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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藻類毒素的免疫化學研究

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計畫類別：■個別型計畫　□整合型計畫
計畫編號：NSC －90－2313－B－040－005
執行期間： 89 年 8 月 1 日至 91 年 7 月 31 日

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計畫參與人員：

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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

藻類毒素的免疫化學研究

Immunochemical studies on selected phycotoxins

計畫編號：NSC 90-2313-B-040-005

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主持人：余豐益　　中山醫學大學生命科學系

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一、中文摘要

微囊藻毒(MCYST-LR)的多株抗體由微囊藻毒與載體蛋白質接合物(MCYST-LR-γ globulin),免疫到兔子體內，接著收集純化免疫血清來得到辨識微囊藻毒的抗體，直接與非直接競爭型酵素免疫分析法(Competitive direct and indirect enzyme-linked imunosorbent assay)被用來測定抗體的特性和分析微囊藻毒在藻株培養液與樣品中的含量。此一直接競爭型酵素免疫分析法對微囊藻毒-LR 與其類似物 RR, YR, 和 nodularin 有交叉反應, 50%抑制濃度分別為 0.10, 0.12, 0.14, 和 0.20 ng/mL, 其最低偵測濃度低於 10 ppb。此一直接競爭型酵素免疫分析法在 25 to 500 ng/g 之分析回收率可達此外 83.7%，分析 11 株藻株培養液與 11 個藻類食品補充品樣品發現有六株藻株是微囊藻毒的產毒株，八個藻類食品補充品樣品含有低於 100 ppb 微囊藻毒的污染。藻株中的微囊藻毒-LR 亦經由高效液相層析法(High performance liquid chromatography)來鑑定確認。

關鍵詞：微囊藻毒、酵素免疫分析法、高效液相層析法

Abstract

Polyclonal antibodies for microcystin-leucine-arginine (MCYST-LR) were generated from rabbits after immunizing the animals with MCYST-LR conjugated with γ-globulin. A competitive direct enzyme-linked immunosorbent assay (cdELISA) and a competitive indirect ELISA (ciELISA) were used for the characterization of the antibodies and for analysis of the toxin in algal cultures and dietary supplements. The concentrations causing 50% inhibition (IC_{50}) of binding of MCYST-horseradish peroxidase (MCYST-HRP) to the solid-phase antibodies by MCYST-LR, MCYST-arginine-arginine variant (MCYST-RR), MCYST-tyrosine-arginine variant (MCYST-YR), and nodularin (NODLN) in the cdELISA were found to be 0.10, 0.12, 0.14, and 0.20 ng/mL, respectively. In the presence of algae matrix, the detection limit is less than 10 ppb. The overall analytical recovery of MCYST-LR (25 to 500 ng/g) added to the algal dietary supplements and then extracted with 0.1M ammonium bicarbonate in the cdELISA was found to be 83.7%. Analysis of MCYSTs in algal cultures and dietary supplements showed that six of eleven cultures produce MCYSTs, and five of algal cultures were not MCYST producers. Eight of eleven tested commercial algal dietary supplements contained with MCYSTs at a level lower than 100 ppb. The presence of MCYST-LR in the *Microcystis aeruginosa* culture was confirmed by high performance liquid chromatography.

Keywords: Enzyme-linked immunosorbent assay, ELISA, microcystins, antibodies, high performance liquid chromatography

二、緣由與目的

Microcystins (MCYSTs) are a group of cyclic peptide hepatotoxins produced by several freshwater cyanobacteria including

Anabaena flos-aquae, Microcystis aeruginosa and *Oscillatoria agardhii* . Contamination of cyanobacteria in water has become a growing public health problem. Drinking of water containing MCYSTs have caused the death of wild and domestic animals worldwide , and also have been implicated in human fatalities. Toxicity of MCYSTs is associated with the specific inhibition of intracellular protein phosphatase 1 (PP1) and 2A both in vivo and in vitro. MCYSTs are found to specifically bind PP1 and PP2A in liver through both non-covalent and covalent binding. This group of low molecular weight cyclic peptides (824 to 1,044 daltons) are chemically stable molecules and cannot be destroyed or removed by conventional water purification methods. Although a guideline for MCYSTs levels in drinking water has recently been introduced by the World Health Organization with a recommended limit of 1 ppb of MCYST-LR equivalents for long-term exposure via drinking water. Potential health risks from exposure to toxins in dietary supplements made from algae have received little attention. The potential for MCYSTs exposure may be substantially greater for the consumers who use algae products. To help avoid the risks of human and animal exposure, it is essential to develop sensitive and specific methods for detection of MCYSTs.

