行政院國家科學委員會專題研究計畫 期末報告

黃麴毒素與微囊藻毒之單株抗體生產及奈米免疫檢測試紙與螢光極化免疫分析法的開發應用(第 2 年)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 99-2313-B-040-002-MY2
執 行 期 間 ：100 年 08 月 01 日至 101 年 10 月 31 日
執 行 單 位 ：中山醫學大學生物醫學科學學系（所）

計畫主持人：余豐益

計畫參與人員：碩士級-專任助理人員：呂權蓁

報 告 附 件 ：出席國際會議研究心得報告及發表論文

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 102 年 01 月 31 日

中文摘要： 黃麴毒素(aflatoxin B1/M1)與微囊藻毒(microcystin)主要由黴菌 Aspergillus 屬與藻類 Microcystis 屬等常產生的黴菌毒素與藻類毒素，此兩類毒素泛存於食品、作物與飲水中，食用遭受污染的食品或飲水會導致人類許多疾病及癌症的生成。我們將黃麴毒素 B1 接合牛血清蛋白金(BSA)抗原打入動物體內以取得黃麴毒素 B1 的抗體，利用此一抗體建立了敏感性高的直接競爭型酵素免疫分析法和以奈米金粒子為標記物的快速免疫層析試紙分析法。直接競爭型酵素免疫分析法中，抑制 50% 的 AFB1-HRP 與抗體結合所需 AFB1 的濃度(IC50)為 0.15 ng/mL。利用這個抗體與奈米金粒子相結合，形成抗體奈米金探針開發出黃麴毒素 B1 的快速免疫層析試紙，利用此一試紙來檢測食品咖啡、飼料中黃麴毒素 B1 的含量，此試紙最低限制為 2 ng/mL，可在 10 分鐘完成檢測結果，不需任何儀器可進行當場的黃麴毒素 B1 檢測。以競爭型酵素免疫分析法與免疫層析試紙分析 36 個食品或飼料樣品中黃麴毒素 B1 的含量，結果顯示 20 個樣品遭受到 1.3~234 ng/g 不等的污染，而且兩種方法得到相當一致的結果。

中文關鍵詞： 黃麴毒素, 微囊藻毒, 酵素免疫分析法, 免疫層析試紙分析法

英文摘要： Aflatoxins and microcystin are toxins that are produced by fungi Aspergillus and algal Microsystis. They are commonly found in foods, cereal products, and water, which cause toxic effects and cancer in human and animal. Antibodies specific to aflatoxin B1 were generated from rabbits immunized with AFB1-bovine serum albumin (BSA). By using these antibodies, this work presents a rapid and sensitive competitive direct enzyme-linked immunosorbent assay (cdELISA) and a gold nanoparticle immunochromatographic strip method for detecting AFB1 in food and feed samples. In the rapid cdELISA, AFB1 at a concentration of 0.15 ng/ml causes 50% inhibition (IC50) of binding AFB1-horseradish peroxidase to the antibodies. Effective on-site detection capability of AFB1 is also developed based on a rapid and sensitive antibody-gold nanoparticle immunochromatographic strip method. This strip has a detection limit of 2.0 ng/ml for AFB1 in food and feed samples. Additionally, the entire analysis is completed within 10 min. Closely examining 36 food

and feed samples by cdELISA reveals that 20 are contaminated with AFB1 from 1.3~234 ng/g. Results of 20 contaminated samples further analyzed with immunochromatographic strip assay correlate well with those obtained from cdELISA. The proposed cdELISA and immunochromatographic strip methods are highly sensitive to the rapid screening of AFB1 in food and feed samples.

英文關鍵詞： aflatoxin B1； microcystin, ELISA, Immunochromatography

附件一

行政院國家科學委員會補助專題研究計畫 □期中進度報告 ■期末報告

黃麴毒素與微囊藻毒之單株抗體生產及 奈米免疫檢測試紙與螢光極化

免疫分析法的開發應用

計畫類別：■個別型計畫 □整合型計畫
計畫編號：NSC 99－2313 －B－040－002－MY2
執行期間： 2010 年 8 月 1 日至 2012 年 10 月 31 日

執行機構及系所：中山醫學大學生物醫學科學系

計畫主持人：余豐益
共同主持人：
計畫參與人員：王敬 之, 呂權蓁

本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：
□移地研究心得報告
□出席國際學術會議心得報告
□國際合作研究計畫國外研究報告

處理方式：除列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年■二年後可公開查詢

中 華 民 國 102 年 01 月 31 日

行政院國家科學委員會專題研究計畫期末報告

黃麴毒素與微囊藻毒之單株抗體生產及奈米免疫檢測試紙與螢光極化免疫分析法的開發應用

計畫編號：NSC 99－2313－B－040－002－MY2

執行期限：99 年 8 月 1 日至 101 年 7 月 31 日

主持人：余豐益　　中山醫學大學生物醫學科學系

中文摘要:

黃麴毒素(aflatoxin B1/M1)與微囊藻毒(microcystin)主要由黴菌*Aspergillus*屬與藻類*Microcystis*屬等常產生的黴菌毒素與藻類毒素，此兩類毒素泛存於食品、作物與飲水中，食用遭受污染的食品或飲水會導致人類許多疾病及癌症的生成。我們將黃麴毒素B1接合牛血清蛋白金(BSA)抗原打入動物體內以取得黃麴毒素B1的抗體，利用此一抗體建立了敏感性高的直接競爭型酵素免疫分析法和以奈米金粒子為標記物的快速免疫層析試紙分析法。直接競爭型酵素免疫分析法中，抑制50% 的AFB1-HRP 與抗體結合所需AFB1 的濃度(IC50)為0.15 ng/mL。利用這個抗體與奈米金粒子相結合，形成抗體奈米金探針開發出黃麴毒素B1 的快速免疫層析試紙，利用此一試紙來檢測食品咖啡、飼料中黃麴毒素B1 的含量，此試紙最低限制為2 ng/mL，可在10 分鐘完成檢測結果，不需任何儀器可進行當場的黃麴毒素B1檢測。以競爭型酵素免疫分析法與免疫層析試紙分析36個食品或飼料樣品中黃麴毒素B1 的含量，結果顯示20個樣品遭受到1.3~234 ng/g 不等的污染，而且兩種方法得到相當一致的結果。

英文摘要:

