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微囊藻毒與蛋白質磷酸酵素間之共價鍵形成的動力學分析

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摘要

在稍早的研究中，已知微囊藻毒與蛋白質磷酸酵素 PP-1/PP-2A 間會形成共價鍵，導致兩者永久性的結合，然在其共價鍵結形成動力學方面的研究結果，卻因分析方法及使用毒素種類的差異而有顯著的不同。在本研究中，嘗試開發基質輔助脫附-飛行時間質譜儀的方法，用以偵測蛋白質磷酸酵素在與微囊藻毒形成共價鍵結時，所出現分子量加成的訊號，以此分析 PP-1 與 MCYST-LR 共價鍵形成之動力學。研究結果顯示，共價鍵結是以十分緩慢的方式形成，每小時形成共價產物的 PP-1 百分比低於 5 %。此與 Craig et al (1996) 的觀察結果近似，同時支持其用以解釋微囊藻毒與 PP-1 作用之"two-step mechanism" 假說，亦即毒素先以非共價鍵力量，快速與 PP-1 結合、抑制後，再逐漸形成共價鍵結的說法；共價鍵結與酵素失活並無直接的關連存在。

ABSTRACT

Formation of microcystin-phosphatase adducts formation is a clear indication of microcystin attach on phosphatases. However, the kinetics of microcystin-phosphatase adduct formation has been controversial due to the uses of different microcystin isoforms and measuring methodologies. Here, we used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to measure the changes in relative intensities of molecular weight signals upon mixing of phosphatase and microcystin, which allowed us to monitor the kinetics of covalent adduct formation. Our data clearly indicated that the shifts of signals upon adduct formation and a slow and steady covalent interaction between MCYST-LR and PP-1c were observed. These observations are in agreement with the results of Craig et al (1996) and support their "two-step mechanism" proposed to elucidate the interaction between PP-1c and microcystins.

一、前言

目前已知會對 PP-1/PP-2A 具有專一抑制活性的天然毒素，除了微囊藻毒之外，尚包括同樣來自藍綠藻的節球藻毒 (nodularin) (Bagu *et al*., 1997)、渦鞭毛藻的黑海綿酸 (okadaic acid, OA) (Bialojan *et al*., 1988; Haystead *et al*., 1989) 、鰭藻毒 (dinophysis toxin, DTX) (Quilliam and Wright, 1995) 、紅藻的 thyrsiferyl-23-acetate (Matsuzawa *et al*., 1994) 、放線菌的 tautomycin (Mackintosh and Klumpp, 1990) 、calyculin (Volter *et al*., 2001; Wakimoto *et al*., 2002) 、fostriecin (Walsh *et al*., 1997) ，以及昆蟲的斑蝥素 (cantharidin) (Li and Casida, 1992; Sodeoka *et al*., 1997; Knapp *et al*., 1998, McCluskey *et al*., 2000) ，微囊藻毒是其中唯一會和 phosphatase 形成共價鍵結的毒素 (Bagu *et al*., 1997)。共價鍵形成的機制，推測為 phosphatase 的 cysteine 所含親核性 (nucleophilic) 的硫基，以 Michael addition 的方式，和微囊藻毒 Mdha 端烯基的碳原子發生反應 (Goldberg *et al*., 1995; Runnegar *et al*., 1995)。微囊藻毒與 PP-1c & PP-2Ac 間共價鍵的形成，除可能造成毒素蓄積時間的延長外，還可能造成毒素蓄積量的低估 (Williams *et al*., 1997)，或是解毒劑於療效上之低落 (Elhanany *et al.* , 2001)，因此有必要針對微囊藻毒與蛋白質磷酸酵素共價鍵結之動力學進行研究。

共價鍵形成的速率目前還有爭議，Craig *et al*. (1996) 利用高效液相層析法，分析 *in vitro* 條件下共價鍵形成的時程，結果發現在反應數小時後，共價產物的含量，以非常緩慢的速度持續的增加。作者因此將微囊藻毒和 PP-1c 的作用稱為”two-step mechanism”，意指毒素先以非共價鍵的方式，快速和酵素接合，抑制其活性後，再逐漸產生共價鍵結；共價鍵的形成與活性抑制之間並無直接的關連。Liu *et al.* (2000) 以類似 Runnegar *et al.* (1995) 的免疫法，分別測試 *in vivo* 與 *in vitro* 條件下共價鍵形成的速率，結果發現在 *in vitro* 條件反應 30 秒後，即有明顯共價產物的出現，而於混合 10 分鐘後，共價鍵的形成即達到飽和。由此作者認為，共價鍵結是發生於毒素與酵素接觸的一刻，同時認為其與酵素活性的喪失有密切的關連。此與上述 Craig *et al*. (1996) 的結果與推論並不一致。

為瞭解上述差異，究竟是來自使用毒素種類，抑或分析方法之不同，本研究嘗試利用共價產物形成時，會在分子量上產生變化 (由 PP-1c 的 37000 增加為 adduct 的 38000) 的現象，另由 MALDI-TOF MS 的偵測加以窺探。MALDI-TOF MS 的技術在過去曾被應用於藥物殘留 (Claffery *et al*., 2001; Elhanany *et al*., 2001) 、蛋白質轉譯後修飾 (post translational modification) (Mills *et al*., 2001) ，有機磷與 acetylcholine esterase 間共價鍵的形成 (Claffery *et al*., 2001) 等有明顯分子量變化的反應探測中，因此或許亦能利用 MALDI-TOF MS 的技

術來分析 PP-1c 與 MCYTS-LR 共價鍵形成速率的研究。和先前分析微囊藻毒的研究一樣，研究中首先亦針對分析條件，包括樣品濃度、去鹽流程、基質種類、樣品與基質的比例，以及共結晶形成條件等進行最適化評估後，再正式進行共價鍵形成的分析，以期能獲得足以進行解析的分析訊號。

二、材料與方法

2-1. 藥品與重要儀器

實驗中所使用的 PP-1c 為來自兔子骨骼肌，並經 *E. coli* 基因轉殖所生產、純化之蛋白質單體，由中正大學分生所黃憲斌教授所提供，並以 2.5 mg/ml 的濃度儲存於含 50 %甘油的緩衝溶液之內。按 Calbiochem 的標準方法，分析所得的酵素比活性為 30000 unit/mg。酵素反應緩衝液中，包括 *p*-NPP、bovine serum album (BSA)、dithiothreitol (DTT)、tris-HCl、$MnCl_2.2H_2O$ 在內的成分均購自 Sigma (St. Louis, MO, USA)。MALDI-TOF MS 分析前處理，所使用的離心透析管 Amicon YM-10 購自 Millipore (Bedford, MA, USA)。樣品共結晶 (co-crystallization) 所使用的基質成分 (matrix) 中，sinapicnic acid 購自 HP (Palo Alto, CA, USA, part number G2038A)，ferulic acid 配方則是參考 Bornsen *et al*. (1997) 的方法，以 50 %的異丙醇水溶液 (v/v) 調配 ferulic acid (Sigma, St. Louis, MO, USA) 成 0.05 g/ml 的濃度而成。

