). The column was Lichrospher®100 NH_2 (5μm, 250 x 2.4 mm). The first peak appeared at 12.6 min was fructose, and the second peak appeared at 15.2 min was glucose, and the last peak appeared at 25 min was sucrose.

pH and conductivity

We surveyed pH by pH meter (Model SP-2000, SUNTEX INSTRUMENT Co., Taipei, Taiwan). We used conductivity meter (Model No.SC-120, SUNTEX INSTRUMENT Co., Taipei, Taiwan) to measure the conductivity of the medium during culture.

Protein extraction

Twenty mg dry weight of suspension cells or 30 mg of hairy roots was mixed with 1.0 mL protein extraction buffer (HEPES 200 mM ; NaCl 150 mM ; EDTA 20 mM ; PMSF 0.1435 mM ; PVP 0.05% ; DTT 1.62 mM ; TritonX-100 0.1 % ; pH 8.0). The mixture was homogenized for 30 sec, and was centrifuged at 13362 ×*g* for 30 min at 4℃ (HERMLE Z323K, Germany), the supernatant was collected.

Western blot

After treatment, we loaded supernatant of protein extract samples to SDS-PAGE (running gel: 15%, stacking gel: 5%) and the electrophoresis was performed. Then, the proteins on the page were transferred to Hybond-C Extra membrane (Amersham) in 85 voltage / 55 minutes, and the membrane was boiled overnight by 5% skim milk. The anti- Der p 2 monoclonal antibody was the 1st antibody, and the rabbit anti-mouse HRP-conjugated IgG was the 2nd antibody for western bolt. Chemifluorescence reagent was used for detection.

Sandwich Enzyme-Linked Immunosorbent Assay

We coated the capture antibody (anti-Der p 2 monoclonal antibody, kindly provided by Professor Chiang) on the 96 wells polystyrene microplate by using coating buffer (Carbonate-bicarbonate pH 9.6). We found that the best blocking reagent was gelatin-NET and the detection antibody was anti-Der p 2 polyclonal antibody from rabbit, and washed by PBST (phosphate buffer saline with 0.05% Tween-20). The TMB substrate expressed blue color when it reacted with HPR. We quantified the Der p 2 concentration by the blue color (OD 650).

Ammonium sulfate precipitation

Ammonium sulfate precipitation was the first step of protein purification. We collected the precipitate in the fraction of

60%~100% by centrifugation and re-suspension by 20mM CH_3COONa buffer, pH 5.0.

Ion exchange chromatography

SP-Sephadex C-25 (Phamarcia Biotech.) was packed into the column for the second step purification. The binding buffer was 20 mM CH_3COONa (pH 5.0) and the elution buffer contained the gradient of 0-250 mM NaCl. The column was regenerated by the binding buffer containing 2 M NaCl.

Affinity column

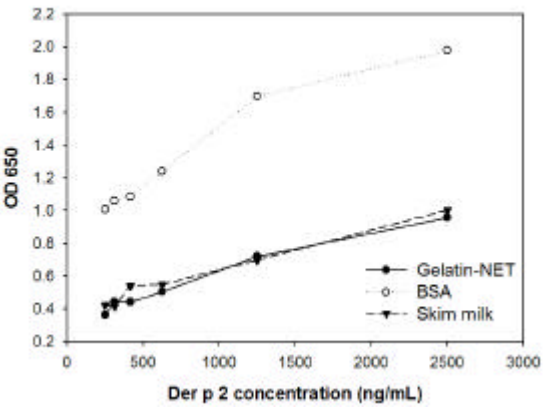
Antibody was purified by HiTrap proteinG column (Amersham). The purified antibody was used for preparation of affinity column. sAfter the antibody mixed with the coupling gel (PIERCE 34028), we added the sample purified from ion exchange column to the affinity column and reaction for overnight at 4°C. Der p 2 proteins were eluted by elution buffer (glycine-HCl, pH 2.7).