Aflatoxins and microcystin are toxins that are produced by fungi *Aspergillus* and algal *Microsystis.* They are commonly found in foods, cereal products, and water, which cause toxic effects and cancer in human and animal. Antibodies specific to aflatoxin B1 were generated from rabbits immunized with AFB1-bovine serum albumin (BSA). By using these antibodies, this work presents a rapid and sensitive competitive direct enzyme-linked immunosorbent assay (cdELISA) and a gold nanoparticle immunochromatographic strip method for detecting AFB1 in food and feed samples. In the rapid cdELISA, AFB1 at a concentration of 0.15 ng/ml causes 50% inhibition (IC_{50}) of binding AFB1-horseradish peroxidase to the antibodies. Effective on-site detection capability of AFB1 is also developed based on a rapid and sensitive antibody-gold nanoparticle immunochromatographic strip method. This strip has a detection limit of 2.0 ng/ml for AFB1 in food and feed samples. Additionally, the entire analysis is completed within 10 min. Closely examining 36 food and feed samples by cdELISA reveals that 20 are contaminated with AFB1 from 1.3~234 ng/g. Results of 20 contaminated samples further analyzed with immunochromatographic strip assay correlate well with those obtained from cdELISA. The proposed cdELISA and immunochromatographic strip methods are highly sensitive to the rapid screening of AFB1 in food and feed samples.

【I】前言與目的:

黃麴毒素(aflatoxins)是一族主要由 *Aspergillus* 屬黴菌所產生的二級代謝產物，此類毒素可能在採收前後或採收後儲藏運送的過程中污染穀物與穀類製品，例如花生及玉米 (IARC, 1993)，根據 FAO (Food and Agriculture Organization) 統計結果指出，世界上有約 25%的農產品遭受黴菌毒素的污染，所以導致農業經濟上嚴重的損失。黃麴毒素共分四大種類，其中以黃麴毒素 B1 最癌性，而且黃麴毒素結構非常穩定，不會因為加熱或是食品加工而失去毒性，因此當母牛食用到遭黃麴毒素 B1 污染的乾草或飼料時，其所分泌的乳汁也會含有代謝產物黃麴毒素 M1 的產生，此種牛奶與奶製品的汙染對於嬰幼兒的健康有極大的威脅。目前國際癌症研究協會 (International Agency for Research on Cancer) 將黃麴毒素 B1 評定為第 1 級的人類致癌物質。目前許多國家對於食物中黃麴毒素 B1 的含量設有 20 ppb 的含量限制。台灣食品藥物管理局在 2009 年抽檢花生製品發現，黃麴毒素 B1 污染污染嚴重者高達 203 ppb 大約高出限制含量 15 ppb 的約 14 倍。2004 年在肯亞也因食用受到黃麴毒素污染的玉米而造成 317 人中毒，最後導致 125 人因為肝癌而死亡的案例 (Eduardo et al., 2005)；此外台灣與美國最近常有報導指出犬類食用遭受污染的飼料而導致腎衰竭與肝臟傷害的事件，其主要污染源經證實為黃麴毒素 B1。最新研究指出黃麴毒素 M1 常常污染牛奶與奶製品並可能與肝癌的盛行具有高相關性 (Peng, 2009; Prandini, 2009)。

微囊藻毒(Microcystin,MCYST)是一種由藍綠藻例如*, Microcystis aeruginosa,* 與 *Anabaena flos-aquae* 等藻類所外泌的藻類毒素(phycotoxins)，此族毒素具有 7 個胺基酸環狀組成(heptacyclic peptide)的結構，分子量大小約為 1000 dalton 左右 (Carmichael, 1994)；此種具有多樣化學結構的小分子毒素常常在池塘、湖泊與水庫等飲水系統中被發現，因而在世界各地一再造成許多動物的中毒與人類的肝臟傷害、腸胃炎、腹瀉等疾病。已知微囊藻毒的致毒機轉藉由抑制蛋白質磷酸酵素(protein phosphatase)作用的致癌物(Mackintosh,1995)。文獻指出巴西曾發生 50 多個洗腎病人死亡 (Jochimsen et al.,1998)及瑞士發生牛群猝死的案例皆是由於 MCYST 毒素污染水源所造成。因此國際癌症研究協會將微囊藻毒 LR 為 2B 族群，也就是一種人類可能的致癌物(possibly carcinogenic to human)。近年來隨著許多人為氮、磷肥料流入湖泊及排水道，造成許多有毒藻類的大量繁殖，所以世界衛生組織 (WHO,1997)已經在飲用水中設立微囊藻毒 1 ppb 的控制含量限制，而美國食品藥物管理局亦對微囊藻毒污染螺旋綠藻(*Spirulina*)類的食品建立監控含量限制為 1 ppm。

二、 材料與方法

2.1 Materials.

Aflatoxin B1, bovine serum albumin (BSA), ovalbumin (OVA), AFB1, AFB2, AFG1 and AFG2 analytical standard solution (10 μg/ml, Certified Reference Material from Supelco; Bellefonte, PA, USA), gelatin, bovine serum albumin (BSA), sodium bicarbonate, N, N-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), and Ferund's complete adjuvant were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Carboxymethoxylamine hemihydrochloride (CMO), ammonium sulfate, sodium chloride, and sodium azide were obtained from Aldrich Chem.Co. (Milwaukee, WI, USA). N, N-dimethylformamide, sodium phosphate, hydrochloric acid, boric acid and Tris were obtained from J. T. Baker (Phillipsburg, NJ, USA). TLC Silica gel 60 F254 plate, Tween 20 and disodium tetraborate (borax) were obtained from Merck (Darmstadt, Germany). Ferund's incomplete adjuvant, goat anti-rabbit IgG -Fc and goat anti-rabbit peroxidase conjugate were obtained from Pierce Chemical Co. (Rockford, IL, USA). Horseradish peroxidase (HRP) was obtained from Roche. HRP substrate solution 3, 3', 5, 5'-tetramethylbenzidine (TMB) was obtained from Neogen Corp. (Lexington, KY, USA). Microtiter plates and strips (low and high protein binding) were obtained from Nunc (Roskilde, Demark). ELx 50 ELISA washer was purchased from Bio-Tek instruments (Winooski, VT, USA). Vmax automatic ELISA reader was purchased from Molecular Devices Co. Colloidal gold (40 nm in diameter) was obtained from BBInternational (Cardiff, United Kingdom). A Easypack Developer's Kit consisted of three pads (sample, conjugate release and absorbent pads) and one nitrocellulose membrane plate with membrane pore size 5

μM and the Easy Printer (model LPM-02) for drawing the test line and control line of membrane were purchased from MDI Membrane Technologies (Ambala, India). All other chemicals and organic solvents used were of reagent grade or better.

