行政院國家科學委員會專題研究計畫 期末報告

伏馬鐮孢毒素與軟海棉酸之單株抗體生產及奈米金快速免疫檢測試紙與電化學免疫感測器分析法的開發應用

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 101-2313-B-040-005-
執 行 期 間 ：101 年 08 月 01 日至 102 年 07 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
碩士班研究生-兼任助理人員：朱光駿

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，1 年後可公開查詢

中 華 民 國 102 年 11 月 05 日

中 文 摘 要 ： 伏馬鐮孢毒素(fumonisin B1)與軟海棉酸(okadaic acid)主要由黴菌 Fusarium 屬與
藻類 Dinophysis 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
水產食品中，食用遭受污染的食品或穀物導致人類許多疾病及癌症的生成。我們將軟
海棉酸接合 r-球蛋白(r-globulin)抗原打入老鼠體內經細胞融合後取得融合瘤細胞株
以生產軟海棉酸的單株抗體，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 okadaic acid 與抗體結合所需 okadaic acid 的濃度(IC50)為 0.77 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軟海棉酸的快速免疫層析試
紙，利用此一試紙來檢測水產食品中軟海棉酸的含量，此試紙最低限制為 5 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軟海棉酸檢測。以競爭型酵素免疫
分析法與免疫層析試紙分析 20 個水產食品中軟海棉酸的含量，結果顯示 10 個樣品遭受
到 0.20~2.06 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： 伏馬鐮孢毒素, 軟海棉酸, 酵素免疫分析, 免疫層析試

英 文 摘 要 ： Fumonisins and okadaic acid (OA) are toxins that are produced by fungi Fusarium and algal
Dinophysis. They are commonly found in foods, cereal products, and seafoods, which cause toxic
effects and cancer in human and animal.. Antibodies specific to okadaic acid were generated from
mouse immunized with OA-ㄓㄩ-globulin. 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 OA in seafood samples. In the
rapid cdELISA, OA at a concentration of 0.077 ng/ml causes 50% inhibition (IC50) of binding
OA-horseradish peroxidase to the antibodies.

Effective on-site detection capability of OA is also developed based on a rapid and sensitive antibody-gold nanoparticle immunochromatographic strip method. This strip has a detection limit of 5.0 ng/ml for OA in seafood samples. Additionally, the entire analysis is completed within 10 min. Closely examining 20 seafood samples by cdELISA reveals that 10 are contaminated with AFB1 from 0.20~2.06 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 OA in seafood feed samples.

英文關鍵詞： fumonisin B1, okadaic acid, ELISA , immunochromatographic strip

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

伏馬鐮孢毒素與軟海棉酸之單株抗體生產及奈米金快速免疫檢測試紙與電化學免疫感測器分析法的開發應用

計畫類別：■個別型計畫　□整合型計畫
計畫編號：NSC　100－2923－B－040－001－MY2
執行期間：　2011 年 8 月 1　日至 2013 年 07 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：洪駿澤, 呂權蓁

本計畫除繳交成果報告外，另含下列出國報告，共 _1_ 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 10 月 31 日

行政院國家科學委員會專題研究計畫期末報告

伏馬鐮孢毒素與軟海棉酸之單株抗體生產及奈米金快速免疫檢測試紙與電化學免疫感測器分析法的開發應用

計畫編號：NSC 101－2313－B－040－005
執行期限：101 年 8 月 1 日至 102 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:

伏馬鐮孢毒素(fumonisin B1)與軟海棉酸(okadaic acid)主要由黴菌*Fusarium*屬與藻類*Dinophysis*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、作物與水產食品中，食用遭受污染的食品或穀物導致人類許多疾病及癌症的生成。我們將軟海棉酸接合γ-球蛋白(γ-globulin)抗原打入老鼠體內經細胞融合後取得融合瘤細胞株以生產軟海棉酸的單株抗體，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okadaic acid與抗體結合所需okadaic acid 的濃度(IC50)為0.77 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軟海棉酸的快速免疫層析試紙，利用此一試紙來檢測水產食品中軟海棉酸的含量，此試紙最低限制為5 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軟海棉酸檢測。以競爭型酵素免疫分析法與免疫層析試紙分析20個水產食品中軟海棉酸的含量，結果顯示10個樣品遭受到0.20~2.06 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:

Fumonisins and okadaic acid (OA) are toxins that are produced by fungi *Fusarium* and algal *Dinophysis*. They are commonly found in foods, cereal products, and seafoods, which cause toxic effects and cancer in human and animal.. Antibodies specific to okadaic acid were generated from mouse immunized with OA-γ-globulin. 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 OA in seafood samples. In the rapid cdELISA, OA at a concentration of 0.077 ng/ml causes 50% inhibition (IC_{50}) of binding OA-horseradish peroxidase to the antibodies. Effective on-site detection capability of OA is also developed based on a rapid and sensitive antibody-gold nanoparticle immunochromatographic strip method. This strip has a detection limit of 5.0 ng/ml for OA in seafood samples. Additionally, the entire analysis is completed within 10 min. Closely examining 20 seafood samples by cdELISA reveals that 10 are contaminated with AFB1 from 0.20~2.06 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 OA in seafood feed samples.