儀器部分，MALDI-TOF MS 的主機為 HP 的 G2025A 型 (Palo Alto, CA, USA)，配置有 337 nm 波長的氮氣雷射，以及 G2024A 的 sample prep accessory system，用以乾燥樣品與基質的混合物。原廠宣稱其具有改善樣品分析效果的能力。

2-2. 樣品製備流程的評估

主要針對基質種類以及樣品塗抹、乾燥的方式等因子，評估在以包括 BSA 水溶液 (2 mg/ml)，以及 PP-1c 酵素液在內的兩種樣品進行分析時，分析訊號的良窳。基質種類方面，針對 sinapicnic acid 商品、自行調配的 ferulic acid，以及額外添加 0.1% TFA 的 ferulic acid 等三種配方進行比較。分析時，統一按樣品/基質比 1/2 (v/v) 的比例混合之後，取其中 0.3 μl 塗抹於探針 (probe) 表面，待其自然乾燥後進行分析。

在樣品乾燥與塗抹的方式部分，統一以含有 0.1% TFA 的 ferulic acid 配方為基質進行測試，比較以自然乾燥，或以 G2024A 抽乾的乾燥方式，以及以一次將所有樣品點在 probe

上，或先在 probe 上塗抹少量樣品，待 probe 表面開始出現些許白色結晶顆粒後，再將其餘樣品塗抹上去的樣品塗抹法進行比較，同時利用顯微鏡，觀察共結晶顆粒的大小與排列的方式。

G2025A 的探針有三種，本研究採用含有 16 個點樣區 (mesas) 的探針進行分析。G2025A 對於該探針的設定，是將單一 mesa 表面細分成 70 個雷射擊發區塊後，以手動或自動模式選取分析區塊，並進行訊號的加成。由過去操作的經驗中，得知以雷射擊發 1 34 區塊時，並無法獲得樣品的訊號，然在對 35 70 區塊的分析中，雖大致能獲得訊號，然不同區塊所獲得的訊號大小與品質有明顯的差異存在。因此在操作策略上，是選取 35 70 號區塊，以每區塊分析一次後累加訊號的方法，獲得兼具再現性及較高訊號雜訊比 (S/N ratio) 的訊號。在樣品完全乾燥後，將探針置入 MALDI-TOF MS 機體內，待真空度低於 10^{-5} torr 後，以 6 μJ 的雷射能量進行分析。

2-3. 共價鍵形成的動力學分析

先後將 555 μl 的緩衝液以及 45 μl 的酵素液，加入含有 30 μg 乾燥 MCYST-LR 的玻璃瓶內 (PP-1c 與 MCYST-LR 的莫爾數比為 1/15)，隨後將樣品置入 30 ^{o}C 恆溫槽內開始進行反應。以和研究一中相同，然不含 BSA 的緩衝液進行實驗。於反應後的第 1、3、6、9、12、15、18、21、24 及 27 小時期間，分別取出 50 μl 樣品進行分析。首先將樣品置入離心透析管內，以 15000 rpm 離心 5 min 後，重複加入兩次的 100 μl 去離子水，以 15000 rpm 離心 10 min，將鹽分洗出。隨後進行倒置離心 (“recovery spin”)，將 PP-1c 樣品由管內取出，此時可獲得約 10 μl 的樣品。取其中 2 μl，與含有 0.1 % TFA 的 ferulic acid 基質配方混合後，取出其中的 0.3 μl 分次塗抹於探針，待其自然乾燥後進行分析。

由於 G2025A 的解析度無法讓 PP-1c 的 $[M+H]^+$ 訊號 (m/z 約 37000) 與共價鍵產物的 $[M+H]^+$訊號 (m/z 約 38000) 達到基線分離的水準，若直接以質譜圖上此兩位置的訊號高度，作為共價產物形成比例的依據時，會出現些許的誤差，因此設計以下的公式進行修正。已知在分析 PP-1c 純質時，其在 m/z = 38000 位置的高度仍有 $[M+H]^+$高度的 0.2 倍，由此設計用以修正訊號高度的二元一次聯立方程式 (simultaneous linear equation in two unknowns) 如下：

m/z 37000 訊號高度 = PP-1c 的實際高度 + 0.2 x 共價產物的實際高度

m/z 38000 訊號高度 = 共價產物的實際高度 + 0.2 x PP-1c 的實際高度

由此，進一步的整理出下列的方程式：

PP-1c 實際高度= (5 x (m/z 37000 訊號高度) - m/z 38000 訊號高度) / 4.8

共價產物實際高度 = (m/z 38000 訊號高度 - 0.2 x (m/z 37000 訊號高度)) / 0.96

由兩種成分實際訊號高度的比例隨反應時間的變化，進行共價產物形成動力學的分析。

三、 結果

3-1. 樣品製備流程的評估

研究結果顯示，基質配方對於蛋白質訊號的良宥具有決定性的影響，塗抹與乾燥的方式亦然。首先在以不同基質配方，分析純水內的 BSA 時，發現 sinapinic acid 配方可獲得優於 ferulic acid 的訊號 (圖一 A, B)，然在分析等體積混合的 PP-1c 與 BSA 樣品時，發現 sinapinic acid 的使用，完全無法測得訊號，然 ferulic acid 仍能測得些許的 BSA 訊號 (圖一 C, D)。由此推測，ferulic acid 對於蛋白質訊號的呈現，較不易受到 PP-1c 樣品內所含甘油及鹽類的干擾，同時 BSA 相對於 PP-1c 易於呈現訊號，因此在後續的分析中，便選擇以 ferulic acid 為基質配方，同時考量將 BSA 成分於酵素反應的緩衝液配方內剔除，以避免其喧賓奪主，對於欲偵測之 PP-1c 訊號產生干擾 (圖二)。

由於無法單憑基質配方的改善來測得 PP-1c 的訊號，因此考量加入去鹽的步驟，將 PP-1c 樣品內所有不利於訊號呈現之甘油與鹽類成分去除之後，再行分析。結果發現離心透析後，PP-1c 訊號獲得明顯的改善。在 ferulic acid 配方加入 0.1% TFA 後，再進行分析的結果，發現 PP-1c 訊號的解析度獲得進一步的提升 (圖三)，因此後續便以含有 TFA 的 ferulic acid 配方，來進行後續共價鍵動力學的研究。