Whereas a number of approaches have been developed for MCYST analysis, most methods have some disadvantages. Chromatographic methods provide good sensitivity but they require highly qualified personnel, extensive sample cleanup as well as expensive equipment The protein phosphatase inhibition assay and mouse assay are toxicologically highly relevant, but they are not sensitive enough to detect toxin concentrations at ppb levels. Development of immunochemical approaches have led to more rapid and sensitive tools for monitoring, detection and quantification of MCYSTs in contaminated algal foods and drinking water supplies. Nevertheless, the sensitivity and applicability of the immunodiagnostic approaches for MCYSTs still require further development to avoid algae matrix interferences and sample cleanup procedures. In the present study, a new method for the production of polyclonal antibodies against MCYSTs was developed, and a sensitive competitive direct ELISA (cdELISA) was established. Details for the production and characterization of these antibodies as well as their use for ELISA for MCYST in algae cultures and dietary supplements are presented herein.

三、完成的結果

Production of Polyclonal Antibodies. Sera collected from rabbits immunized with MCYST-γ-globulin or with MCYST-KLH were subjected to the indirect ELISA. Typical titration curves of antibody titers obtained from a MCYST-γ-globulin immunized rabbit over a period of 18 weeks are shown in Figure 1. Antibodies against MCYST-LR were detected in the sera of rabbits as early as 6 weeks after initial immunization. The antibody titer increased progressively with time and the highest titer was found in the sera of rabbits around 18 weeks after immunization. The antibody titers of the rabbits immunized with MCYST-KLH were found to be considerably lower than those immunized with MCYST-γ-globulin (data omitted).

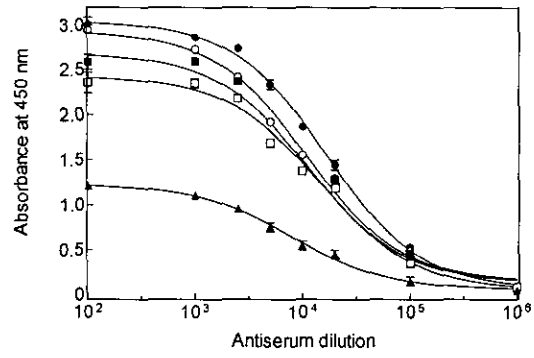

Figure 1. Determinant of antibody titers for a representative rabbit after immunization with MCYST-LR-γ-globulin by an MCYST-LR-PLL-based indirect ELISA. The antiserum were obtained 0 (▲), 6(□),9(■), 12(○), and 18(●) weeks after immunization.

Characterization of Antibodies.

Both the cdELISA and ciELISAs were used to determine the specificity of antibodies. Because the antibody amount required to coat the microtiter plates in the cdELISA was much less for the antiserum obtained from rabbits immunized with MCYST-γ-globulin than those immunized with MCYST-KLH (data not shown), the antiserum obtained from rabbits immunized with MCYST-γ-globulin was used in the subsequent studies. In the cdELISA, the concentrations causing 50% inhibition (IC_{50}) of binding of MCYST-HRP with the antibodies by MCYST-LR, MCYST-RR, MCYST-YR, and nodularin were found to be 0.10, 0.12, 0.14, and 0.19 ng/mL, respectively (Figure 2). The relative cross-reactivity of the antibodies to MCYST-LR, MCYST-RR, MCYST-YR, and nodularin was calculated to be 100, 83, 71, and 53, respectively. Such result indicates that the antibody has similar affinity for MCYST-LR, MCYST-RR, and MCYST-YR. However, the affinity toward nodularin was less. Similar results were obtained in the ciELISA where MCYST-polylysine was coated to the wells of ELISA plates to serve as solid-phase antigen (Figure 3). The concentrations causing 50% inhibition of binding of antibodies to the solid-phase MCYST-polylysine by free MCYST-LR, MCYST-RR, MCYST-YR, and nodularin were found to be 0.21, 0.28, 0.30 and 0.42 ng/mL, respectively. The relative cross-reactivity of the antibodies to MCYST-LR, MCYST-RR, MCYST-YR, and nodularin in the ciELISA was calculated to be 100, 75, 70, and 50, respectively. Okadaic acid, a potent PP1 and PP2A inhibitor , at a concentration of 500 ng/mL did not inhibit the binding of the marker antigen with the antibodies in either ELISA.

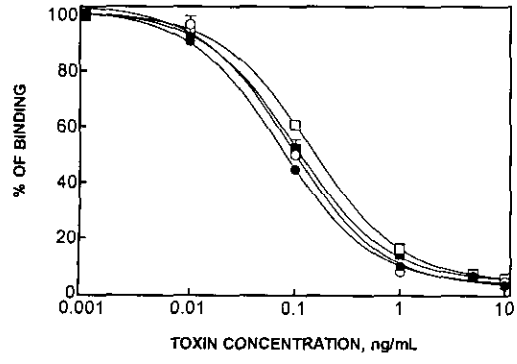

Figure 2. Cross-reactivity of anti- MCYST antibodies with MCYST-LR (●) MCYST-RR(○), MCYST-YR(■), and Nodularin(□) in a competitive direct ELISA(cdELISA).