【I】前言與目的:

伏馬鐮孢毒素(fumonisins)是一族於 1988 年由南非研究者發現的真菌毒素，主要由常見的 *Fusarium* 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食品與與穀類製品，此類真菌毒素泛存於玉米、高粱、水稻及甘蔗等經濟作物中(Gelderblom et al., 1988)。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，人類或動物食用遭受污染的食物或穀物，導致各類疾病包括癌症之發生，所以導致農業經濟上嚴重的損失。目前已有超過 10 種伏馬鐮孢毒素類似物被發現，主要的伏馬鐮孢毒素分 FmB1、B2 和 B3 三大種類，其中以伏馬鐮孢毒素 B1（Fumonisins B1; FmB1）是伏馬鐮孢毒素中最常見的一種毒素，而且最具毒性與致癌性，而且 FmB1 結構非常穩定，不會因為加熱或是食品加工而失去毒性，FmB1 已經被證實是一種癌症的促進物(cancer promoter)，食用遭受此類毒素污染的食品及飼料會導致人類及動物體的許多疾病包括人類的食道癌(esophageal cancer)，豬的肺水腫(pulmonary edema in swine)、馬的腦神經白質化(equine eukoencephalomalacia)等(Gelderblom et al., 1988)。國際癌症研究協會(International Agency for Research on Cancer)將鐮孢菌(*F. moniliforme*)所產生的毒素評定為 2B 族群，也就是可能是人類致癌物(a possible human carcinogen) (IARC, 1993)，目前美國家對於伏馬鐮孢毒素在食品及飼料中分別設有 2 ppm 及 4 ppm 的含量限制(regulatory level)，瑞士對於伏馬鐮孢毒素設有 1 ppm 及的含量限制(Wild, 2010)，台灣則尚未定有含量限制。Wang et al.(2007) 調查大陸 Huaian 區域食道癌盛行與居民食用玉米遭受伏馬鐮孢毒素污染的相關情形，結果顯示 117 個樣品中有 112 個遭受伏馬鐮孢毒素的污染，平均受污染濃度高達 2. 84 ppm。台灣 Tseng et al.(1999)以 HPLC 方法針對玉米樣品中伏馬鐮孢毒素做過分析調查，結果顯示 110 個樣品中有高達 49 個樣品污染伏馬鐮孢毒素從 109~1148 ppb，由此可見玉米遭受伏馬鐮孢毒素的汙染可謂相當嚴重。

軟海綿酸 (Okadaic acid, OA) 是一種常見於貝類中的藻類毒素，是由 *Dinophysis* 和 *Prorocentrum* 這兩屬渦鞭毛藻所產生的次級代謝物，經由濾食性貝類食用而累積於體中，特別是二枚貝，像是紫貽貝與海扇貝等等。研究顯示 OA 及其經生物代謝後之衍生物 dinophysistoxins (DTXs) 為引起下痢性貝毒 (diarrhetic shellfish poisoning，DSP) 的主要毒素，在誤食的幾小時內會產生腹瀉、噁心嘔吐與腹痛等症狀並持續三到四天 (Torgersen et al.,2005)。OA 的分子式為 $C_{44}H_{68}O_{13}$，分子量為 804.5，屬於類脂溶性之長鏈聚醚毒素，對熱穩定但對光較敏感。OA 會抑制蛋白質磷酸酶 (protein phosphatases 1 and 2A) 的作用，使蛋白質磷酸化增加，研究指出 OA 主要影響的器官為腸道，會造成脂質的過氧化 (Guzman and Castro, 1991)。此外，OA 被懷疑會促進消化道腫瘤之生成。歐盟對 OA 的限制含量為 160 ng/g (ppb)，超過此含量就容易造成急性中毒 (Cordier et al.,2000)。

二、 材料與方法

2.1 Materials.

Materials Okadaic acid (OA) and dinophysistoxin-1 (Fig. 1) were purchased from Taiwan Algal Science (Tauyuan, Taiwan). An analytical standard solution of OA at 20 μg/mL was purchased from Calbiochem (San Diego, CA). Bovine serum albumin (BSA), γ-globulin, gelatin, ovalbumin (OVA), ammonium biocarbonate, Tween 20, dimethyl sulfoxide (DMSO), 1,1′-carbonyldiimidazole (CDI), 1-ethyl-3- [3-dimethylaminopropyl]- carbodimide (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti-mouse-peroxidase conjugate and keyhole limpet hemocyanin (KLH) were obtained from Pierce Chemical Co. (Rockford, IL). Horseradish peroxidase (HRP) was obtained from Roche (Mannheim, Germany). HRP substrate solution 3, 3', 5, 5'-tetramethylbenzidine (TMB) was

obtained from Neogen Corp (Lexington, KY). Ammonium sulfate, absolute ethanol and HPLC grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). 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). Vmax automatic ELISA reader was purchased from Molecular Devices Co. (Menlo Park, CA). Polyethylene glycol (PEG 1500), hypoxanthine (H), aminopterin (A), and thymidine (T) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). BM Condimed H1 Hybridoma Cloning Supplement (BMH1) was purchased from Roche Applied Science (Mannheim, Germany). Freund's incomplete adjuvant, Dulbeco Modified Eagle's Medium (DMEM), fetal calf serum, and penicillin–streptomycin were obtained from GIBCO Laboratories (Grand Island, NY). A mouse mAb isotyping kit was obtained from Roche (Mannheim, Germany). Virus-free, 9–10-week-old, female BALB/c mice were obtained from National Animal Research Center (Taipei, Taiwan). The murine myeloma cell line P3/NS–1/1–AG4–1 (NS–1) was obtained from Bioresources Collection and Research Center in Taiwan. Gold nanoparticle (20 nm and 40 nm in diameter) purchased from BBInternational (Cardiff, United Kingdom). A Easypack Developer's Kit consisted of three pads (sample, conjugate release and absorbent pads), cover tape and one nitrocellulose membrane plate with membrane pore size 5 μM and 15 μM. The Double Axes Programmable Control Printer (model P-602) for drawing the test line and control line of membrane were purchased from Troy Technology (Taichung, Taiwan). The 0.45 μm syringe filter was obtained from Gelman Science (Ann Arbor, MI). All other chemicals and organic solvents used were of reagent grade or better.

Preparation of Various OA Conjugates.