Results

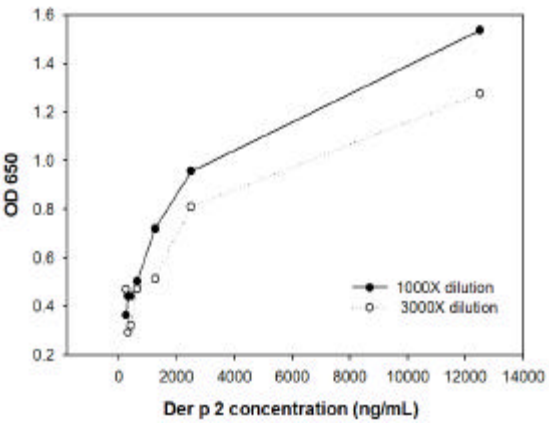
Establishment of ELISA

We tried to find the best conditions for quantification of Der p 2 in ELISA assay. The recombinant Der p 2 produced by yeast was to be the standard. The monoclonal antibody used as the capture antibody at the concentration of 1:1000 (v:v) showed an better changes between OD values and Der p 2 concentration than polyclonal antibody (data not shown), and gelatin-NET was better than BSA and skim milk in this ELISA system of Der p 2 assay **(Fig. 2A)**. The better detection antibody was rabbit polyclonal antibody in the concentration of 1:1000 (v:v) **(Fig. 2B)**. The enzyme-conjugated antibody for lightening was Goat anti-rabbit HRP conjugated IgG, and showed a better assay result at the concentration of 1:3000 (v:v) than 1:5000 (v:v) **(Fig. 2C)**.

(A)



(**B**)



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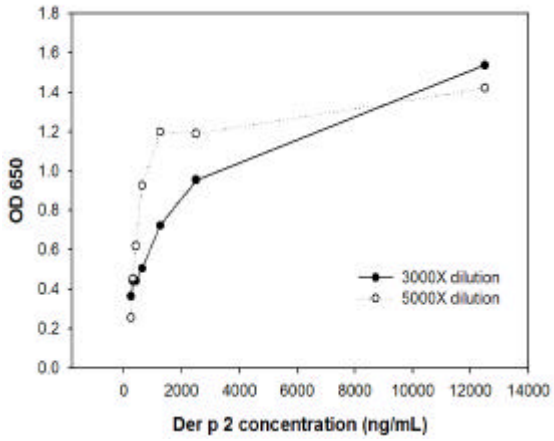


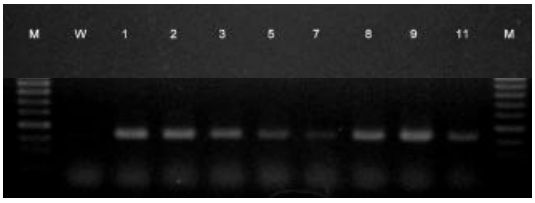
Fig. 2 (A) Measurement of Der p 2 in sandwich-ELISA. 1:100 (v:v) diluted monoclonal capture antibody. We used three kinds of blocking reagent: gelatin-NET, BSA, and skim milk. The concentration of standard was 250~2500 ng/mL. The detection antibody was 1000X dilution, and the Goat anti-rabbit HRP conjugated IgG was 3000X dilution. (B) Different dilution concentration of detection antibody: Rabbit anti-Der p 2 in sandwich-ELISA.The capture antibody was 100X dilution of monoclonal antibody. The concentration of standard was 0~12000 ng/mL, and the blocking buffer was gelatin-NET. The goat anti-rabbit HRP conjugated IgG was 3000X dilution. (C) Different dilution concentration of Goat anti-rabbit HRP conjugated IgG in sandwich-ELISA. sThe capture antibody was 100X dilution of monoclonal antibody. The concentration of standard was 0~12000 ng/mL, and the blocking buffer was gelatin-NET. The detection antibody was 1000X dilution.

Suspension cell lines in flask cultures

The CaMV 35S promoter/Der p 2 tobacco transgenic plantlet was used to induce the formation of calli and hairy roots. Eight cell lines of suspension cells were obtained. Confirmation of Der p 2 gene by PCR and western blot of Der p 2 protein was analyzed **(Fig.3).** These cell lines showed the good accumulated biomass in 15 d. The highest dry weight was 15.47 mg/mL for S8. However, the S3 had the highest Der p 2 content in 8 cell lines, and the Der p 2 content reached 600.8 ng/mL in 17 d. The accumulation of biomass and Der p 2 protein was not parallel in flask cultures.

S1 and S3 were chosen for further research. Samples were taken at set intervals in 31 d cultivation, and pH and conductivity of medium were also measured. In this work, the S3 showed a higher biomass 16.4 mg/mL of dry weight and 788.3 ng/mL of Der p 2 content in 16 d, than the S1 **(Fig. 4A)**. The pH value of the S1 culture increased significantly after 20 d, lysis of the cultivated cells was observed **(Fig. 4B)**. The decline of conductivity of medium meant the nutrient (inorganic salt) in the medium was exhausted. Accompany with the decline of conductivity through the cultivated time, accumulation of biomass was observed. The conductivity of medium finally increased because of lysis of cells. In S3 culture, the ratio of Der p 2 in total protein increased with the culture time, finally reached 1.27 ng/mL after 29 d **(Fig. 5A)**. Profile of tobacco suspension cell in flasks was shown in **Fig. 5B**.

