附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

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執行期間：　89 年　8 月　1 日至　91 年　7 月　31 日

計畫主持人：劉秉慧

共同主持人：

計畫參與人員：林姵岑

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□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：中山醫學大學生命科學系

中　華　民　國　91 年　10 月　28　日

行政院國家科學委員會專題研究計畫成果報告

黃麴毒素調控蛋白質 AFLR 之免疫化學及分子功能性研究

計畫編號：NSC 90-2313-B-040-003
執行期限：89 年 8 月 1 日至 91 年 7 月 31 日

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一、中文摘要

黃麴毒素是一族致癌及致突變的真菌毒素，為了瞭解在黃麴毒素的生化合成途徑中，調控基因 *aflR* 所扮演的角色，首先將 *aflR* 的 cDNA 在大腸桿菌中表達成為 AflR 蛋白質，並且以此為 Balb/c 老鼠的免疫原產生五株穩定的融合瘤細胞株並且予以定性。此外，利用特定序列的 DNA 親和層析法將分子量 47.5 kDa 的 AflR 蛋白質自產毒 *A. parasiticus* 菌株中純化出來；純化後的蛋白質能夠與兩段分別位於 *aflR* 基因和黃麴毒素合成基因的啟動區域在活體外形成複合物；然而來自於非產毒菌株的 *A. oryzae* 的蛋白質萃取物則完全無法辨識該兩段區域；研究結果顯示在黃麴菌屬中。AflR 與特定 DNA 區域的結合能力與菌株是否會產生黃麴毒素的特性應有直接的相關性，

關鍵詞：黃麴毒素生合成。AflR 蛋白質。單株抗體，。DNA 的結合能力

Abstract

To investigate the role of regulatory protein AflR in aflatoxin biosynthetic pathway, recombinant AflR was expressed in E.coli and purified homogeneously. With recombinant AflR as immunogen, five stable hybrodoma cell lines were generated and characterized. On the other hand, the native AflR was purified from *A. parasiticus* by sequence-specific DNA affinity chromatography. Purified AflR with a molecular weight of 47.5 kDa not only bound the AflR binding site (5'-TTAGGCCTAA-3') found upstream of the aflR gene, but also bound the sequence II (5'-TCGNNNNNCG A-3') in the promoter region of the structural genes. In contrast, partially purified protein extracts from nonaflatoxigenic *A. oryzae* did not recognize either of the DNA sequences as compared to aflatoxigenic *A. parasiticus* AflR in electrophoretic mobility shift assays. These findings suggest that the sequence-specific DNA binding ability of AflR is essential for the activation of aflatoxin biosynthetic pathway.

Key words: aflatoxin biosynthesis, AflR protein, monoclonal antibody, DNA binding ability

二、計劃緣由與目的

黃麴毒素(Aflatoxins)是一族主要由 *Aspergillus parasiticus*，*A. flavus* 和 *A. nomius* 等真菌所產生的二級代謝物；其中又以黃麴毒素 B1(Aflatoxin B1)的致肝癌性最強，目前已知至少有十七種不同的酵素在真菌體內參與黃麴毒素的化學合成，其中已知一個命名為 *aflR* 的調控基因(1)對於數個黃麴毒素合成途徑中酵素基因的表現具有轉錄層次上的活化啟動功能(2)，此 *aflR* 基因可轉譯成為一個 47 kDa 大小的 AFLR 蛋白質(3)，並且該衍生蛋白質序列含有一個 GAL-4 type binuclear zinc finger motif 的 DNA 結合區(2)。

雖然在典型產毒菌株中，AFLR 蛋白質的存在為黃麴毒素產生的必要因素，但是往往在某些不產毒菌株中(包括 *A. oryzae* 和 *A. sojae*)，不僅偵測到 *aflR* 基因並且發現了 *aflR* mRNA 和 AFLR 蛋白質的存在(4，5)，而原本預測會受到 AFLR 蛋白質活化的合成酵素基因卻沒有因此而被啟動，因而沒有毒素生成，這個結果與自產毒菌株中所得到的結果剛好相反；為了釐清這