Conjugation of OA to γ-globulin. OA was conjugated to γ-globulin in the presence of EDC under the following conditions[31]. The EDC solution (1.0 mg of EDC in 0.02 mL of DMSO) and NHS solution (1.0 mg of NHS in 0.02 mL DMSO) were freshly prepared and then added to an OA solution (1.0 mg of OA in 0.2 mL of DMSO). The mixture was added slowly to 2.0 mg of γ-globulin, which was dissolved in 0.4 mL of 0.1 M carbonate buffer (pH 9.6) and kept at room temperature for 2 h. After reaction, the mixture was dialyzed against 2 L of 0.01 M phosphate buffer containing 0.15M NaCl (PBS, pH 7.5) for 72 h with two exchanges of PBS and then lyophilized for storage at –20 ^{o}C.

Conjugation of OA to OVA/KLH for Indirect competitive ELISA. OA was conjugated to OVA or KLH by the EDC and NHS method and used as a solid-phase antigen for the indirect competitive ELISA[31]. In a typical reaction, 0.5 mg of OA in 0.1 mL of DMSO was mixed with 2.5 mg of OVA or KLH first, and then 1 mg of EDC and 0.75 mg of NHS were added to the mixture with constant stirring. After the coupling reaction was carried out at 25 ^{o}C for 2h, the mixture was dialyzed against PBS for 72 h and then lyophilized for storage.

Preparation of OA-Peroxidase. Conjugation of OA to HRP was achieved by the he EDC and NHS method [26, 31]. Briefly, 0.2 mg of OA in 0.1 mL of DMSO was mixed with 0.6 mg of EDC and 0.4 mg of NHS, and then a HRP solution (0.8 mg of HRP in 0.3 mL of 0.1 M carbonate buffer, pH 9.6) was added. After being stirred at room temperature for 2 h, the mixture was dialyzed against PBS for 72 h and then lyophilized.

Production of Monoclonal Antibody (mAb).

Immunization. For generating mAbs against OA, four female BALB/c mice (9–10 weeks of age) were each immunized with 40 μg of OA-γ-globulin in PBS that had been emulsified with an equal

volume of Freund's complete adjuvant. Four weeks after the initial intraperitoneal immunization, weekly booster injections were made with the same amount of immunogen in PBS containing no adjuvant. Blood samples were collected from the tail of each mouse at weekly intervals after each booster injection. A competitive indirect ELISA (ciELISA) as described below was used to determine the antibody specificity in the serum.

Fusion and cloning.

The mouse with the highest antibody specificity (9 weeks after the initial immunization, including four booster injections) was selected for fusion reaction. Four days before fusion, the mouse was primed with a total of 50 μg of immunogen. The mouse was euthanized 3 days after the final immunization and the entire spleen was aseptically removed and mashed with a glass pestle. The spleen cells were then passed through a cell dissociation sieve-tissue grinder kit packed with mesh 80 (CD-1, Sigma) to produce a single-cell suspension, which was then mixed with 1 x 10^7 of myeloma cells. The cells was centrifuged, suspended in 0.2 mL of HT medium, and then fused by gradually adding 1 mL of PEG 1500 in 1 min into the cell pellet. HT medium was used to do slow dilution of PEG. The cell pellet was rotated for further 1 min, then added 1 mL medium over 1 min, 2 mL medium over 2 min and waited 2 min, 4 mL medium over 1 min and waited 4min. Finally, added 8 mL medium over 1 min. After centrifugation at 300 g for 5 min, the cells were pelleted again, resuspended in hypoxanthine, aminopterin, and thymidine (HAT) medium plus BMH1 medium to a final concentration of 10%, and plated into 96-well tissue culture plates. The colonies were fed every fifth day with freshly prepared HAT medium. When the colonies reached at least half-confluence in the well, hybridomas were screened for specific antibodies against OA acid using a ciELISA described later. Three hybridoma cell lines from the mouse immunized with OA-γ-globulin were obtained. Wells containing positive cells were cloned by the limiting dilution method into 96-well tissue culture plates[32].

Production of ascites fluid.

Female BALB/c mice, 10 weeks old, were injected intraperitoneally with 0.5 mL pristane 7 days before receiving an intraperitoneal injection of 2 x 10^6 hybridoma cells suspended in DMEM. Ascites fluid developed 2–3 weeks after the injection of the cells and was collected every other day for 6 days. The ascites fluid was centrifuged at 7000 rpm (5900 *g*) for 5 min to remove cell debris. The IgG from the cleared ascites fluid was purified by ammonium sulfate precipitation (50% saturation for the final solution) twice and then stored at –70°C.

Characterization of Monoclonal Antibodies

Determination of isotype.

A mouse mAb isotyping kit was used to determine the isotypes of mAb. Identification of specific immunoglobulin was carried out according to the manufacturer's protocol.

Competitive Indirect ELISA (ciELISA) A competitive indirect ELISA was used to characterize each mAb. Briefly, each well of a microtiter plate was coated with 0.1 mL of the OA-OVA and kept at 4 ^{o}C overnight. After the plate had been washed four times with Tween-PBS (0.35 mL per well; 0.05% Tween 20 in PBS) using an automated ELISA washer, 0.17 mL of gelatin-PBS (0.17 mL per well; 0.1% gelatin in PBS) was added and allowed to incubate at 37 ^{o}C for 30 min. The plate was washed again and 0.05 mL of OA standard with concentrations from 0.01 to 100 ng/mL in PBS or extracted samples were added to each well, and then the anti-OA mAb (25 ng/mL in PBS, 0.05 mL per well) was added to all wells and incubated at 37 °C for 50 min. After incubation, the plate was washed

four times with Tween-PBS, and 0.1 mL of goat anti-mouse IgG-HRP conjugate (1:20000 dilution) was added and incubated at 37°C for 45 min. The plate was washed four times with Tween-PBS, and 0.1 mL of TMB substrate solution was added. After 10 min of incubation at room temperature, 0.1 mL of 1 N hydrochloric acid was added to stop the reaction. Absorbance at 450 nm was determined in a Vmax automatic ELISA reader.