(A)



(B)

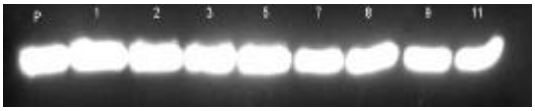
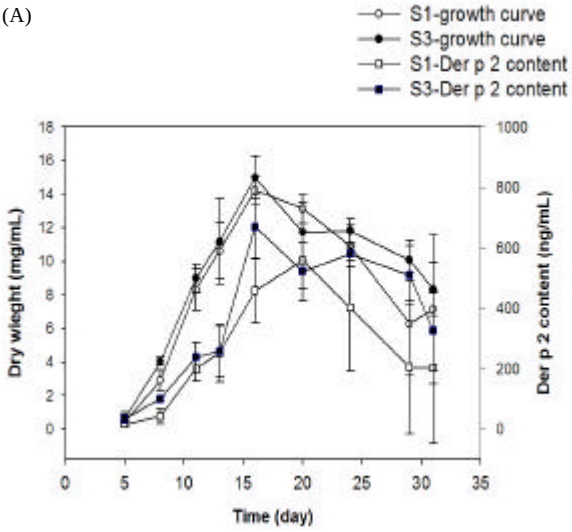


Fig. 3 (A) PCR for Der p 2 gene in genomic DNA of tobacco. The symbols are M: Marker ; W: wild type ; Cell line: S1-S11. The S4, S6, and S10 were contaminated in cultivation. The length of PCR fragment was 417 bp. (B) Western blot for Der p 2 in 8 suspension cell lines. P: Positive; Cell lines: S1-S11. The positive control: Der p 2 from yeast.

(A)



(B)

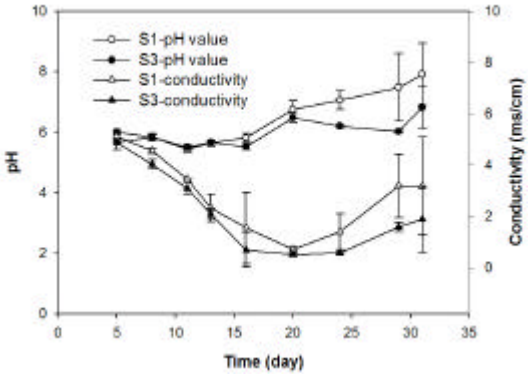
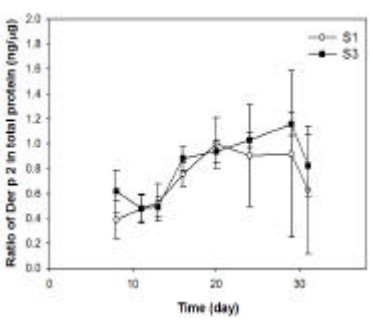


Fig. 4 (A) Growth and Der p 2 content of S1 & S3 in flask cultures. The S1 and S3 cultures were sampled every 4 or 5 d. The Der p 2 content was determined by ELISA. Every cell line replicated at three times (n=3). The conditions of culture were at 27℃, 100 rpm, in the dark. (B) pH and conductivity of S1 & S3 in flask cultures. The S1 and S3 cultures were sampled every 4 or 5 d. Every cell line replicated at three times (n=3). The pH value was determined by pH meter and the conductivity was determined by conductivity meter by measuring the medium of culture.

(A) (B)



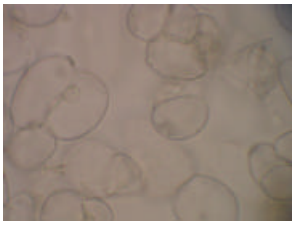


Fig. 5 (A) Ratio of Der p 2 in total protein in flask cultures. The total protein was determined by method of Bradford, and the Der p 2 content was determined by ELISA. (B) Profile of tobacco suspension cells in flask. (400X)

Suspension cells cultivated in bioreactor

S1 and S3 were further cultivated in bioreactors. In **Fig. 6A**, the accumulated biomass (dry weight) of S1 continuously increasing to 10.7 mg/ml in 19 d. Der p 2 content reached the maximum 442.3 ng/mL in the 13th d. The same pattern was also observed in S3 culture: the highest Der p 2 content (515.1 ng/mL) at the 8th d **(Fig. 6B)**. The content of Der p 2 in cells increased with the sucrose hydrolyzed into glucose and fructose **(Fig. 6C, 6D)**. By comparing S1 and S3, we found the cultivation of S3 grew faster than S1 in bioreactor, and when carbon source was exhausted completely, the content of Der p 2 began to decline at the same time.