項疑點並且提供一個簡單而靈敏的工具作為日後深入研究 AFLR 蛋白質調控機制之用，本計畫的目標之一就是希望藉由產生對AFLR蛋白質具有高度專一性的單株抗體來對數種產毒及不產毒菌諸進行免疫化學方面的研究。

在分子功能的研究方面，AFLR 蛋白質已知可和一段特定的 DNA 序列I(5'-TTAGG CCTAA-3')相結合，但是這段序列 I 只位在 *aflR* 基因轉錄起始點的上游區域，卻無法在任何已知之合成酵素基因的上游被發現(2)，接著以 *Aspergillus nidulans* 為題材的研究小組在 *A. nidulans* 中發現一種與 AFLR 之間約有 31 %氨基酸同源性的類似物，此類似物能夠與一段特定 DNA 序列 II(5'-TCGN5CGA-3')相結合，但是卻無法辨認上述之 DNA 序列I(6)，進一步分析數個合成酵素基因的 5'端上游區域，結果發現這些上游區域都含有該特定 DNA 序列II，雖然已知並不具有 DNA 序列I。至於位於 *aflR* 基因 5'端的 DNA 序列I以及合成酵素基因 5'端的 DNA 序列II在黃麴毒素的生化合成途徑中究竟扮演著什麼樣的角色至今仍不清楚。因此本計劃的的最終目標在於探討 AFLR 蛋白質與兩段 DNA 序列(I 和 II)在活體外的結合能力（在此指專一性 specificity）是否與菌株的產毒性相關。

III. Result and Discussion

Extensive efforts have been made to try to generate momoclonal antibodies specific to AflR protein. In addition, to evaluate the binding ability of AflR to DNA sequence I and II, AflR preparations were affinity-purified from *A. parasiticus* 2999, a standard aflatoxin-producing strain, and electro-phoretic mobility shift assays (EMSA, also called DNA binding assay) was conducted. Results are shown as the followings.

1. Expression and purification of partial-length recombinant AflR protein

Plamid pET29b(+)-aflR was designed so that the AflR fusion protein contained the putative zinc finger binding domain (amino acids 29 to 56) and a (His)6 domain at the N-terminal end of the protein to allow purification with Ni-NTA. The size of the purified AflR was approximately 32 kDa on SDS-PAGE (Fig 1, lane 5), which is in agreement with the size expected on the basis of the predicted amino acid composition. In the absence of IPTG, no such band was induced (Fig 1, lane 3), further indicated that the 32-kDa band results from expression of the recombinant gene.

2. Production and characterization of monoclonal antibodies (mAb)

Three female Balb/c mice were each immunized and boostered with purified recombinant AflR. Ten days after fusion, an average of 0.6 colony/well was found. Initial screening of 957 wells showed 5 stable master cell lines(1G8, 6D8, 7B4, 8D3 and 14F5) producing antibodies that were strongly reactive with purified recombinant AFLR coated on the ELISA plate (Fig 2). Isotyping of mAbs from various hybridoma cell lines showed that all of them containing IgG heavy chain and kappa light chain (Table 1).

To further characterize the mAbs, Hybridoma cell line 14F5 was chosen to generate ascites fluid. Ascites fluid developed 2-3 weeks after injection was collected from Balb/C mice and purified by ammonium sulfate precipitation. The specificity of ascites fluid was determined by Western blotting (Fig. 3)

3. DNA affinity purification of AflR

A purification procedure for AflR was developed using a specific DNA affinity resin derived from the AflR recognition sequence 5'-TTAGGCCTAA-3', which is located 120 bp upstream of the AflR translation start site. After the affinity chromatography steps were repeated two consecutive times, the highly purified

sample of AflR was analyzed by SDS gel electrophoresis (Fig. 4A). The purified sample consisted predominantly of the 47.5 kDa AflR, as recognized by anti-AflR antibodies in Western blotting (Fig. 4B). With the application of EMSA, the purified AflR formed a protein-DNA complex with oligonucleotide AF-2, which contains the AflR binding sequence 5'-TTAGGCCTAA-3' (Fig. 5B).