在樣品乾燥與塗抹的方式部分，自然乾燥者所獲得之訊號，明顯較真空乾燥時為佳，而在自然乾燥條件下，分次塗抹所獲得的訊號，遠較一次塗抹時為佳 (圖四) 。在顯微鏡的觀察下，發現真空乾燥時會出現不具任何晶形之凝聚物，自然乾燥時，則可觀察到明顯的結晶顆粒，然隨塗抹方式之不同，而在晶形及其分佈上有所不同。一次塗抹後乾燥，所獲得的結晶呈現較粗的長條型，排列較為鬆散且不均勻，分段塗抹所獲得的結晶則十分細緻，同時會均勻的散佈在整個 probe 的表面 (圖五)。

3-2. 共價鍵形成動力學的分析

在 PP-1c 與 MCYST-LR 混合後的第 1、3、6、9、12、15、18、21、24 及 27 小時，所測得的 MALDI-TOF MS 的質譜訊號如圖六所示。PP-1c 形成共價產物的比例，隨反應時間變化的關係如圖七所示。由此推估初反應速率接近 5% hr^{-1} (每小時形成共價產物的 PP-1c 比例)，同時隨反應時間的進行而逐漸降低。

四、討論

MALDI-TOF MS 的技術，被視為分析蛋白質分子量技術之一大突破。目前除了廣泛應用於蛋白質體 (proteomics) 的研究之外 (van Adrichem *et al*., 1998)，其他相關於蛋白質分子量變化的研究，諸如蛋白質 post-translational modifications 的種類與部位 (Mills *et al*., 2001)，蛋白質於電泳前所進行的 alkylation 反應效率 (Galvani *et al*., 2001)，acetylcholinesterase 與有機磷化合物的作用 (Elhanany *et al*., 2001)，以及安非他命於頭髮內殘留的檢測 (Claffey *et al*., 2001) 等領域等均有應用。本研究初步的構想即來自於此，然在初步的研究中發現，或許是因為 PP-1c 純質的量太少、溶解度差，加上酵素液內所含鹽類及甘油雜質的比例極高等因素，造成質譜訊號的難以呈現。雖已知質譜訊號的獲取和樣品分子離子化及脫附的能力有關，理應可由離子化及脫附理論的研究著手，然由於尚無明確的理論依據能完整說明整個過程，可能影響分析結果的因子又極多 (karas and kruger, 2003)，因此不得不依據傳統 trial and error 試誤法則，針對許多已知能影響訊號的分析條件一一加以評估與修正。本研究嘗試由基質配方、樣品塗抹及乾燥的方法，以及去鹽方法的使用與否等因子著手，進行初步的測試。

首先實驗結果說明，基質配方確實是影響 PP-1c 質譜訊號良窳的關鍵因子之一。實驗中所使用的三種基質配方，sinapicnic acid 為常見的商品化配方，ferulic acid 為 Westman *et al.* (1998) 的研究中，最能降低鹽類雜質干擾程度之配方，而在基質中額外加入 TFA 的作法，則是參考質譜儀的使用說明，其宣稱能增加部分蛋白質的溶解度，因而改善質譜的訊號。在分析溶於純水的 BSA 樣品時，sinapicnic acid 配方所能獲得的訊號雖稍優於 ferulic acid，然在進行 PP-1c 酵素原液與 BSA 水溶液混合物的分析時，卻完全無法獲得訊號。反倒是 ferulic acid 配方至少能測得清晰的 BSA 訊號。此說明 sinapicnic acid 基質，較易受到其他雜質的干擾而導致分析訊號低落。由於絕大多數的蛋白質樣品並非都和 BSA 一樣都能溶於純水中，加上來自生物體的蛋白質樣品，多少會含有一些鹽類或是 detergent 的成分，因此如何選取較不易受到鹽類干擾的基質配方，實為以 MALDI-TOF MS 分析蛋白質的一大考量點。

不同的基質配方之所以對於鹽類雜質干擾的容忍程度不同，除了和基質本身的特性有關之外，Bornsen *et al*. (1997) 認為還和基質的溶劑息息相關。作者認為 ferulic acid 配方，之所以能容忍鹽類雜質的干擾，應和異丙醇/水=1/1 (v/v) 的基質溶劑有關。鹽分與甘油造成分析訊號低劣的原因，在於其會嚴重干擾蛋白質與基質的共結晶形成 (Westman *et al*., 1998)。由於配方是基質的飽和溶液，在與樣品混合乾燥，乃至共結晶的過程中，最早析出的應是基質本身；隨著乾燥的持續，分析物亦隨之析出，進而與基質間形成共結晶。當配方中含有較多水分時，推測鹽類分子會因較易停留在未乾的溶液內延遲析出，而減緩其對共結晶的干擾。相較於 ferulic acid 配方，sinapicnic acid 商用品配方的溶劑組成為乙氰/甲醇/水= 60/36/8 (v/v/v) ，相較於 ferulic acid 配方的含水量極低，因此進一步的推測，含水量的多寡或許才是真正影響基質對於鹽類雜質干擾容忍程度的因子。

樣品塗抹於 probe 表面及乾燥的方法，則是另一個會影響訊號良宥之關鍵所在。由實驗的結果推測，此應和共結晶晶形的適切與否有關。真空乾燥時，容易形成分佈不均且晶形不明顯的塊狀凝集，呈現的分析訊號最差。將全數 0.3 µl 體積的樣品全數點於 probe 上，待其自然乾燥的作法，將形成許多較粗長、同時分佈均勻度稍差的晶體，呈現的分析訊號稍佳於前者。分次塗抹後自然乾燥的作法能獲得分佈均勻且細緻的晶形，訊號亦最理想。由此歸納出，當共結晶的晶形細緻且均勻時，能夠獲得最為理想之訊號，而乾燥、塗抹的方法對於晶形的良宥會有決定性的影響。Westman *et al*. (1998) 宣稱在以其所謂” seed layer method “的方法，先在 probe 表面塗上少量基質，待其乾燥之後，再小心塗上分析樣品與基質混合物的作法，能獲得最理想的訊號，或是其他利用類似真空鍍膜的作法，將樣品與基質的混合液均勻的噴灑在 probe 上的作法 (Dr Wu, personal communication) ，想必亦為獲取細緻且均勻的共結晶有關，和本實驗的作法有異曲同工之妙。

共結晶顆粒的大小與均勻度對於樣品訊號影響的規則，不僅適用於蛋白質樣品的分析，在分析諸如微囊藻毒的小分子時亦同樣適用。因此藉由共結晶顆粒細緻度與均勻度的改善，或許有助於提升 MALDI-TOF MS 對於微囊藻毒的偵檢極限，實質改善其於藻毒分析的能力。