cdELISA. The anti-OA mAb from ascites was diluted in PBS (1:10000 dilution; 1 μg/mL) and 0.1 mL of the diluted solution was used to coat each well. After the plate had been incubated at 4 ^{o}C overnight, it was washed with Tween-PBS followed by blocking with BSA-PBS at 37 ^{o}C for 30 min. The plate was washed again with Tween-PBS four times, and then OA standard (0.05 mL per well in PBS) concentrations from 0.01 to 100 ng/mL or samples together with the OA-HRP conjugate (10 ng/mL, in PBS, 0.05 mL per well) was added and incubated at 37^{o}C for 50 min. The plate was washed four times with PBS-Tween, and 0.1 mL of TMB substrate solution was added. After incubation 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.

cdELISA of Shellfish Samples Contaminated with OA.

Twenty shellfish samples, including clam, scallop, mussel and oyster, purchased from local food stores were used to determine the OA levels. Briefly, each sample (5 g) was homogenized with 20 mL of extraction solvent (methanol/water, 80/20, v/v) for 5 min [24, 33]. After centrifugation at 10000 rpm (14000 g) for 10 min, 1 mL of supernatant was added with 1 mL of hexane by vortex mixing for 1 min and then 3 mL of chloroform was added by vortex for 2 min. After centrifugation for 5 min to separate the layers, the lower chloroform layer was aspirated and dried under rotary vacuum evaporator. The dried sample was reconstituted with 1 mL of PBS and directly subjected to cdELISA and for the immunochromatographic strip assay.

Preparation of Antibody-Gold Nanoparticle Probe

OA mAbs were dialyzed against boric acid-borax buffer for 24 h at 4 ^{o}C and then centrifuged at 10000 rpm (14000 g) for 10 min to get clear supernatant for conjugation. The pH of the gold nanoparticle (40 nm or 10 nm in diameter) solution was adjusted to pH 9.0 with 0.1 M K_2CO_3 (pH 11.5) for conjugation with OA mAb[27, 28]. The 5 μg of anti-OA mAb was added dropwise to the 2 mL of pH-adjusted gold nanoparticle solution with gentle stirring. The mixture was reacted for 1 h at room temperature and blocked by 10% (w/v) filtered BSA for 30 min. This mixture was centrifuged at 14000 rpm (19000 g) for 30 min at 4^{o}C, and then the supernatant solution was removed, the gold pellets were resuspended by adding 450 μL of 20 mM Tris-buffered saline (pH 8.0) with 1% BSA and 0.1% sodium azide. These OA mAb-gold nanoparticle probes were stored at 4 ^{o}C until use.

Preparation of Immunochromatographic Strip

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 drew with 0.5 μL of OA-KLH (0.8 mg/mL) conjugate and 0.5 μL of rabbit-anti-mouse IgG antibody (1 mg /mL), respectively. The treated nitrocellulose membrane was dried for 10 min at room temperature. The OA mAb-gold nanoparticle conjugate (5 μL/strip) was added to an untreated glass-fiber membrane to be used as a conjugate release pad. The conjugate pad was air dried for 5 min. 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 pasted on the top of the membrane sheet. The whole assembled sheet was cut lengthways with a automatic cutter and divided into strips (5 mm × 75 mm).

Assay of OA in Shellfish Samples with Immunochromatographic Strip

Three hundred microliters of extraction sample solutions and the different concentration of OA analytical standard solution (0-50 ng/mL) in PBS were added into per well. Subsequently, one immunochromatographic strip was dipped into per well vertically. The extracted samples or OA standard solution was migrated upward the membrane. The strip test was allowed to developed color for 10 min and test results were determined visually.

三. 結果(Results)

Monoclonal Antibody Production

Mice were injected with OA-γ-globulin and boosted for 4 times with the same antigen, the mouse with serum that had the highest affinity for OA was sacrificed for a hybridoma screening. After spleen/NS-1 cell fusion and cloning, the ciELISA with OA-OVA was used as coated reagent for screening the hybridoma cells, which produced mAbs specific to OA. In the about 500 examined wells, three clones showed strong positive signals in the ciELISA; of these, the clone 6B1 showed the highest affinity for OA. Therefore, the supernatant of the 6B1 culture was aspirated from the fusion well and subjected to limiting dilution for hybridoma selection. After limiting dilution and ELISA screening, clone 6B1A3, which showed the highest affinity for OA, was used to produce culture supernatant and ascites fluid.

Characterization of Antibody.

Determination of isotype. The mAb isotype produced by cell line 6B1A3 was identified as immunoglobulin G1, κ-light chain.

Competitive direct and indirect ELISA. The specificity of 6B1A3 mAb was measured by both cdELISA and ciELISA. As shown in Fig. 2A, the concentrations causing 50% inhibition (IC_{50}) of binding of OA-HRP with the mAb by OA and DTX-1, were found to be 0.077 and 1.870 ng/mL, respectively in cdELISA. The ciELISA revealed similar results for OA-OVA coated onto the wells of ELISA plates to serve as solid-phase antigen. The IC_{50} values for mAb binding to OA-OVA by free OA and DTX-1 were 0.58, and 2.0 ng/mL, respectively (Fig. 2B). However, other phycotoxins such as saxitoxin and domoic acid did not inhibit mAb binding to the marker antigen (OA-HRP or OA-OVA) in either ELISA system, even at concentrations as high as 10 μg/mL.

Analysis of OA in Shellfish Samples with mAb-based cdELISA.