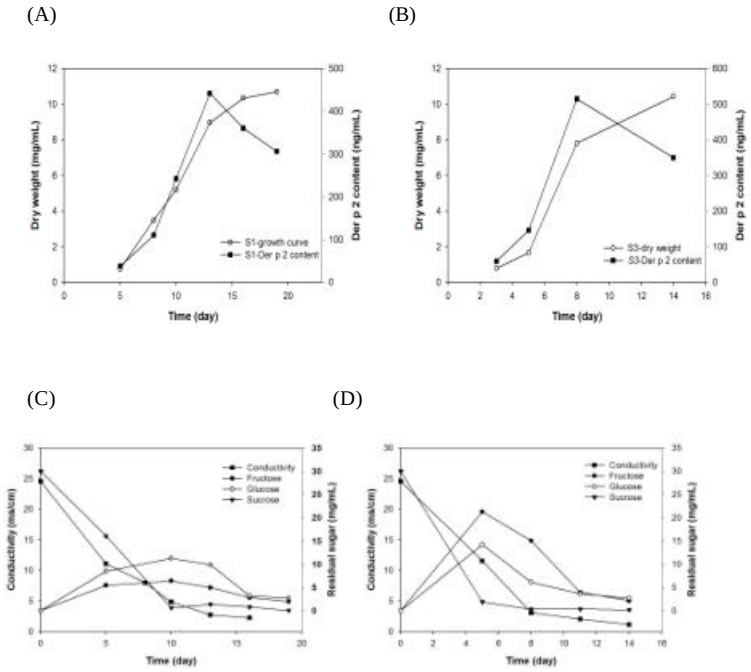


Fig. 6 (A) Growth and Der p 2 content of (A) S1 and (B) S3 cultivated in 5L bioreactor. Residual sugar and conductivity of (C) S1 cultivated and (D) S2 in 5L bioreactor. The cell lines were sampled every 4 or 5 d. The Der p 2 content was determined by ELISA. The conductivity was determined by measuring the medium of culture. The residual sugars were determined by HPLC. The conditions of culture were at 27℃, 120 rpm, with 0.15 vvm aeration in the dark.

Expression of CaMV35S promoter/Der p 2 gene in hairy roots

After induction of hairy roots from transgenic tobacco, 24 cell lines were established. The R17 showed the highest accumulation of biomass: 158.5 mg / 100 mL. Three cell lines contained ratio of Der p 2 in total protein higher than 1.0 ng/μg. The R19 was the highest, reached 1.47 ng/μg. Hairy roots contained higher ratio of Der p 2 in total protein than in suspension cells was observed, but the accumulated biomass of suspension cells was significant higher than hairy roots in the same culture period. The Der p 2 content reached 475.5 ng/mL in hairy roots of R19 **(Fig. 7)**. The R19 had the highest Der p 2 content, but the R17 possessed both high Der p 2 content and higher growth rate, therefore, the R17 expressed the

best productivity among the 24 cell lines.

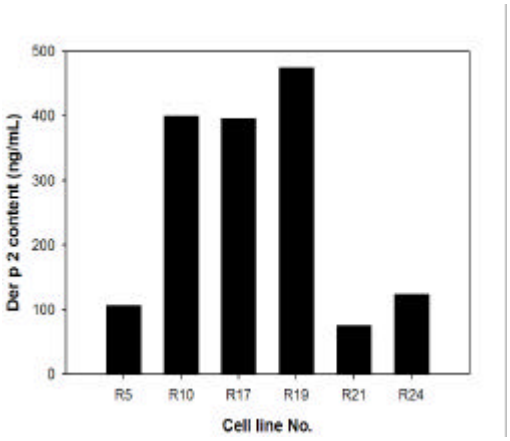


Fig. 7 Der p 2 content of hairy roots. The CaMV 35S promoter/Der p 2 gene hairy roots cultured in 30 d and the medium of culture was 100 mL. We chose R5, R10, R17, R19, R21 and R24 for detecting the Der p 2 content. The Der p 2 content was determined by ELISA.

Purification of Der p 2

Der p 2 was extracted and precipitated by 60-100 % ammonium sulfate. Then, the precipitate was re-dissolved and further purified by ion exchange column (SP-Sephadex). The chromatography was eluted from 40 mM to 60 mM NaCl. The yield of the Der p 2 was 52.8% **(Table 1)**. Finally, we used the monoclonal anti-Der p 2 affinity column (self-established) to purify the Der p 2, and the yield was 19.7 %. The purity of Der p 2 in every step was evaluated in the SDS-PAGE **(Fig. 8)**, and was confirmed by western blot.