除了善用基質種類的選取以及樣品乾燥、塗抹的方法外，樣品內的鹽類及甘油等成分亦應盡量排除，以避免其造成分析訊號的低落。Millipore 曾為此設計出 zip-tip 的工具，為充填少量 C18 材質的微量吸管，利用其對蛋白質的吸附性及可被沖洗的特性，來達到排除雜質及濃縮樣品的效果。理論上其具有快速簡便及適用範圍廣泛之優點，然實際測試時，

卻發現 PP-1c 並未能成功的被吸附或洗出，因此才改用傳統的離心透析法進行去鹽。在以適當的離心透析管材質，搭配適切的離心力、離心時間以及清洗次數的前提下，能獲得十分良好的 PP-1c 訊號，然離心透析的操作過程實則須掌握的極為嚴格，無論是稍增或稍減去離子水的體積、多洗或少洗一次，或以過高、過低的轉速進行清洗等，均不易獲得良好的訊號。由此歸納出，適切的去鹽為分析 PP-1c 另一個重要的因子。過度的去鹽之所以會影響 PP-1c 的訊號，推測和 PP-1c 溶解度的降低有關。在無鹽的情況下，幾乎所有蛋白質的溶解度均會降低，而不溶的蛋白質，會因無法和基質間形成均勻的共結晶而導致分析訊號的低劣。PP-1c 的溶解度原本就奇差無比 (Dr Huang，personal communication)，在接近無鹽的條件下想必更差，因此相信因此而比其他蛋白質更難獲得理想的訊號。在 ferulic acid 配方內添加少量 TFA 的作法，之所以有助於改善 PP-1c 或其他蛋白質分析訊號的原因，應和其對蛋白質溶解度的改善有關。此和 van Adrichem *et al.* (1998) 研究中，在基質內加入少量介面活性劑後，脂溶性蛋白質的訊號獲得改善的原理類似。而在實驗中，BSA 的訊號之所有遠比 PP-1c 為理想，可能亦和 BSA 的溶解度較佳有關。

本研究的結果顯示，在 *in vitro* 的條件下，MCYST-LR 和 PP-1c 之間的作用模式，為毒素先很快的以非共價鍵的作用力和 PP-1c 接合，造成酵素活性的喪失後，再以十分緩慢的速率形成共價鍵，此與 Craig *et al* (1996) 研究結果的推測一致，然與 Liu *et al*. (2000) 的推論有極大的差異。Craig *et al* (1996) 的研究是以 MCYST-LL 與 PP-1c 在 30 ^{o}C 下混合、反應 16 小時後，利用 HPLC 進行分析的結果，發現有接近 65 %的 PP-1c 形成共價產物，此和本研究在相同溫度下, 以 MCYST-LR 進行反應, 所測得 60 % 的數據十分近似. Liu *et al*. (2000) 的作法，則是將 MCYST-LR 在室溫下與 PP-1c 進行反應後，加入介面活性劑並加以煮沸，將未形成共價鍵的 MCYST-LR 與 PP-1c 分開後進行電泳，隨後以 MCYST-LR 的單株抗體偵檢是否有 adduct 產物的形成。結果在反應 30 秒後即測出共價鍵產物的出現，而在混合 10 分鐘後共價產物的量即已不再增加。作者因此推測在 *in vitro* 條件下，毒素與酵素之間共價鍵結的形成速率極快。Liu *et al*.的研究結果，推測和酵素免疫法的靈敏度太高有關，以致於在反應 30 秒後，共價產物仍極少量的情況下即能被測出，同時其所使用抗體量或是酵素呈色反應的反應劑使用量不足，導致在反應十分鐘後即無法測得更高濃度共價產物，進而導致誤判。

後續將以相同的方法，研究其他不同種類微囊藻毒與 PP-1c 形成共價鍵的動力學是否與 MCYST-LR 有所差別，或是是否能將本法應用於 *in vivo* 的分析。