2.2 Preparation of various aflatoxin B1 conjugates

Preparation of Aflatoxin B1-CMO

The method used by Chu, Hsia, and Sun (1977) for preparing aflatoxin-CMO was modified to allow for conjugating to carrier protein or enzyme. A typical experiment began with 10 mg aflatoxin B1 plus 15 mg carboxymethoxylamine hemihydrochlortide (CMO), which was dissolved in 6 ml reflux solution (1.0 ml pyridine, 4.0 ml methanol, 1.0 ml water) in a round bottom flask. After the reaction mixture was gently refluxed for 2.5 hr with continuous magnetic stirring, the flask was wrapped in aluminum foil and kept at room temperature overnight. A rotary evaporator was used to concentrate the reaction mixture to about 1 ml, then the remaining 1 ml mixture and aflatoxin B1 standard were spotted onto TLC plate. TLC plate were developed in chloroform:methanol (9:1) plus 1.5% acetic acid, and observed under UV light at 365 nm long wavelength. The derivative was then removed from the TLC plates and re-dissolved in chloroform, which was centrifuged for 5 min to remove silica gel residues. The aflatoxin B1-CMO was air dried for conjugation.

Conjugate of aflatoxin B1 to BSA, OVA and HRP for ELISA

Aflatoxin B1-CMO was conjugate to OVA and HRP by adding water-soluble carbodiimide (EDC) and NHS under the following conditions. Freshly prepared EDC (1.0 mg of EDC in 0.01 ml of DMF) and NHS (0.8 mg of NHS in 0.01 ml DMF) solutions were prepared and added to an aflatoxin B1-CMO solution (0.5 mg aflatoxin B1-CMO in 0.13 ml of DMF).The reaction was kept at room temperature for 2 hr with continuous magnetic stirring. Than, the mixture was divided into two vials each contained 75 μl of reaction mixture. The BSA or OVA (1.5 mg of BSA or OVA in 1.0 ml of 0.1 M $NaHCO_3$, pH 8.3) and HRP (1.5 mg of HRP in 1.0 ml of 0.1 M $NaHCO_3$, pH 8.3) solutions were prepared and dividedly added to two vials dropwise, and kept at room temperature for another 2 hr with continuous magnetic stirring. After the reaction, the above two reaction mixtures were dialyzed against 2 liter of 0.01M phosphate buffer containing 0.15 M NaCl [phosphate-buffered saline (PBS), pH 7.5] for 72 hr with two exchanges of buffer. After the dialysis, the mixture was collected for the ELISA analysis.

2.3. Production of polyclonal antibody

The schedule and methods of immunization were the same as those described by Yu, Chi, Liu, and Su (2005). The rabbit was injected intradermally at multiple sites on the shaved back (about 30 sites) with 500 μg of immunogen (aflatoxin B1-BSA conjugate) in 1 ml of 0.01M PBS mixed with 1 ml of Freund Complete adjuvant. For booster injections, the same amount of immunogen was mixed with an equal volume of incomplete Freund adjuvant and injected subcutaneously at 4 sites on the thigh of the rabbit. The antisera were precipitated with $(NH_4)_2SO_4$ to a final concentration of 50% and 35% in sequence by using a 100% saturated $(NH_4)_2SO_4$ solution. The precipitate was redissolved in distilled water equal to half of the original volume and than dialyzed against 2 l of PBS for 72 hr at 4 ^{o}C with two changes of buffer. Final added 0.01 M PBS to the original volume.

2.4. Characterization of polyclonal antibodies

ciELISA

A ciELISA was used to characterize anti-aflatoxin B1 antiserum. Briefly, each well of a microtiter plate (plate 269620; Nunc) was coated with 0.1 ml of aflatoxin B1-OVA (1：10,000 dilution in 0.01 M PBS) and kept at 37°C 1 hr or at 4 ^{o}C overnight. After the plate has been washed with PBS-Tween (0.35 ml per well; 0.05% Tween 20 in 0.01 M PBS) using an automated ELISA washer (Elx 50, Bio-Tek), blocking the plate by added 0.17 ml of gelatin-PBS (0.17 ml per well; 0.1 % gelatin in 0.01 M PBS) and incubated at 37 ^{o}C for 30 min. The plate was washed as described above, and 0.05 ml of aflatoxin B1, B2, G1 and G2 standard with concentrations from 0.01 ng/ml to 10 ng/ml or samples were added to each well, and then, the anti-aflatoxin B1 antiserum (1:5000 dilution in 0.01 M PBS; 0.05 ml per well) was added to all wells and incubated at 37°C for 1 hr. After incubation, the plate was washed again, and 0.1 ml of goat anti-rabbit IgG-HRP conjugate (1:20000 dilution) was added and kept at 37°C for 50 min. The plate was washed four times with PBS-Tween again, and 0.1 ml of TMB substrate solution was added. After about 10 min kept at room temperature, 0.1 ml of 1 N hydrochloric acid was to stop the reaction. Absorbance at 450 nm was

determined in a Vmax automatic ELISA reader.

Rapid cdELISA

The anti-aflatoxin B1 antibody was diluted in 0.01 M PBS (1:10000, 1 μg/ml), and 0.1 ml of the diluted form was coated onto each well. After the plate had incubated at 37°C for 1 hr or at 4°C overnight, it was washed four times with PBS-Tween followed by blocking with BSA-PBS (0.17 per well; 0.1 % BSA in 0.01 M PBS) at 37 °C for 20 min. The plate was washed again and 0.05 ml of aflatoxin B1, B2, G1, and G2 standards with concentrations from 0.01 ng/ml to 10 ng/ml or samples were added to each well, and than, the aflatoxin B1-HRP conjugate (1:10,000 dilution in 0.01 M PBS; 0.05 ml per well) was added to all wells and incubated at 37°C for 15 min. The plate was washed four times with PBS-Tween, and 0.1 ml of TMB substrate solution added. After kept at room temperature in the dark for 10 min, the reaction was terminated by adding 0.1 ml of 1 N HCl. The absorbance at 450 nm was determined in the Vmax automatic ELISA reader.