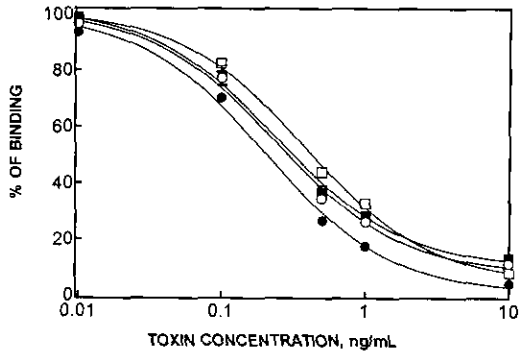

Figure 3. Cross-reactivity of anti- MCYST antibodies with MCYST-LR (●) MCYST-RR(○), MCYST-YR(■), and Nodularin(□) as determined by an competitive indirect ELISA(ciELISA).

Analytical Recovery of MCYST-LR Added to Algal Dietary Supplement by cdELISA.

Results for the analytical recovery of MCYST-LR added to the algae samples by cdELISA are presented in Table 1. The analytical recovery for the sample spiked with MCYST-LR at a level of 10 ng/g was more than 100%. Therefore, data from this group was excluded from the calculation of the overall analytical recovery. In the concentration of 25 to 500 ng/g, the overall average of analytical recovery for the samples was found to be 83.7% (CV, 9.9%).

Analysis of MCYSTs in Cyanobacteria Cultures with cdELISA

To test the efficacy of cdELISA for MCYSTs in cyanobacteria isolates and related algal products, various clones of *Microcystis* cultures were subjected to cdELISA. A wide range of levels of MCYSTs was detected in these cultures by cdELISA and the results are presented in Table 2. Six of the eleven strains tested were found to be MCYSTs producers, but 5 were not. Two of the heavy producers, MTY-1 and MTN-2, yielded as much as 1,073 ppm (2 $x10^{-4}$ ng/cell) and 1,466 ppm (3 x 10^{-4}

ng/cell), respectively. MTN-3 and MTN-4 produce lower amounts of toxins with 555 ppm (8×10^{-5} ng/cell) and 593 ppm (8×10^{-5} ng/cell), respectively; MTY-2 and MCY-1 produce the least amounts of toxins at concentrations of 382 ppm (3×10^{-5}) and 149 ppm (3×10^{-5}), respectively.

Analysis of MCYSTs in Algal Dietary Supplement Products with cdELISA

Eleven algal dietary supplement products with *Spirulina*, blue-green algae or *Chlorella* as major components were collected from health food stores and subjected to cdELISA for MCYSTs. The results were presented in the Table 3. Eight of the eleven samples were found to be MCYSTs positive with the levels below 78 ppb. All of the examined *Spirulina* and blue-green algae products contained MCYSTs in various amounts. Among them, sample #1 was found to have the highest level MCYSTs of 78 ppb. Among the six *Chlorella* products, sample #6 contained measurable MCYSTs of 36 ppb, and two others, sample #7 and #8 were less than 20 ppb. Three of the rest of *Chlorella* products were found to be MCYSTs free.

Confirmation of the presence of MCYST-LR by HPLC

HPLC chromatograms for the analysis of MCYST-LR standard and MCYST-LR in *M. aeruginosa* culture (MTY-1) sample are shown in Figure 5A and 5B, respectively. MCYST-LR was well identified under the isocratic elution. The extraction of MTY-1 showed a major peak with a retention time of 6.6 min comparable with standard MCYST-LR, which had a retention time of 6.7 min. Some small peaks, which may be associated with other MCYST-LR toxin analogs, were also detected.

四、計畫成果自評

本研究的主要目的是針對藻類中的微囊藻毒生產抗體並且與建立酵素免疫分析法來快速檢測分析藻源食品及藻株中此一微囊藻毒分佈與污染的情形，由於此微囊藻毒的抗體具有很好的專一性，因此建立之直接競爭酵素免疫分析法是目前已發表文章分析微囊藻毒是最靈敏的方法，可提供微囊藻毒一快速而且簡便之分析方法。。此一文稿亦已經發表在美國化學學會期刊 J. Agric. Food. Chem. 2002. Vol (50): 4176-4182。利用此一直接競爭酵素免疫分析法方法分析藻類樣品與飲用水樣品，不僅分析樣品可不經前處理而且短時間可分析大量樣品。因此此一研究計畫成果相當卓著。

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