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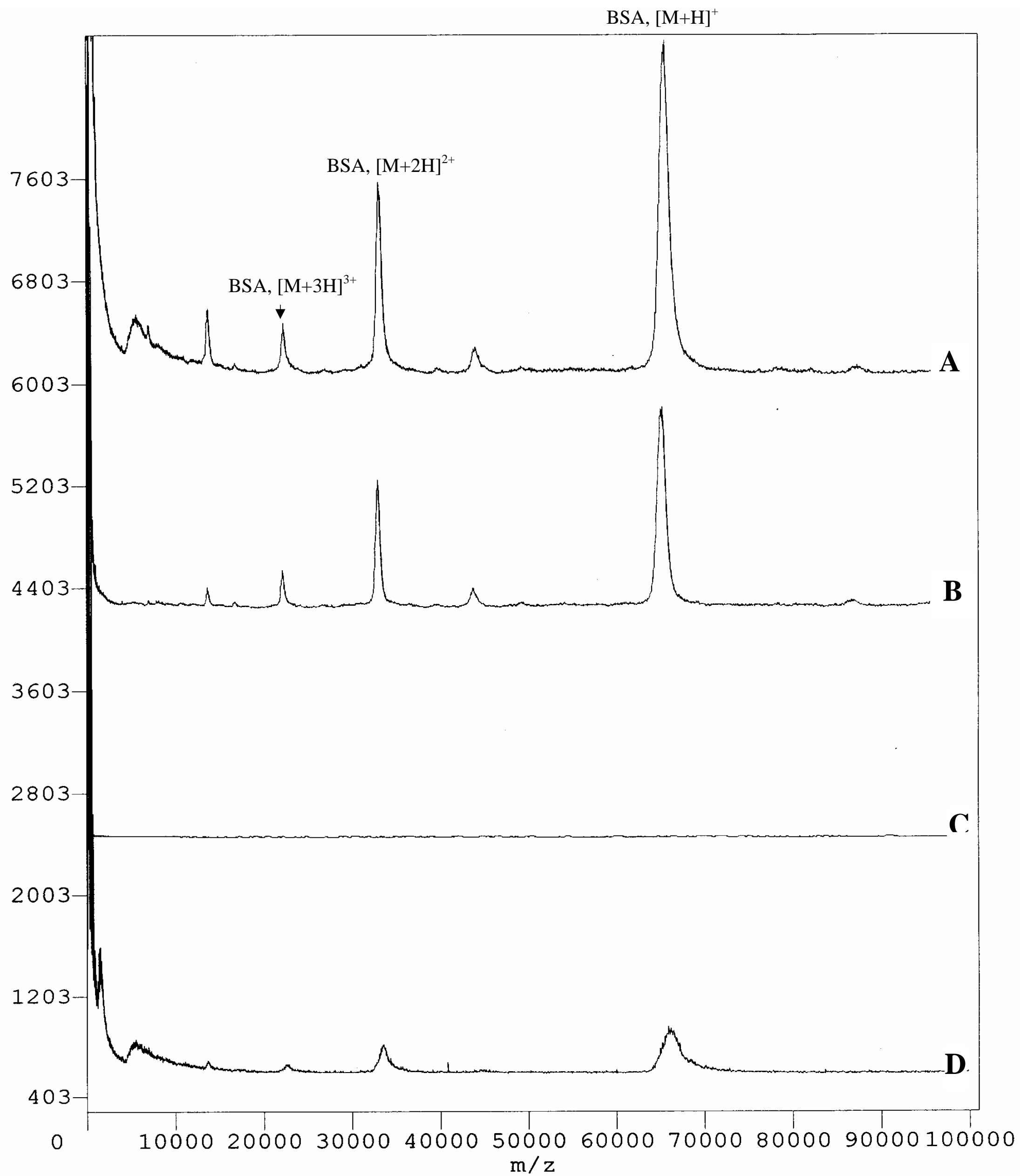
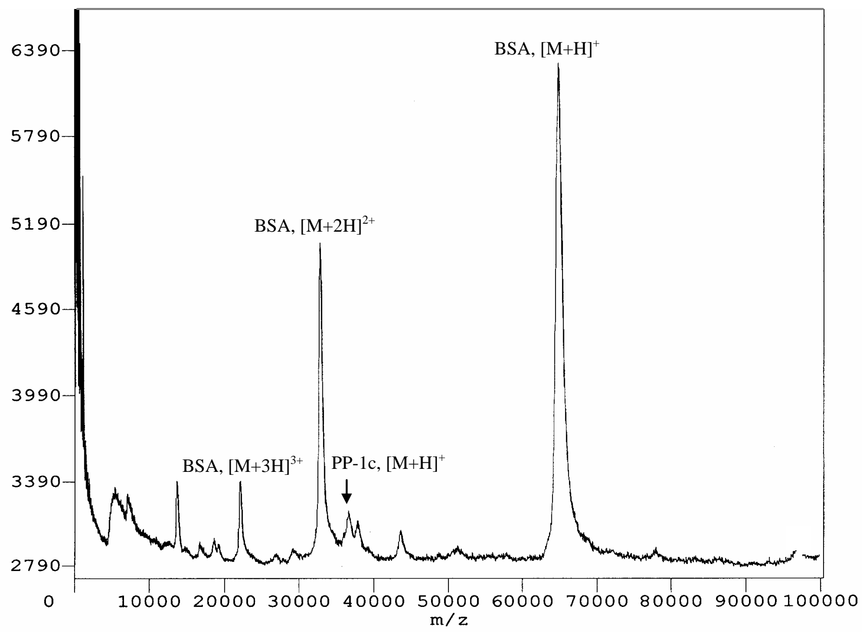
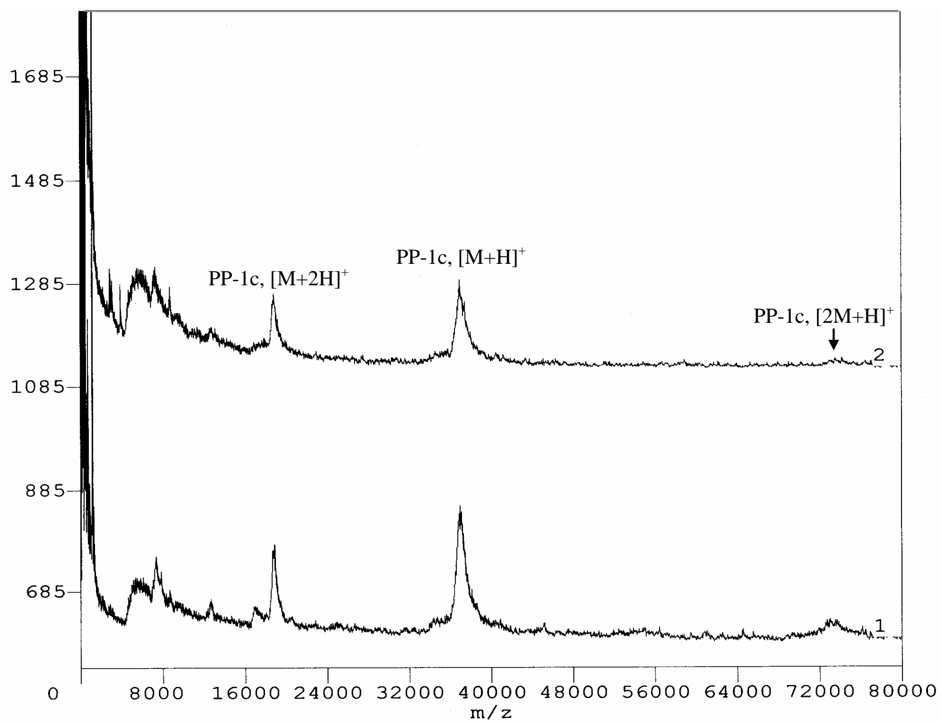


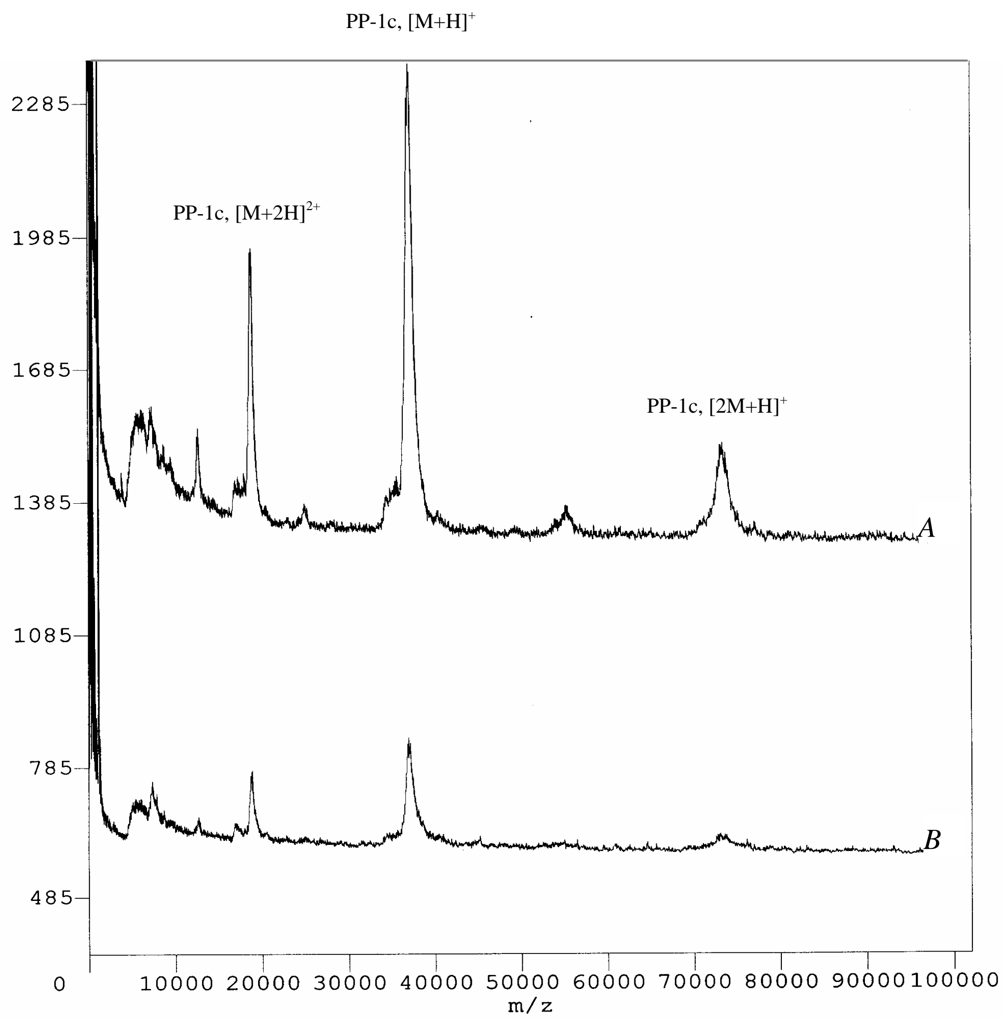
圖一、基質成分對於質譜訊號的影響。A、B： BSA 水溶液分別以 sinapicnic acid 及 ferulic acid 基質分析，C、B：BSA 與 PP-1c 的混合液，分別以 sinapicnic acid 及 ferulic acid 基質進行分析