2.5. cdELISA of food and feed samples contaminated with aflatoxins

Thirty six different brand names of food and feed samples including coffee, red yeast rice and corn based feed purchased from local stores were subjected to determine their AFB1 levels. Briefly, each grinding sample (10 g) was shaken with 100 ml of extract solvent (methanol/water, 80/20, v/v) for 10 min. After centrifugation at 14000 x g for 5 min, the supernatant solution was aspirated was passed through a 0.45 μm syringe filter and 1 ml of clear extract, diluted with 9 ml of 0.01 M PBS was directly subjected to cdELISA. For on-site immunochromatographic strip assay, the 0.1 ml of clear middle solution was aspirated and diluted with 0.2 ml of PBS for the test.

2.6. Preparation of antibody-gold nanoparticle probe

The 5 μl of anti-aflatoxin B1 antibody (1 mg/ml) was dissolved in 1 ml of boric acid-borax buffer (pH 8.0), and this antibody solution was added dropwise to 2 ml of the gold nanoparticle (40 nm in diameter) solution with gentle stirring. The mixture was reacted for 1 hr at room temperature and blocked with 400 μl filtered BSA for 30 min. After the reaction, the mixture was centrifuged at 14,000 rpm for 30 min at 4°C, and then the supernatant was discarded, and the gold pellets were resuspended by adding 200 μl of 20 mM Tris-buffer saline (pH 8.0) with 1 % BSA and 0.1 % sodium azide. These anti-aflatoxin B1 antibody-colloidal gold probes were stored at 4°C until use.

2.7. Preparation of immunochromatographic strip for AFB1

An immunochromatographic strip consisted of three pads (sample, conjugate release, and absorbent pads) and one nitrocellulose membrane with test and control zones. The test and control zones of the nitrocellulose membrane were drawn with 0.25 μl of AFB1-OVA (0.1 mg/ml) conjugate and 0.5 μl of goat anti-rabbit IgG antibody (0.1 mg/ml), respectively. The treated nitrocellulose membrane with a plastic backing plate was dried for 10 min at the room temperature. The AFB1 antibody-gold nanoparticle probe (3 μl/strip) was added to an untreated glass-fiber membrane to be used as a conjugate release pad. The conjugate release pad was air-dried for 5 min at 37°C. The release pad was pasted on the plate by over-crossing 4 mm with the NC membrane. The sample pad was also pasted on it by over-crossing 6 mm with release pad. The absorbent pad was passed on the other side of the plate. The whole assembled plate was cut lengthways with an automatic cutter and divided into strips (4 mm x 75 mm) by cutting machine . These assembled strips were kept in 4°C refrigerator in a sealed plastic bag until use.

2.8. Assay of aflatoxin B1 in food and feed samples by Immunochromatographic strip

The assay was carried out by adding sample solution, and the certificated standard aflatoxin B1 solution by serial dilution (0-10 ng/ml) into microplate wells. Subsequently, the immunochromatographic strips were dipped into the wells vertically. In order to characterize and define the cutoff level for each selected concentration, more than n=5 measurements were tested in our strip detection system. The sample solution or standard aflatoxin B1 solution (0.3 ml) were applied to the sample pad and migrated up the membrane. The strip test was allowed to develop color for 10 min, and the test results were determined visually.

三. 結果(Results)

3.1 Production and characterization of antibodies.

Antibody titers were determined using an indirect ELISA with AFB1-OVA coated onto ELISA plates. Antibodies against AFB1 were initially detected in the sera of rabbits after the 6th week of the first immunization. The antibody titers increased gradually over time, and the highest antibody titer was observed in the sera of rabbits 15 weeks after two subsequent immunizations. Additionally, the specificity of the antibodies was determined using both the competitive indirect and direct ELISA methods. According to Fig. 1A, the antibodies displayed a higher affinity for AFB1, Moreover, according to this figure, the concentrations causing 50% inhibition (IC_{50}) of binding of AFB1-HRP with the antibodies by AFB1, AFB2, AFG1, and AFG2 were 0.15, 0.55, 0.32 and 3.83 ng/ml , respectively. Based on the IC_{50} values, the relative cross-reactivity of antibodies to AFB1, AFB2, AFG1 and AFG2 was determined as 100, 28, 49, and 4, respectively. A similar pattern of cross reactivity was obtained in the ciELSA, in which AFM1-OVA were coated onto the wells of the ELISA plate to function as the solid-phase antigen. Finally, the IC_{50} values of binding of antibodies to AFB1-OVA by free AFB1, AFB2, AFG1 and AFG2 were obtained to be 0.30, 1.32, 0.78 and 5.68 ng/ml, respectively (Fig. 1B).

3.2. Analysis of AFB1 in food and feed samples with cdELISA

The efficacy of cdELISA for monitoring AFB1 in food and feed products was evaluated by collecting 36 brand names of food and feed samples from local stores and then subjecting them to cdELISA. Table 1 summarizes the cdELISA results, indicating that 20 of the 36 examined samples were AFB1 positive with levels ranging from 1.3~234 ng/g. Of those, sample 3 contained with the highest toxin level at 234 ng/g. Additionally, sample 9 had the lowest toxin level at 1.3 ng/g. Additionally, sample 1, 2, 3, 4, 18, and 19 were contaminated with a toxin level ranging from 65.0~234 ng/g, which exceeded the US-FDA (20 ng/g) and Taiwan-FDA (15 ng/g) regulatory limits of AFB1 in food.

3.3. Construction of the immunochromatographic strip method

A one-step immunochromatographic strip method for on-site detection of AFB1 in food and feed samples was constructed using antibodies specific to AFB1. This method assumes that aflatoxins in the sample compete with AFB1-OVA conjugate for binding with the colored antibody-gold nanoparticles. A situation in which the aflatoxins concentration in the sample exceeds a specified amount implies that the toxin occupies all binding sites of antibody-gold conjugates in the “conjugated pad” area. Consequently, in terms of the test zone, no free antibody-gold conjugate is available for binding with AFB1-OVA. Therefore, the red line on the test zone is absent, indicating a positive result. Next, in this study whether the assay performed properly is verified by constructing a control zone with a goat-anti-rabbit secondary antibody. Under accurate operations, the control zone should always display a red color line, regardless of the presence or absence of aflatoxins. Therefore, an aflatoxin-free sample should display two red lines on the membrane, whereas a toxin positive sample should have only one red line (Fig. 2.)