Twenty shellfish samples were collected from local food stores and subjected to cdELISA to measure OA contamination; Table 1 presents the measurement results. The proposed detection system revealed that ten of the twenty examined samples were OA-positive. Sample 7 showed the highest OA levels (0.517 ng/mL; 2.068 ng/g). Samples 9 and 13 also had the OA levels higher than 1.0 ng/g. The OA levels in the remaining seven positive samples were lower than 1.0 ng/g. However, all the oyster samples are free of OA contamination.

Construction of Immunochromatographic Strip

The mAb was used to fabricate an immunochromatographic strip, in which the OA-KLH conjugate competes with OA in the sample solution for the antibody-gold nanoparticle label. Figure 3 is a schematic depiction of the immunochromatographic strip test format. In the absence of OA in the sample solution, the antibody-gold nanoparticle conjugate bound and trapped by the OA-KLH conjugate formed a red line. In contrast, a sufficient OA concentration in the sample solution caused the toxin to occupy the antigen binding sites on the antibody-gold nanoparticle conjugates, which prevented the limited antibody-gold nanoparticle conjugates from binding with the OA-KLH conjugate in the test zone. The absence of the red line in the test zone indicated a positive result (Fig. 3). The control zone was coated with rabbit-anti-mouse secondary antibody to confirm that the assay

had been performed properly; regardless of the presence of OA. An OA-free sample shows two red on the membrane whereas a positive sample with OA presents only one red line.

Characterizaion of OA Immunochromatographic Strip.

Various concentrations of OA standard solution (0-50 ng/mL) were subjected to immunochromatographic strip test. The assay required no more than 10 min, and the detection limit of immunochromatographic strip test for OA approached 5 ng/mL (Fig. 4). To characterize and define the cutoff level for each selected concentration, at least 5 measurements were tested by the immunochromatographic strip detection system. At an OA concentration above 5 ng/mL, all antibody-gold nanoparticle conjugates were occupied, which prevented the antibody-gold nanoparticle conjugates from binding with the OA-KLH in the test zone, However, the capture of the antibody-gold nanoparticle conjugates by the rabbit anti-mouse antibody on the membrane resulted in only one red line in the control zone.

Analysis of OA in Shellfish Samples by Immunochromatographic Strip Assay

Immunochromatographic strip was further used to analyze OA contamination in shellfish samples. Table 1 shows the analytical results. Ten contaminated shellfish samples with OA levels ranging from 0.051 ng/mL to 0.517 ng/mL according to cdELISA (Table 1) had two red lines on the immunostrip membranes, which indicated a negative outcome (Fig 5). However, in one spiked sample (S) containing 5.0 ng/mL of OA in shellfish extract, one red line in the test zone disappeared, which indicated that the sample was OA-positive (Fig 5).

四、計畫成果自評

本研究主要目的是將建立融合瘤細胞株生產伏馬鐮孢毒素(fumonisin B1)與軟海棉酸(okadaic acid)的單株抗體，原來三年計畫僅核一年執行，目前有關軟海棉酸的單株抗體已經產生，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，此一研究成果已經投稿至 Journal of Agricultural and Food Chemistry。此外對於馬鐮孢毒素融合瘤篩選方面，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一年期計畫，因此電化學免疫分析法無法順利執行。

五、參考文獻

(1) Codd, G. A.; Ward, C. J.; Bell, S. G. Cyanobacterial toxins:occurrence, mode of action, health effects and exposure routes. *Arch. Toxicol. Suppl.* **1997**, 19, 399-410.
(2) Scoging, A.; Bahl, M. Diarrhetic shellfish poisoning in the UK. *Lancet* **1998**, 352, 117.
(3) Armi, Z.; Turki, S.; Trabelsi, E.; Ceredi, A.; Riccardi, E.; Milandri, A. Occurrence of diarrhetic shellfish poisoning (DSP) toxins in clams (*Ruditapes decussatus*) from Tunis north lagoon. *Environ. Monit. Assess.* **2012**, 184, 5085-5095.
(4) Hossen, V.; Jourdan-da Silva, N.; Guillois-Becel, Y.; Marchal, J.; Krys, S. Food poisoning outbreaks linked to mussels contaminated with okadaic acid and ester dinophysistoxin-3 in France, June 2009. *Euro. Surveill.* **2011**, 16, Article 3
(5) Li, A.; Ma, J.; Cao, J.; McCarron, P. Toxins in mussels (*Mytilus galloprovincialis*) associated with diarrhetic shellfish poisoning episodes in China. *Toxicon.* **2012**, 60, 420-425.
(6) Carvalho, P. S.; Catian, R.; Moukha, S.; Matias, W. G.; Creppy, E. E. Comparative study of Domoic Acid and Okadaic Acid induced-chromosomal abnormalities in the Caco-2 cell line. *Int. J. Environ. Res. Public Health* **2006,** 3, 4-10.
(7) European Commission. Commission Regulation (EU) amending Regulation (EC) No 2074/2005 as regards recognized testing methods for detecting marine biotoxins in live bivalve molluscs.