圖二、酵素緩衝液內 BSA 的成分對於 PP-1c 訊號的干擾

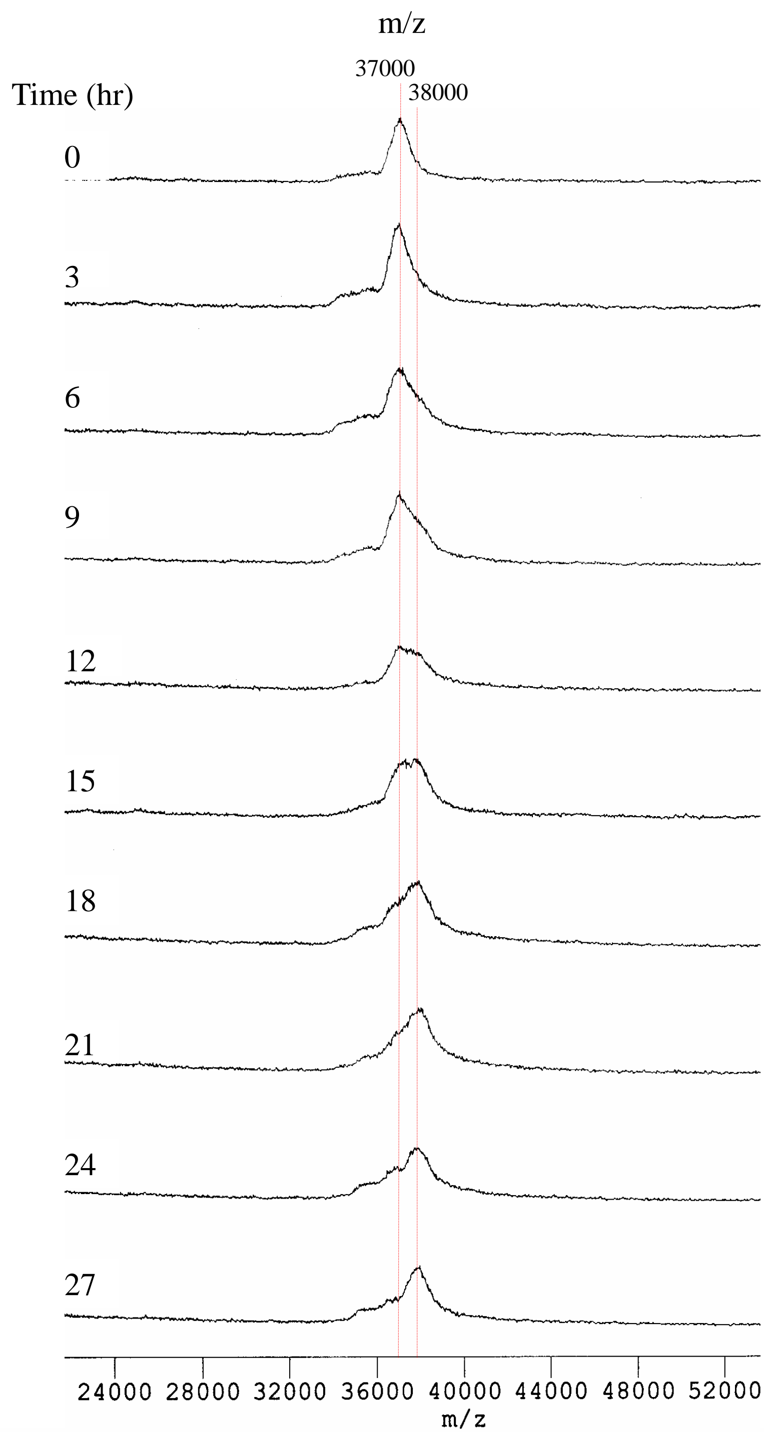


圖三、TFA 的有無對於 PP-1c 分析訊號的影響。1 為含 TFA，2 為不含 TFA 時的分析訊號。

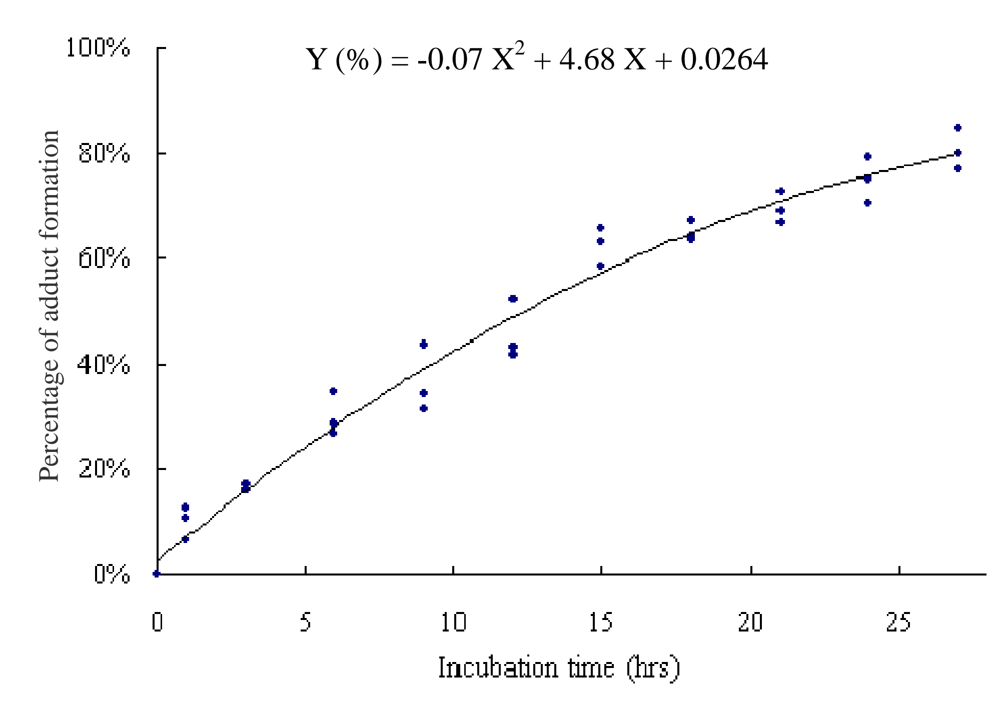


圖四、樣品塗抹方法的不同對於 PP-1c 訊號的影響。A、分次塗抹，B、一次全數塗抹

圖五、各種樣品製備流程下所呈現的晶型，A、全數塗抹後真空乾燥者，B、全數塗抹後自然乾燥，C、分次塗抹後自然乾燥



圖六、共價產物動力學分析中，不同反應時間點所測得之質譜圖



圖七、 PP-1c 共價產物形成的動力學。

附件：相關著作

註： 群體計畫 (PPG) 者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料。
若為群體計畫，請勾選本表屬於：■ 子計畫；或 □ 總計畫 (請自行整合)。

1.列出貴計畫於本年度中之所有計畫產出於下表，包含已發表或已被接受發表之文獻、已取得或被接受之專利、擬投稿之手稿 (manuscript) 以及專著等。

2.「計畫產出名稱」欄位：請依「臺灣醫誌」參考文獻方式撰寫；

3.「產出型式」欄位：填寫該產出為國內期刊、國外期刊、專利、手稿或專著等。

4.「SCI」欄位：Science Citation Index，若發表之期刊為 SCI 所包含者，請在 欄位上填寫該期刊當年度之 impact factor。

5.「致謝與否」欄位：請註明該成果產出之致謝單位。若該成果產出有註明國科會資助字樣者，請以 NSC 註明。

序號	計　畫　產　出　名　稱	產出型式	SCI*	致謝與否
1.	Chen Y. M., Chou H. N., Bai J. U., Lee T. H. and Huang R., 2005. Comparison of protein phosphatase inhibition assay with the mouse and *Artemia* toxicity assays in the toxic *Microcystis* screening. *Aquatic Toxicol.*, in revision.	國外期刊	是 (2.2)	有
2.	Chen Y. M., Huang R. and Chou H. N., 2004. Application of protein phosphatase 1 inhibition assay and a reversed phase liquid chromatography for the detection of microcystins in algal dietary supplements. *J. Food Drug Anal.*, in press.	國內期刊	是 (0.6)	有
3.	Chen Y. M., Huang, H. B., Lee S. J., Lee, T. H., and Chou, H. N., 2005. Application of MALDI-TOF MS on Monitoring of Microcystin-phosphatase Adduct Formation. *Toxicon*, manuscript in preparation.	國外期刊	是 (2.0)	有
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Comparison of protein phosphatase inhibition assay with the mouse and *Artemia* toxicity assays in the toxic *Microcystis* screening

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Abstract

The inhibitory activity of microcystins on protein phosphatase 1 (PP1) was applied for the screening of toxic *Microcystis*. Activity of protein phosphatase 1 was measured by the absorption at 405 nm of the *p*-nitrophenol, the enzyme reaction product of *p*-nitrophenyl phosphate. Inhibitory activities were represented by the ratio of *p*-nitrophenol absorption, with and without the presence of microcystin-LR or crude extract of *Microcystis*. Eight strains of *M. aeruginosa*, isolated from various aquaculture ponds and water reservoirs in Taiwan have been cultured for toxicity analysis. Extracts of the cyanobacteria were subjected to the mentioned enzyme inhibition assay as well as mouse and brine shrimp toxicity assays. Extracts from 200 ng dry cells of the toxic strains were found to be quite enough to observe the inhibition on protein phosphatase activity. Ranking of the