3.4. Detection limit of immunochromatographic strip method for AFB1

Various concentrations of the AFB1 analytical standard (0-10 ng/ml) were applied on immunochromatographic strips, in which AFB1-OVA was absorbed on the test line. The cutoff level of AFB1 for our strip was characterized and defined by measuring each toxin concentration at least five times. According to Fig. 3, a positive sample with a concentration exceeding 2 ng/ml fails to produce a visual red line in the test zone. This finding suggests that AFB1 at a concentration as low as 2 ng/ml is sufficient to occupy all antibody-gold conjugates and prevent the conjugates from binding with AFB1-OVA in the test zone. Conversely, a sample containing AFB1 less than 1.0 ng/ml forms a visible red line on the test zone, implying that the detection limit of strip is approximately 1~2 ng/ml. The control line always has a red line regardless of the presence of aflatoxins in samples, thus confirming the development of a correct test strip.

3.5. Analysis of AFB1 in food and feed samples with immunochromatographic strip assay method

The immunochromatographic strip assay performed in this was completed within 10 min. AFB1 contamination in the food and feed samples examined by cdELISA were analyzed by applying strips. According to Fig. 4, samples 1, 2, 3, 4 and 18, which displayed AFB1 levels ranging from 7.24 ng/ml (72.4 ng/g) to 23.4 ng/ml (234 ng/g) in cdELISA (Table 1), yielded a positive result, with only one red line on the control zone of the immunostrip. Sample 19, which contained 6.5 ng/ml (65 ng/g) of AFB1, displayed a weak red line in the test zone and a clear red line in the control zone, indicating positive/negative symbols. (Table 1), Above analytical results of food and feed samples in Fig. 4 are expressed as positive/negative

symbols in Table 1 for comparison with cdELISA.

四、計畫期中成果自評

本研究主要目的是將建立融合瘤細胞株生產黃麴毒素 B1 與微囊藻毒的單株抗體，目前有關黃麴毒素的抗體已經產生，並且利用此一抗體建立了敏感性高的直接競爭型酵素免疫分析法及以奈米金粒子為標記物的快速免疫層析試紙分析法，此一研究成果已經發表於 2013 年 Food Control 30:184-189。此外對於微囊藻毒融合瘤篩選方面，進行的並不算順利。Balc/c 老鼠品質不穩定，老鼠血液中無法得到高專一性抗體以進行融合瘤篩選。本計畫原計畫為三年期計畫被刪減成二年期計畫，因此第三年螢光極化免疫分析法無法順利執行。

五、參考文獻

Anfossi, L., Darco, G., Calderara, M., Baggiani, C., Giovannoli, C., & Giraudi, G. (2011). Development of a quantitative lateral flow immunoassay for the detection of aflatoxins in maize. *Food Additive and Contamination Part A.***,** 28, 226-234.

AOAC Official Method 999.07. (2000). Aflatoxin B1 and total aflatoxins in peanut butter, pistachio paste, fig paste, and paprika powder-immunoaffinity column LC with post-column derivatization. *AOAC Int. Official Methods of Analysis* (17th Ed.) Chapter 49 (Supplement). Gaithersburg, MD.

Castells, M., Marin, S., Scanchis, V., & Ramos, A. J. (2008). Distribution of fumonisins and aflatoxins in corn fractions during industrial cornflake processing. *International Journal of Food Microbiology,* 123, 81–87.

Chu, F. S., Hsia, M. T. S., & Sun, P. (1977). Preparation and characterization of aflatoxin B1-O-carboxymethyloxime. *Journal of AOAC (Association of Official Analytical Chemists),* 60, 791-794.

Commission Regulation (EC) (2004). No 683/2004 of 13 April 2004 as regards aflatoxins and ochratoxin A in foods for infants and young children. *Official Journal of European Union, L106,* 3-5.

Creppy, E. E. (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters.* 127, 19-28.

Delmulle, B. S., De Saeger, S. M., Sibanda, L., Barna-Vetro, I., & Van Peteghem, C. H. (2005). Development of an immunoassay-based lateral flow dipstick for the rapid detection of aflatoxin B1 in pig feed. *Journal of Agricultural and Food Chemistry***,** 53, 3364-3368.

IARC (2002). Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, IARC monographs on the evaluation of carcinogenic Risks to Humans. *International Agency for Research on Cancer,*82, 171-300.

Khayoon, W. S., Saad, B., Yan, C. B., Hashim, N. H., Ali, A. S. M., & Salleh, M. I., S. B.(2010). Determination of aflatoxins in animal feeds by HPLC with multifunctional column clean-up. *Food Chemistry,* 118, 882-886.

Kolosova, A. Y., Shim, W. B., Yang, Z. Y., Eremin, S. A., & Chung, D. H. (2006). Direct competitive ELISA based on a monoclonal antibody for detection of aflatoxin B1. Stabilization of ELISA kit components and application to grain samples. *Analytical and Bioanaytical Chemistry,* 384, 286-294.

Lattanzio, V. M. T., Solfrizzo, M., Powers, S., & Visconti, A. (2007). Simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in maize by liquid romatography/tandem mass spectrometry after multitoxin immunaffinty clean up. *Rapid Communications in Mass Spectrometry*, 21, 3253–3261.t

Lee, N. A., Wang, S., Allan, R. D., & Kennedy, I. R. (2004). A rapid aflatoxin B1 ELISA: development and

validation with reduced matrix effects for peanuts, corn, pistachio, and Soybeans. *Journal of Agricultural and Food Chemistry*, 52, 2746-2755.

Li, F., Yoshizawa, T., Kawamura, O., Luo, X., & Li, Y. (2001). Aflatoxins and fumonisins in corn from the high-incidence area for human hepatocellular carcinoma in Guangxi, China. *Journal of Agricultural and Food Chemistry*, 49, 4122–4126.

Liu, B. H., Tsao, Z. J., Wang, J. J., & Yu, F. Y. (2008). Development of a monoclonal antibody against ochratoxin A and its application in enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip. *Analytical Chemistry,* 80, 7029–7035

Nguyen, M., Tozovanu, M., Tran, T., & Pfohl-Leszkowicz, A. (2007). Occurrence of aflatoxin B1, citrinin and ochratoxin A in rice in five provinces of the central region of Vietnam. *Food Chemistry*, 105, 42-48.

Paek S. H,, Lee, S. H., Cho, J. H., & Kim, Y. S.(2000). Development of rapid one-step immunochromatographic assay. *Methods*, 22, 53-60.

Rustom, I. Y. S.(1997). Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. *Food Chemistry,* 59, 57-67.

Shiu, C. M., Wang, J. J., Yu, F. Y. (2010). Sensitive enzyme-linked immunosorbent assay and rapid one-step immunochromatographic strip for fumonisin B1 in grain based food and feed samples. *Journal of Science of Food and Agriculture* 90, 1020-1026.