Official Journal of the European Union **2011**, 15, 3-6.
(8) Gerssen, A.; Mulder, P. P.; McElhinney, M. A.; de Boer, J. Liquid chromatography-tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. *J. Chromatogr. A.* **2009,** 1216, 1421-1430.
(9) Louppis, A. P.; Badeka, A. V.; Katikou, P.; Paleologos, E. K.; Kontominas, M.G. Determination of okadaic acid, dinophysistoxin-1 and related esters in Greek mussels using HPLC with fluorometric detection, LC-MS/MS and mouse bioassay. *Toxicon*. **2010**, 55, 724-733.
(10) Suzuki, T.; Quilliam, M. A. LC-MS/MS analysis of diarrhetic shellfish poisoning (DSP) toxins, okadaic acid and dinophysistoxin analogues, and other lipophilic toxins. *Anal. Sci.* **2011**, 27, 571-584.
(11) Stobo, L. A.; Lacaze, J. P.; Scott, A. C.; Gallacher, S.; Smith, E. A.; Quilliam, M. A. Liquid chromatography with mass spectrometry--detection of lipophilic shellfish toxins. *J. AOAC Int.* **2005**, 88, 1371-82.
(12) Draisci, R.; Croci, L.; Giannetti, L.; Cozzi, L.; Lucentini, L.; De Medici, D.; Stacchini, A. Comparison of mouse bioassay, HPLC and enzyme immunoassay methods for determining diarrhetic shellfish poisoning toxins in mussels. *Toxicon.* **1994**, 32, 1379-1384.
(13) Hokama, Y. Recent methods for detection of seafood toxins: recent immunological methods for ciguatoxin and related polyethers. *Food Addit. Contam.* **1993**, 10, 71-82.
(14) Kreuzer, M. P.; O'Sullivan, C. K.; Guilbault, G. G. Development of an ultrasensitive immunoassay for rapid measurement of okadaic acid and its isomers. *Anal. Chem*. **1999**, 71, 4198-4202.
(15) Lamas, N. M.; Stewart, L.; Fodey, T.; Higgins, H. C.; Velasco, M. L.; Botana, L. M.; Elliott, C. T. Development of a novel immunobiosensor method for the rapid detection of okadaic acid contamination in shellfish extracts. *Anal. Bioanal. Chem.* **2007**, 389, 581-587.
(16) Lu, S. Y.; Zhou, Y.; Li, Y. S.; Lin, C.; Meng, X. M.; Yan, D. M.; Li, Z. H.; Yu, S. Y.; Liu, Z. S.; Ren, H. L., Production of monoclonal antibody and application in indirect competitive ELISA for detecting okadaic acid and dinophytoxin-1 in seafood. *Environ. Sci. Pollut. Res. Int.* **2011**, 19, 2619-2626.
(17) Lu, S.Y.; Lin, C.; Li, Y. S.; Zhou, Y.; Meng, X. M.; Yu, S. Y.; Li, Z. H.; Li, L., Ren, H. L.; Liu, Z.S. A screening lateral flow immunochromatographic assay for on-site detection of okadaic acid in shellfish products. *Anal. Biochem*. **2012**, 422, 59-65.
(18) Matsuura, S.; Hamano, Y.; Kita, H.; Takagaki, Y. Preparation of mouse monoclonal antibodies to okadaic acid and their binding activity in organic solvents. *J. Biochem*. **1993**, 114, 273-278.
(19) Mountfort, D. O.; Kennedy, G.; Garthwaite, I.; Quilliam, M.; Truman, P.; Hannah, D. J. Evaluation of the fluorometric protein phosphatase inhibition assay in the determination of okadaic acid in mussels. *Toxicon*. **1999**, 37, 909-922.
(20) Stewart, L. D.; Hess, P.; Connolly, L.; Elliott, C. T. Development and single-laboratory validation of a pseudofunctional biosensor immunoassay for the detection of the okadaic acid group of toxins. *Anal. Chem.* **2009**, 81, 10208-10214.
(21) Stewart, L. D.; Elliott, C. T.; Walker, A. D.; Curran, R. M.; Connolly, L., Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors. *Toxicon.* **2009**, 54, 491-498.
(22) Tsumuraya, T.; Fujii, I.; Hirama, M. Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. *Toxicon.* **2010**, 56, 797-803
(23) Vale, P.; Sampayo, M. A., Comparison between HPLC and a commercial immunoassay kit for detection of okadaic acid and esters in Portuguese bivalves. *Toxicon.* **1999**, 37, 1565-1577.
(24) Wang, L., Sang, Y. X., Wang, X. H. Enzyme-linked immunosorbent assay for okadaic acid: investigation of analytical conditions and sample matrix on assay performance. *J. AOAC Int.* **2011**, 94, 1531-1539.

(25) Paek, S. H.; Lee, S. H.; Cho, J. H.; Kim, Y. S. Development of rapid one-step immunochromatographic assay. *Methods* **2000**, 22, 53-60.
(26) Liu, B. H., Tsao, Z. J., Wang, J. J., Yu, F. Y. Development of a monoclonal antibody against ochratoxin A and its application in enzyme-linked immunosorbent assay and gold nanoparticle immunchromatographic strip. *Anal. Chem.* **2008**, 80, 7029-7035.
(27) Tsao, Z. J.; Liao Y. C.; Liu, B. H.; Su, C. C.; Yu, F. Y. Development of a monoclonal antibody against domoic acid and its application in enzyme-linked immunosorbent assay and colloidal gold immunostrip. *J. Agric. Food Chem.* **2007**, 55, 4921-4927.
(28) Ciminielo, P.; Dellaversano, C.; Fattorusso, E.; Forino, M.; Magno, S.; Santelia, F. Tsoukatou, M. Investigation of the toxin profile of Greek mussels Mytilus galloprovincialis by liquid chromatography-mass spectrometry. *Toxicon.* **2006**, 47, 174-181.
(29) Lee, K. J.; Mok, J. S.; Song, K. C.; Yu, H.; Lee, D. S.; Jung, J. H.; Kim, J. H. First detection and seasonal variation of lipophilic toxins okadaic acid, dinophysistoxin-1, and yessotoxin in Korean gastropods. *J. Food Prot.* **2012**, 75, 2000-2006.
(30) Vale, P.; Veloso, V.; Amorim, A. Toxin composition of a *Prorocentrum lima* strain isolated from the Portuguese coast. *Toxicon.* **2009**, 54, 145–152.
(31) Yu, F. Y.; Liu, B. H.; Chou, H. R.; Chu, F. S. Development of a sensitive ELISA for the determination of microcystins in algae. *J. Agri. Food Chem.* **2002**, 50, 4176- 4182.
(32) Oi, V. T.; Herzenberg, L. A. In *Selected Methods in Cellular Immunlogy*: Mishell, B. B.; Shiigi, S. M. Eds.; Freeman: San Francisco, CA, **1980**; pp 351-371.
(33) Kelly, S. S.; Bishop, A, G.; Carmody, E. P.; James, K. J. Isolation of dinophysistoxin-2 and the high-performance liquid chromatographic analysis of diarrhetic shellfish toxins using derivatisation with 1-bromoacetylpyrene. *J. Chromatoatog. A.* **1996**, 749, 33-40.