enzyme inhibitory potency of the strains was found almost in accordance with that in mouse or brine shrimp toxicity assay. A conversion of IC_{50} from PP1 inhibition activities and LD_{50} or LC_{50} from animal toxicities of *Microcystis* extracts to the contents of microcystin-LR equivalent was very useful for such comparison. The toxin contents of microcystin-LR equivalent in *M. aeruginosa* strains from this conversion agreed well with the microcystin composition data from HPLC analysis. Phosphatase inhibition assay has been proved an easy and reliable method in screening of the toxic blue-green populations in the field by its high sensitivity, high throughput and good correlation to the toxicity on mice and brine shrimp larvae.

***Keywords*: *Microcystis aeruginosa*; microcystin; protein phosphatase 1; enzyme inhibitor**

1. Introduction

Blooms of *Microcystis* occur worldwide in fresh and brackish water (Hallegraeff, 1993; Falconer et al., 1999). They usually cause serious environmental hazards, not only forming the anoxia condition in the water, but also producing toxins. The toxins produced by *Microcystis* are hepatotoxic microcystins (Park and Watanabe, 1996), which have been implicated in the death of livestock (Kaya et al., 1996), aquaculture fish (Anderson et al., 1993) and wild animals (Murphy et al., 2000), as well as illness (Falconer et al., 1983) and death (Pouria et al., 1998) of human. The highly toxic effect of microcystins as well as their apparent tumor promoting activity in liver is suspected the result from their inhibition on protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), known as the major serine/threonine protein phosphatases in eukaryotic cells (Mackintosh et al., 1990; Nishiwaki-Matsushima et al., 1992; Toivola et al., 1999).

Microcystins are cyclic heptapeptides that share a general structure of cyclo-D-Ala-L-**X**-D-methyl-Asp-L-**Z**-Adda-D-Glu-*N*-methyl-dehydroalanine (Fig. 1), where **X** and **Z** represent the single letter abbreviation of variable amino acids inserted in the sequence, and Adda represents 3-amino-9-methoxy-2,6,8- trimethyl-10-phenyldeca-4,6-dienoic acid. Among the composing amino acids, *N*-methyl-dehydroalanine is usually abbreviated as Mdha. The term 'microcystin (MCYST)' plus the suffix XZ are thus applied for the different compounds in this group (Carmichael et al., 1988). Minor modifications, such as demethylation or isomerization, and the numbering of amino acids affected are prefixed to microcystin (Namikoshi et al., 1992). Structures of microcystins studied in this research are given in Fig. 1.