4. Purified AFLR bound specifically to the AnAFLR binding site

Fernandes et al. (1998) have documented the specific binding site of A. nidulans AflR, AnAflR(5'-TCG(N5)CGA-3'). To determine whether the purified AflR bound to the AnAflR binding site, a double stranded 20 bp oligonucleotide, OM-2, containing the putative AnAFLR binding site was used in EMSA. The OM-2 oligonucleotide formed a band shift in the presence of purified AflR, indicating the formation of a DNA-protein complex (Fig 5A).

5. DNA binding ability of partially purified A. oryzae

A. oryzae is a well-known non- aflatoxigenic species. To determine whether there is any protein in *A. oryzae* extracts showing binding ability to AflR binding site, protein extracts were particially purified from *A. oryzae* NRRL 451 mycelia. Although *A. parasiticus* protein fraction formed DNA-protein complexes with either AF-2 or OM-2 by EMSA, the A. oryzae fraction did not bind to either oligonucleotide. (Fig 6)

IV. Self assessment of experimental results

Part of the above results has been published (Liu, B. H. and F. Y. Yu. 2001. DNA affinity-purified AflR binds to the 5'upstream region of the aflatoxin gene cluster. *Food Science and Agricultural Chemistry*, 3, 84-90). More effors will be make to collect publishable data in the area of monoclonal antibody production.

V. Reference

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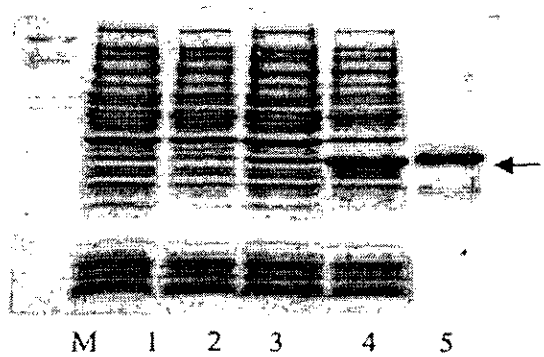

Fig. 1.(A) SDS-polyacrylamide gel electrophoresis of affinity-purified AflR visualized by silver staining. Lane 1, hydroxylapatite-purified (HA) fraction; Lane 2-3 represent the patterns of proteins obtained after two consecutive passes over the sequence-specific DNA affinity resin. (B) Western blot analysis of the purified AflR fractions using anti-AflR antibodies as the probes.

cell line	1G8	6D8	7B4	8D3	14F5
Heavy chain	IgG_{2a}	IgG_1	IgG_1	IgG_1	IgG_1
Light chain	κ	κ	κ	κ	κ

Table 1. Isotyping of mAbs from various hybridoma cell lines

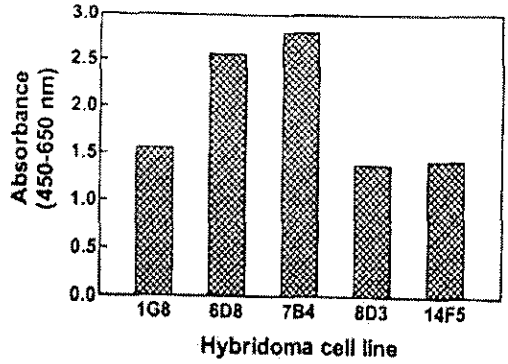

Fig. 2. Affinity of mAbs generated from the supernatant fluids of stable hybridoma cell lines. ELISA was conducted using Ni-NTA purified recombinant AflR (0.02 μ g ml^{-1}, 0.1ml/well) as the coating antigen.

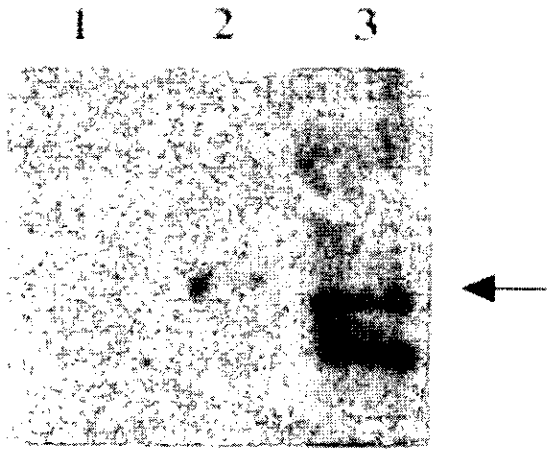

Fig. 3. Determination of the specificity of ascites fluid from hybridoma 14F5 with immunoblots. Lane 1, IPTG-induced *E. coli* containing pET29b(+); Lane2, extracts from *E. coli* containing pET29b(+)-*aflR* insert without IPTG induction; Lane 3, IPTG-induced *E. coli* containing pET29b(+)-*aflR* insert