Tudorache, M. & Bala, C. (2008) Sensitive aflatoxin B1 determination using a magnetic particles-based enzyme-linked immunosorbent assay. *Sensors*, 8, 7571-7580

Wang, J. J., Liu, B. H., Hsu, Y. T., & Yu, F. Y. (2011). Sensitive enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip for detecting aflatoxin M1 in Milk. *Food Control,* 22, 954-959.

Wild, C. P., Gong, Y. Y. (2010) Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis.* 31,71-82.

Wood, G. E. (1992). Mycotoxins in foods and feeds in the United States. *Journal of Animal Science*, 70, 3941-3949.

Wu, H. C., Wang, Q., Yang, H. I., Ahsan, H., Tsai, W. Y., Wang, L.Y., Chen, S. Y., Chen, C. J., & SAntella, R. M. (2009) Aflatoxin B1 exposure, hepatitis B virus infection, and hepatocellular carcinoma in Taiwan. *Cancer Epidemiology& Biomarkers Prevention,* 18, 846-853.

Xiulan, S., Xiaolian, Z., Jian, T., Zhou, J., & Chu, F. S.(2005) Preparation of gold-labeled antibody probe and its use in immunochromatography assay for detection of aflatoxin B1. *International Journal of Food Microbiology*, 99, 185-194.

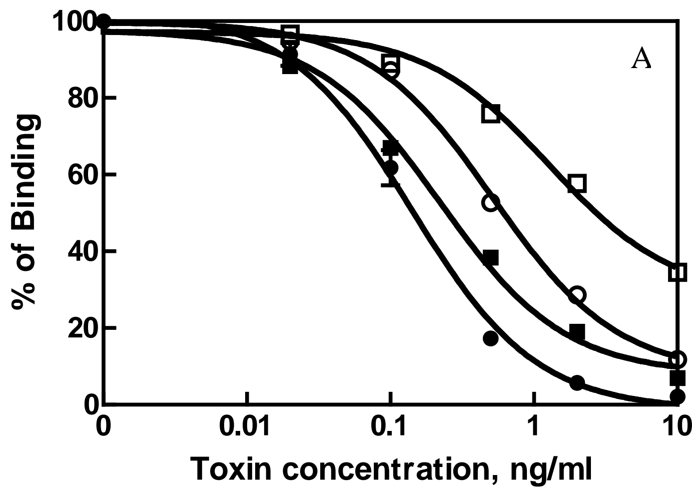
Yu, F. Y., Chi, T. F., Liu, B. H., & Su, C. C. (2005). Development of a sensitive enzyme-linked immunosorbent assay for the determination of ochratoxin A. *Journal of Agricultural and Food Chemistry* 53, 6947-953.

Table 1. ELISA and Immunochromatographic Strip Analysis of AFB1 in Food and Feed Samples

Samples	ELISA (ng/ml)	ELISA(ng/g) [a]	Immunochromatographic strip assay[a]
1.	9.21	92.1	+
2.	11.20	112.0	+
3.	23.40	234.0	+
4	8.44	84.4	+
5	0.17	1.7	–
6	0.42	4.2	–
7	0.82	8.2	–
8	0.29	2.9	–
9	0.13	1.3	–
10	0.62	6.2	–
11	0.56	5.6	–
12.	0.71	7.1	–
13	0.97	9.7	–
14	0.96	9.6	–
15	0.75	7.5	–
16	0.39	3.9	–
17	0.67	6.7	–
18	7.24	72.4	+
19	6.50	65.0	±
20	0.93	9.3	–

[a] Samples were duplicate and each sample extract was analyzed in triplicate.

[b] 1 ml of aliquot of extract solution contained 0.1 g of sample.



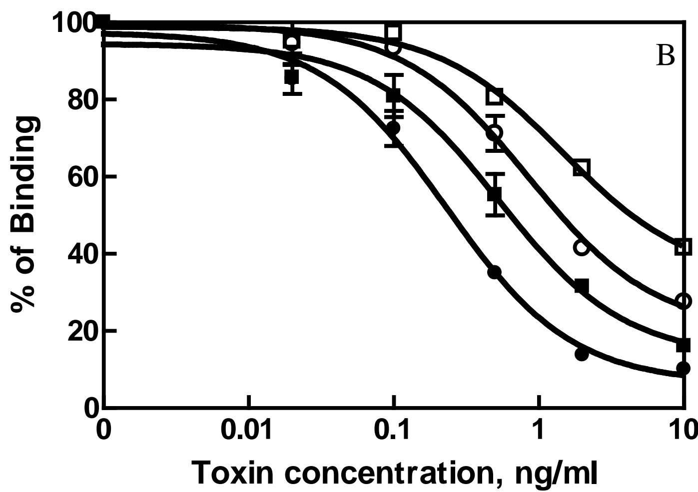


Figure 1. (A). Cross-reactivity of anti-aflatoxin B1 antibodies with aflatoxin B1 (●) , aflatoxin B2 (○) aflatoxin G1(■) and aflatoxin G2 (□) in a competitive direct ELISA (cdELISA). All data were obtained based on the average of three sets of experiments. The absorbance of the control, A0, with no toxin present, was 2.4. **(B).** Cross-reactivity of aflatoxin B1 antibodies with aflatoxin B1 (●) , aflatoxin B2 (○) aflatoxin G1(■) and aflatoxin G2 (□) as determined by a competitive indirect ELISA (ciELISA). All data were obtained based on the average of three sets of experiments. The absorbance of the control, A0, with no toxin present, was 2.0.

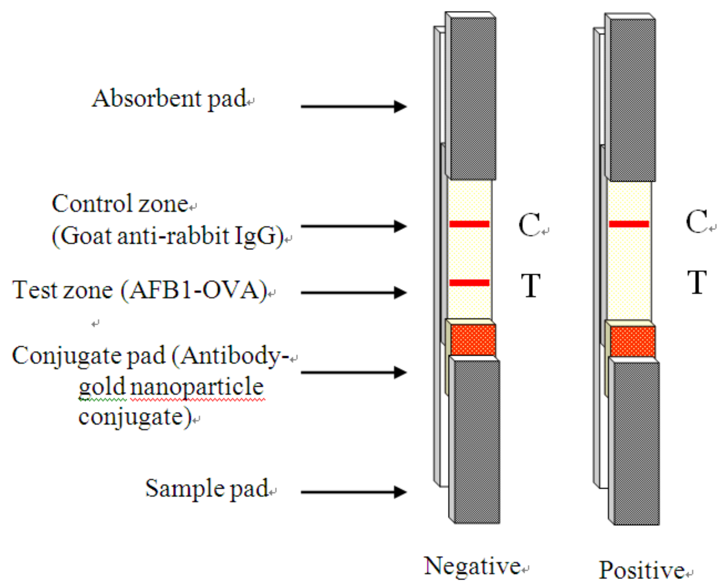


Figure 2. Schematic illustration of immunochromatographic strip. C, control zone (Goat anti-rabbit IgG); T, test zone (AFB1-OVA).