Table 1. Purification of *Der p* 2.

Step	Total protein (μg)	Der p 2 (μg)	Ratio (%)	Purification (fold)	Yield (%)
Ammonia sulfate precipitation	1095.691	2.53	0.238	1	100
SP Sephadex	541.1429	1.336	0.246	1.03	52.8
Monoclonal anti-Der p 2 affinity column	20.3	0.5	2.46	10.3	19.7

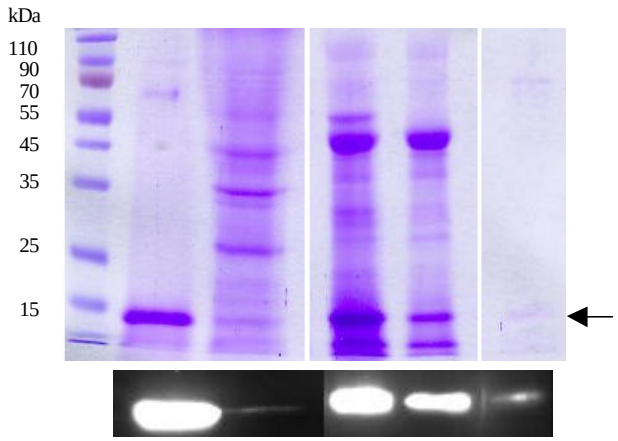


Fig. 8 SDS-PAGE and Western blot of Der p 2. The protein of Der p 2 was 14 kDa. **Lane 1**: Marker; **2**: Standard; **3**: Crude extraction supernatant; **4**: After ammonium sulfate precipitation; **5**: After ion exchange column; **6**: After affinity column.

Discussion

In this study, the sandwich ELISA system for the detection of Der p 2 was established. The gelatin-NET was found a proper blocking buffer than BSA. The reason may be caused by the antibodies used in this study were different to others. The r^2 value in our test reached 0.98. The range of efficient detection was found between 31.25 ng/mL ~ 12.5μg/mL.

The yield of Der p 2 was 50 mg/L in *E. coli* (Mueller et al., 1997), and was 7.6 (±2.9) mg/L in yeast (Hakkrt et al., 1998). The ratio of Der p 2 in total protein reached 0.01% in plantlet of tobacco and potato (Wang et al., 2002). In our study, the yield of Der p 2 was 788.3 μg/L and the ratio of Der p 2 in total protein was 0.11%. The ratio of Der p 2 in total protein was 10-fold higher in plant cells than it in plantlet.

We found that Der p 2 protein could be expressed successfully both in suspension cells and hairy roots. The ratio of Der p 2 in total protein were almost the same in the two systems, but the Der p 2 content in suspension cells was significant higher than it in hairy roots. Besides, the accumulated biomass of suspension cells was also higher than it of hairy roots. Therefore, the suspension cell culture is more suitable than hairy roots for Der p 2 production. The suspension cells cultured in flasks and in bioreactor reached their maximum content 788.3 ng/mL after 16 d and 515.1 ng/mL after 8 d, respectively. Although the content of Der p 2 in suspension cells cultivated in flasks was higher than it in bioreactor, the suspension cells cultivated in bioreactor got a good productivity in a shorter time. In the batch culture of bioreactor, carbon source was exhausted rapidly at a shorter time because cells grow faster, and the protein in cells was depleted to maintain the viability of cells when nutrition was not limited. Fed-batch in bioreactor culture will be necessary for improvement of cell density and exogenous protein accumulation in the further study.

Regarding the induction rate of hairy roots, the factors such as the infection ability of *A. rhizogenes* and the age of tobacco plantlet will affect the induction results. The clones of hairy roots were induced from the same leaf of transgenic plant, however, the ability for Der p 2 production was quite different. It was though to be caused by the different copy numbers and the diversity locus of T-DNA in genome.

We purified the Der p 2 by ammonium sulfate precipitation, ion exchange and affinity column of monoclonal anti-Der p 2. The yield of affinity column was as low as in 1.89 %. In order to get a lot of purified Der p 2 for further research, the conditions, such as the preparation of affinity column, to replace the monoclonal antibody by polyclonal antibody, the binding time of the antibody with antigen, and the pH of the elution buffer in affinity column, should be investigated.

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成果自評

本研究結果與計畫預期之達成率為百分之百。本內容以煙草毛狀根進行外源蛋白質塵? 過敏原 Derp2 **之表現與生產，具有學術與應用價值。**