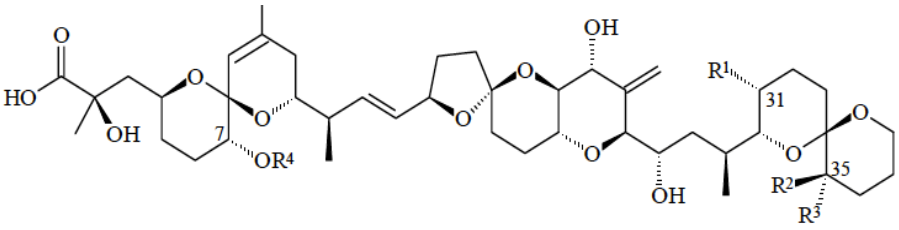
Table 1. ELISA and Immunochroatographic Strip Analysis of OA in Shellfish Samples

No.		ELISA [a] (ng/mL ± SD)	ELISA [a] (ng/g± SD) [b]	Immunochromatographic strip [a]
1.	clam	0.051± 0.008	0.204± 0.032	—
2.	clam	0.143 ±0.005	0.572±0.020	—
3.	mussel	0.106 ± 0.004	0.424 ± 0.016	—
4	clam	ND	ND	
5	oyster	ND	ND	
6	scallop	0.196 ± 0.018	0.784 ± 0.072	—
7.	clam	0.517 ± 0.004	2.068 ± 0.016	—
8.	clam	0.195 ± 0.012	0.780 ± 0.048	—
9.	clam	0.307 ± 0.012	1.228± 0.048	—
10.	oyster	ND	ND	
11	scallop	ND	ND	
12	scallop	ND	ND	
13.	clam	0.388 ± 0.005	1.552 ± 0.020	—
14 .	mussel	ND	ND	
15.	clam	ND	ND	
16.	scallop	0.216 ± 0.026	0.864 ± 0.104	—
17.	crab	ND	ND	
18.	clam	0.112 ± 0.023	0.448± 0.092	—
19.	oyster	ND	ND	
20.	oyster	ND	ND	

[a] Each sample was extracted twice and each extract was analyzed in triplicate.

[b] One mL extract solution contains 0.25 g of shellfish samples.

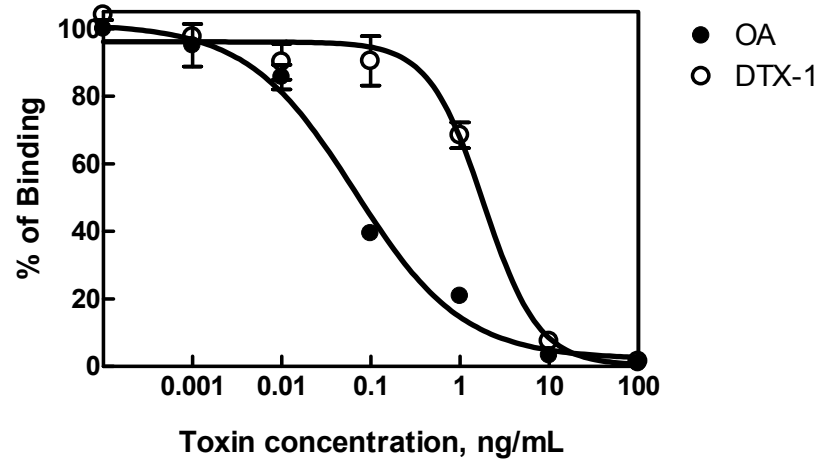
[c] ND, not detectable



	R^1	R^2	R^3	R^4
OA	CH_3	H	H	H
DTX-1	CH_3	CH_3	H	H

Figure 1. Structures of Okadaic acid (OA) and dinophysistoxin-1(DTX-1)

(A)



(B)

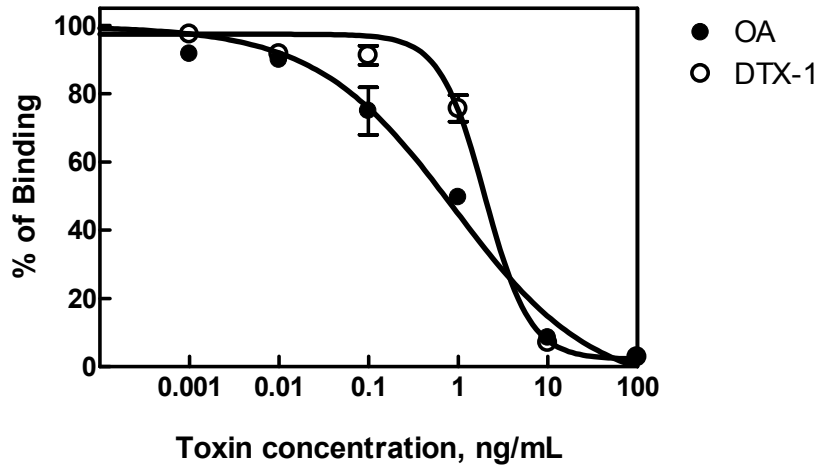


Figure 2. A. Cross-reactivity of anti-OA antibodies with OA (●) and DTX-1 (○) in a cdELISA. **B.** Cross-reactivity of anti-OTA antibodies with OA (●) and DTX-1 (○) as determined by a ciELISA. All data were obtained based on the average of three sets of experiments. The absorbance of the control, A0, with no toxin present, was 2.0.