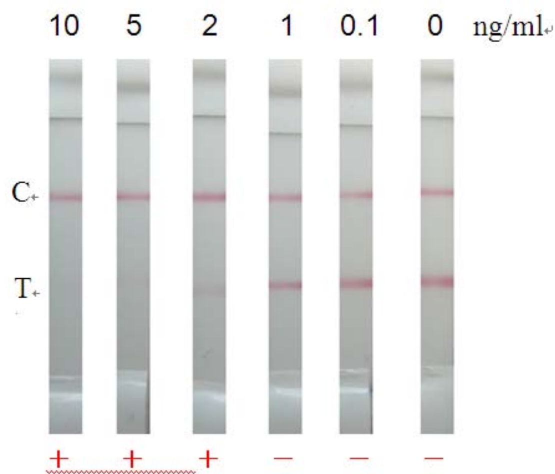


Figure 3. Detection limit of aflatoxin B1 with immunochromatographic strip. A series of dilution (0-10 ng/ml) of certificated standard AFB1 was dissolved in PBS. A concentration higher than 2.0 ng/ml of AFB1 led to the disappearance of a red line at the test zone.

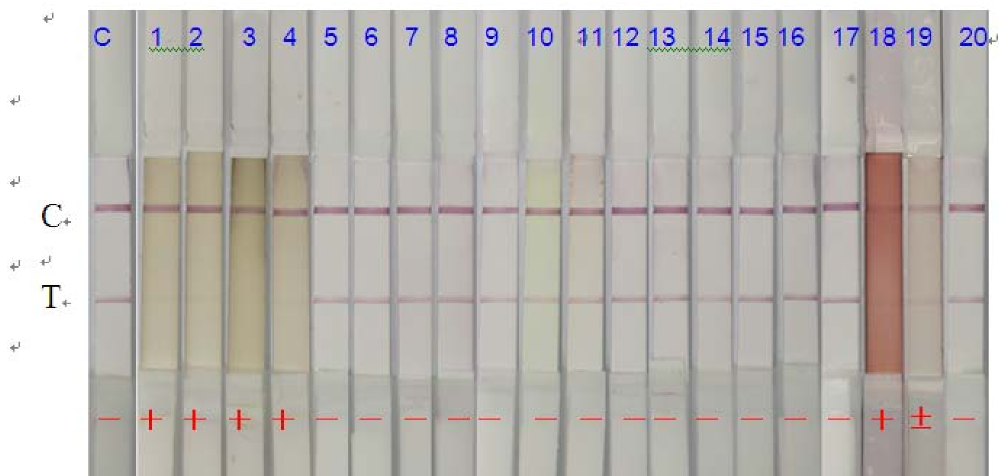


Figure 4. Detection of AFB1 with immunochromatographic strip in control and 20 contaminated samples. A control strip containing PBS indicates that two red line on the membrane, verifying that it is AFB1 negative. Sample 1, 2, 3, 4, and 18 containing AFB1 higher than 2.0 ng/ml showed only one red line on the membrane indicating that they are AFB1 positive.

行政院國家科學委員會補助國內專家學者出席國際學術會議報告

101 年 5 月 10 日

報告人姓名	余豐益	服務機關及職稱	中山醫學大學生物醫學科學系教授
會議 時間 地點	101 年 3 月 11~15 美國舊金山	本會核定補助文號	NSC-99-2313-B-040-002-MY2
會議名稱	(中文) 第 51 屆毒理學會年會 (英文) 51th Society of Toxicology Annual Meeting		
發表論文題目	(中文) 軟海綿酸單株抗體之製備並將其應用於酵素連結免疫分析法及奈米金粒子免疫層析試紙分析法之開發 (英文) Development of a monoclonal antibody against okadaic Acid and its application in enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip		

一、參加會議經過

第 51 屆毒理學會年(Society of Toxicology, SOT)會於 2011 年 3 月 11 至 3 月 15 日於美國舊金山舉行，會議舉行地點位於舊金山 downtown 的 Moscone 展覽與會議中心進行，筆者於 3 月 8 日先行搭機抵達舊金山，前兩日適逢週末便參觀了舊金山市區與加州大學-柏克萊分校與史丹福大學。會議於 3 月 11 日開始報到，筆者於當日前往會場報到並繳交所需註冊費，展開五天的年會議程。由於展覽與會議中心於市區內，交通相當方便，但是也因為位於大城市中，各項消費均不便宜。今年的議程與往年相似，主要分為 keynote 演講，口頭報告與海報發表兩部份，還有展覽場各廠商展覽各種新式儀器。本年主題 microRNAs 為一主要方向，包括 Role of microRNAs in control gene expression in human physiology and pathology 藉由口頭報告可使與會人士對主講者的相關研究得到一個有系統的介紹新知識，而透過海報發表可以藉由面對面的提問與討論方式增加意見交流。筆者此次參與是: Food Safety and Nutrition I 於 3 月 14 日早上舉行，藉由海報與其他學者交流，此次本分項大約有 50 篇壁報展示。本次會議總計有約有 30 個不同領域分散於各個個會場同時舉行，其中包含數百場的口頭報告及數千篇的海報發表。

二、與會心得

本次會議總計有個不同領域分散於展覽與會議中心各個會場同時舉行，由於不同領域口頭報告場次重疊，所以無法一一參與，以下僅就部份場次略作心得與討論。在 30 個領域中，以 Food Safety and Nutrition 與 Nanotoxicology 與筆者研究較為相近；因此在會議期間主要是參與這兩個領域的的一些演講。藉由聽講過程中不僅獲得許多新穎的觀念與技術，相信對於研究上能提供一些創新想法，對於往後實驗技術與方法能更上一層樓。