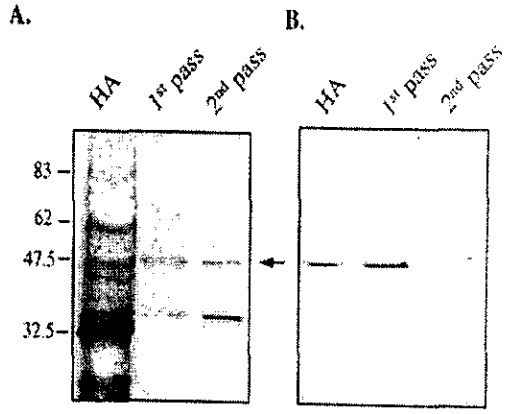

Fig 4. SDS-PAGE showing expression and purification of AflR fusion protein. Lane 1 and 2, protein extracts of *E.coli* containing pET29b(+) vector without the *aflR* insert; Lane 3 and 4, protein extracts of *E.coli* containing pET29b(+) vector with the *aflR* insert. Only the extracts in lane 2 and 4 were from E. coli induced with 1mM IPTG. Lane 5, purified AflR from Ni-NTA resin.

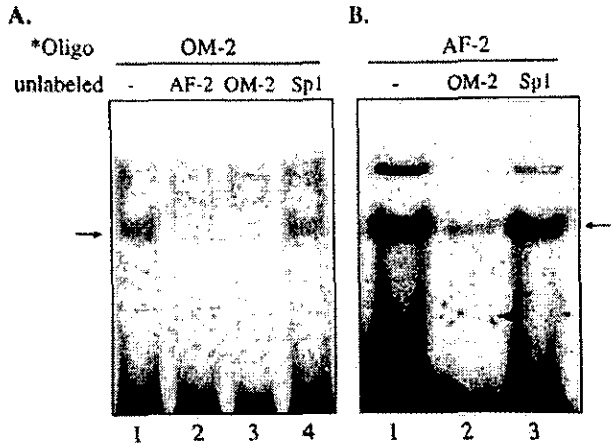

Fig. 5. Purified AflR specifically bound to the AnAflR binding site in OM-2 oligonucleotide. (A) EMSA with ^{32}P-labeled OM-2 and purified AflR. Binding reactions were carried out without the unlabeled competitor (lane 1) or with unlabeled AF-2 (lane 2), OM-2 (lane 3), and Sp1 (lane 4). (B) EMSA with AF-2 and purified AflR. Binding reactions were carried out without the unlabeled competitor (lane 1) or with unlabeled OM-2 (lane 2), and Sp1(lane 3).

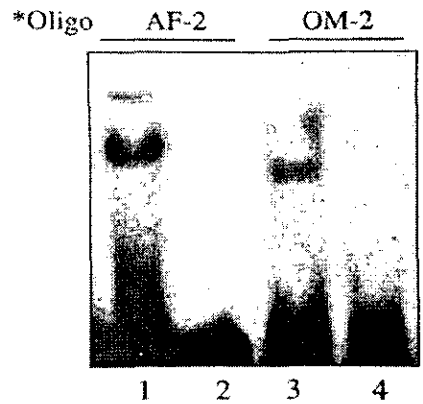

Fig. 6. DNA binding ability of *A. oryzae* protein extracts by EMSA. 10 μg of HA-purified proteins of *A. parasiticus* (lanes 1,3)and 10 μ g of HA-purified proteins of *A. oryzae* (lanes 2, 4) were incubated with the labeled AF-2 (lanes 1,2) or labeled OM-2 (lanes 3, 4) in binding reactions.