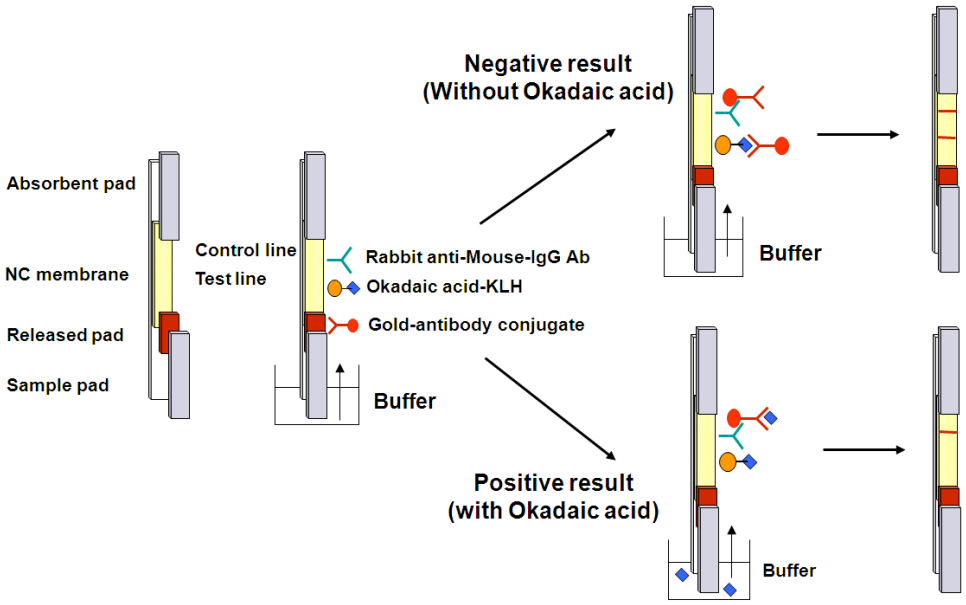


Figure 3. Schematic illustration of immunochromatographic strip. C, control zone (Rabbit anti-mouse IgG); T, test zone (OA-KLH).

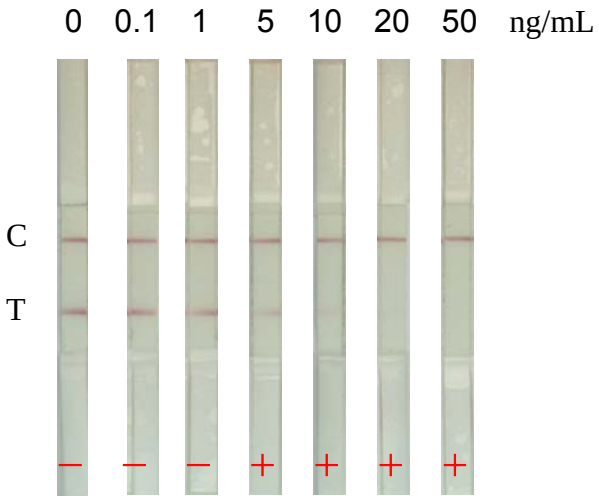


Figure 4. Detection limit of OA with immunochromatographic strip. A series of dilution (0-50 ng /mL) of certificated standard OA was dissolved in PBS. A concentration higher than 5 ng/mL of OA was found to cause a disappearance of a red line at the test zone.

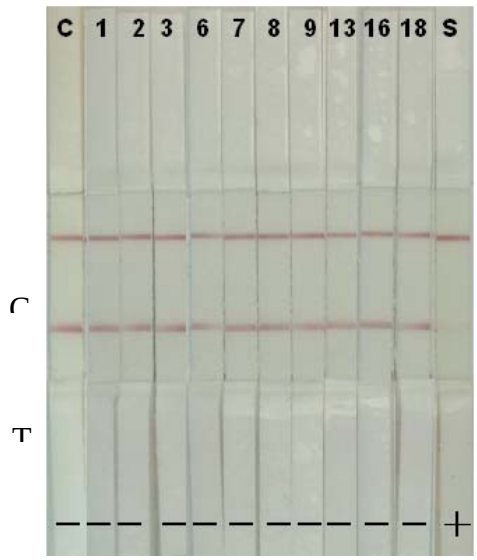


Figure 5. Detection of OA with immunochromatographic strip in control (C), spiked (S) and 10 shellfish samples. A spiked strip containing 5 ng/mL of OA in shellfish extract indicates that a red line disappeared in the test zone, verifying that it is OA positive. All samples containing OA lower than 5 ng/mL showed two red lines on the membrane indicating that they are OA negative.

國科會補助計畫衍生研發成果推廣資料表

日期:2013/10/31

國科會補助計畫	計畫名稱: 伏馬鐮孢毒素與軟海棉酸之單株抗體生產及奈米金快速免疫檢測試紙與電化學免疫感測器分析法的開發應用
	計畫主持人: 余豐益
	計畫編號: 101-2313-B-040-005- 學門領域: 食品及農化

無研發成果推廣資料

101 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：101-2313-B-040-005-
計畫名稱：伏馬鐮孢毒素與軟海棉酸之單株抗體生產及奈米金快速免疫檢測試紙與電化學免疫感測器分析法的開發應用	

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	1	1	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%	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)

已發表一篇軟海棉酸之冷光分析法於 2013 年 SCI 期刊 Talanta 116:343-346, 另一篇投稿中.

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)

本計畫生產出軟海棉酸之專一性單株抗體, 並開發其酵素免疫分析法及快速免疫檢測層析試紙.

在學術上發表 High impact factor SCI paper,技術上尚屬新穎並且具有高度產業可應用性.

本計畫可應用於海鮮食品受到軟海棉酸汙染之快速檢測.