由於毒理學會年會所涵蓋之範圍相當廣,除了一般壁報之展示與相當多精采之演講，各領域專家學者無不將其研究之精華，於會中和大家分享，使個人瞭解現階段各種毒理學的研究方向及一些成果與討論，由不同領域之專家學者從不同角度思考問題，更能激發各種可能性，對於研究方面能有不同的想法。從這次毒理學會所舉辦的年會，可以了解目前 microRNAs 為一主流方向,利用 microRNAs 可以 as biomarkers for organ damage 或 epigenetic and miRNA regulation of carcinogenesis 等方向，目前一直以來都是各國研究者所注重視的方向，也視為一種新技術觀摩的會議，會議中所發表的論文堪稱最前瞻的研究成果，因此頗具參考價值。

三、建議

由於此次出國與會是筆者第一次參加毒理學(SOT)會的年會，在壁報現場發現大多數參與者皆是大陸來美就讀之博士生獲博士後研究，基本上已經沒有台灣來美就讀之博士生，顯示台灣留美學生已經非常稀少，長遠來看對台灣學術發展是一大隱憂。總之感謝國科會的經費補助，此次與會在各方面多是新奇的經驗，覺得收穫良多，期待下次有機會再度參與此一盛會。

To: Dr. Feng-Yih Yu
From: William Slikker Jr., Scientific Program Committee Chair
Date: December 1, 2011

Congratulations! You are the designated contact person for the abstract listed below, which has been accepted for a poster presentation during the 51st Annual Meeting of the **Society of Toxicology, March 11-15, 2012 at the Moscone Convention Center in San Francisco, CA.**
Please note the following important information about your presentation:
Presenters should display posters only on the assigned date and time, which is listed below in this confirmation. Diagrams of the poster session layouts, for both morning and afternoon sessions, will be included in the Final Program. These diagrams will note the abstract final ID number followed by the poster board number. Your poster board number follows the abstract final ID number below.

ABSTRACT INFORMATION:
Abstract Number/Poster Board number: 2027 Poster Board -419
Abstract Title: Development of a Monoclonal Antibody against Okadaic Acid and Its Application in Enzyme-linked Immunosorbent Assay and Gold Nanoparticle Immunochromatographic Strip
Presenting Author: Feng-Yih Yu
Session Title: Food Safety and Nutrition I
Presentation Date & Time: March 14, 2012 from 9:00 AM to 12:30 PM
Presentation Location: Exhibit Hall

As the contact person, you are the only person who will receive this notification of abstract acceptance. If you are not the presenter, it is important to provide this information to the person who will present this scientific research. Instructions for preparing a poster presentation can be found on the SOT Web site at http://www.toxicology.org/ai/meet/am2012/present.asp.
If circumstances prevent attendance, the presenting author must arrange for the paper to be given by a substitute. Once in San Francisco, if the assigned presenter cannot attend his/her session, the presenter must leave a message in the SOT office at the convention center explaining the problem.

Please visit the SOT Annual Meeting Web site at
http://www.toxicology.org/ai/meet/am2012/sanfrancisco.asp for up-to-date information on the planned featured lectures and special events.
The poster sessions will follow the schedule listed below for the 2012 SOT Annual Meeting:
Monday Morning 9:30 AM to 12:30 PM
Monday-Wednesday Afternoon 1:00 PM to 4:30 PM
Tuesday and Wednesday Morning 9:00 AM to 12:30 PM
Thursday Morning 8:30 AM to 12:00 Noon
If you have questions, please contact Nichelle Sankey (nichelle@toxicology.org) at SOT Headquarters. We look forward to seeing you in San Francisco, CA!

Development of a Monoclonal Antibody against Okadaic Acid and Its Application in Enzyme-linked Immunosorbent Assay and Gold Nanoparticle Immunochromatographic Strip

Chun-Tse Hung, Biing-Hui Liu, Feng-Yih Yu*
Department of Biomedical Sciences, Chung Shan Medical University, Taichung, Taiwan

Abstract:

Okadaic acid (OA) is a toxin that accumulates in bivalves and causes diarrheal shellfish poisoning. A monoclonal antibody (mAb) specific to okadaic acid (OA) was produced from a stable hybridoma cell line, 6B1A3, generated by the fusion of P3/NS1/1-AG4-1myeloma cells with spleen cells isolated from a BALB/c mouse immunized with OA-γ-globulin. The 6B1A3 mAb belongs to the immunoglobulin G1 (K chain) isotype. A competitive direct enzyme-linked immunosorbent assay (cdELISA) and a competitive indirect ELISA were established for antibody characterization. The concentrations causing 50% inhibition of binding of OA-horseradish peroxidase to the antibody by OA were found to be 0.075 ng/mL in the cdELISA. A sensitive and rapid mAb-based gold nanoparticle immunochromatographic strip was also developed using this mAb. This strip has a detection limit of 5 ng/mL for OA and can be completed in 10 min. Closely examining 20 seafood samples by cdELISA revealed that 17 were slightly contaminated with OA, with a mean concentration of 0.45 ng/mL. Analysis of OA in seafood samples revealed that data obtained from immunochromatographic strip were in a good agreement with those obtained from cdELISA. The mAb-based cdELISA and immunochromatographic strip assay established in this study were sensitive and accurate for rapid screening of OA in seafood samples.

國科會補助計畫衍生研發成果推廣資料表

日期:2013/01/30

<table>
<tr><td rowspan="3">國科會補助計畫</td><td>計畫名稱: 黃麴毒素與微囊藻毒之單株抗體生產及奈米免疫檢測試紙與螢光極化免疫分析法的開發應用</td></tr>
<tr><td>計畫主持人: 余豐益</td></tr>
<tr><td>計畫編號: 99-2313-B-040-002-MY2　　　學門領域: 食品及農化</td></tr>
<tr><td colspan="2">無研發成果推廣資料</td></tr>
</table>

99年度專題研究計畫研究成果彙整表

計畫主持人：余豐益　　**計畫編號**：99-2313-B-040-002-MY2

計畫名稱：黃麴毒素與微囊藻毒之單株抗體生產及奈米免疫檢測試紙與螢光極化免疫分析法的開發應用

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	1	1	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	1	1	100%		
國外	論文著作	期刊論文	2	2	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

本計畫計畫開發黃麴毒素與微囊藻毒之單株抗體並開發奈米金免疫檢測試紙以快速檢測食品與飼料中黃麴毒素之污染. 其中黃麴毒素之奈米金免疫檢測試紙已開發完成, 此一技術具有

速度快, 不需任何儀器設備可現場檢測等優點.