Protein phosphatase inhibition assay using PP1 and PP2A with the radioactive enzyme substrate has been developed for quantitative analysis of microcystins (Craig et al., 1993; Lambert et al., 1994). Recently chromatic and fluorescent methods using *p*-nitrophenyl phosphate (*p*-NPP) (An and Carmichael, 1994; Ash et al., 1995; Tubaro et al., 1996; Rivasseau et al., 1999; Wong et al., 1999), 4-methylumbelliferyl phosphate (4-MUP) and fluorescein diphosphate (FDP) (Vieytes et al., 1997; Fontal et al., 1999) as enzyme substrates are replacing radioactive detections in protein phosphatase inhibition assays. In this research we applied the multi-well microplate and the chromatic methods in PP1 activity analysis to screen the enzyme inhibition activity of the various strains of *Microcystis aeruginosa* in comparison with their toxicities on mice and brine shrimp larvae. Various concentrations of Microcystin-LR were applied as positive controls in these phosphatase inhibition assay and animal toxicity assay. Toxin profiles of these *M. aeruginosa* strains were also analyzed by high performance liquid chromatography (HPLC) to confirm the relationship between their microcystin content and animal toxicity.

2. Materials and methods

Eight strains of *Microcystis aeruginosa* were isolated from freshwater ponds or water reservoirs at various locations in Taiwan during 1989-1993 (Lee et al., 1998; 1999). All strains were cultured in modified Fitzgerald media (Hughes et al., 1958) at 25±1 ^{o}C and illuminated at 25 μEinm^{-2}s^{-1} with a fluorescent light for 12 h a day. Cell mass of each strain was collected in its late exponential phase by continuous centrifugation and followed by lyophilization. Extracts of

each strain were prepared by mixing 200 mg lyophilized cells in 40 ml methanol with a Vortex and centrifuged to collect the supernatant. Cell residues were repeated for another two extractions by 40 ml methanol. All three methanolic extracts were combined and ready for the preparation of various concentration solutions used in the following assays.

2.1. Mouse toxicity assay

From the ethanolic extract of each *M. aeruginosa* strain, 14.4, 7.2, 3.6, 1.8, 0.9, 0.45 and 0.225 ml were taken out and transferred into separate vials and evaporated to dryness. These dry residues were re-suspended in 3 ml saline solution (0.95%) for intra-peritoneal injection of mice (ICR strain, male, 20±1 g, from Animal Supply of National Taiwan University Hospital, Taipei, Taiwan), 1 ml each and three duplicates for each dose. This makes seven doses equivalent to 400, 200, 100, 50, 25, 12.5 and 6.25 mg dry cells per kg mouse for mouse toxicity assay. Four hours after each injection, the mortality of each dose was recorded (Lee et al., 1999). Toxicities of each *M. aeruginosa* strain, represented by LD_{50}, were then calculated by probit analysis accordingly (Finney, 1963).

Toxin standard MCYST-LR, prepared as in Lee and Chou (2000) was applied as control in the same assay for its LD_{50} on ICR strain mice. Six doses equivalent to 200, 100, 50, 37.5, 25 and 12.5 μg per kg mice were prepared and 6 duplicates for each dose.

2.2. Artemia toxicity assay

Larvae of *Artemia salina* were prepared from dried eggs (Ocean Star International Inc.,

Snowville, UT, USA) one day ahead of the toxicity assay following the method in Lee et al. (1999). Live larvae were suspended in seawater in a concentration of about 250 individuals per ml and transferred to 96-well-microplates, 50 μl per well. Forty-eight milliliters of the ethanolic extracts from each *M. aeruginosa* strains were extracted twice with 24 ml *n*-hexane to remove the oil soluble components and then evaporated to dryness. Dried residues of each sample were re-dissolved in 0.5 ml seawater to give a concentration of 160 mg/ml in terms of lyophilized cell mass. Further dilutions to concentrations of 80, 40, 20, 10 and 5 mg/ml were also prepared for every strain. Fifty microliters of each sample solutions were added to each well containing 50 μl of larvae suspension, 4 duplicates of each sample. Mortalities of each well in brine shrimp assay were recorded after 24 hr treatment and toxicities of each *M. aeruginosa* strains, represented as LC_{50}, were also calculated by probit analysis (Finney, 1963).

Another batch of the same volume of sample extracts were prepared through the same de-fat procedure as described above and then went through a solid phase extraction (SPE) operation to remove any components that may interfere the death of brine shrimp caused by microcystins. The SPE operation used a column of 0.6 cm i.d. that was packed with 0.1 g silica gel (Baker silica gel 40 μm flash chromatography packing, J. T. Baker, Phillipsburg, NJ, USA). Columns were preconditioned with 5 ml of ethyl acetate/isopropanol 4/3 (v/v). De-fatted sample extracts were re-dissolved in 1 ml conditioning solution and top-loaded on the SPE column before they were washed by 5 ml of the same solution. Microcystin fractions were then eluted by 2 ml MeOH and dried by evaporation. These methanolic extracts of each strain were

re-dissolved in 0.5 ml seawater and then diluted to give concentrations of 160, 80, 40, 20, 10 and 5 mg/ml in terms of lyophilized cell mass separately. Brine shrimp larvae toxicity was thus performed with these solutions in wells on microplate as described above. Toxicities of each *M. aeruginosa* strains were calculated and represented by LC_{50}. These data were compared with the data obtained from the same assay without SPE operation.

LC_{50} of toxin standard, MCYST-LR, was also determined as a control in brine shrimp larvae toxicity assay for methanolic extracts of *M. aeruginosa* strains. Five concentrations of MCYST-LR, 20, 10, 5, 2 and 1 μg/ml were applied in the same assay.

2.3. Protein Phosphatase 1 inhibition assay

The PP1 inhibition assay used *E. coli* recombinant protein phosphatase 1 α-isoform of rabbit muscle (Cat. No. 539493, CalBiochem, San Diego, CA, USA), in a buffer solution containing 50 mM Tris-HCl, pH7.0, 0.1 mM EDTA, 5 mM dithiothreitol, 0.2 mM $MnCl_2$ and BSA 0.2 mg/ml, following the protocol kindly supported by Dr. Chang (Technical Service, CalBiochem). Enzyme activity assay used *p*-nitrophenyl phosphate (*p*-NPP) as substrate and detected by the 405 nm absorption of the reaction product *p*-NP with a SPECTRA Fluor Plus microplate reader (Tecan, AG, Hombrechtikon, Switzerland).

All of the *Microcystis* extracts and microcystin-LR solutions in 30 μl MeOH of different concentrations were mixed with 970 μl buffer to make the sample solutions. Enzyme solution was prepared by adding 1 μl of the mentioned protein phosphatase 1 (2500 unit/ml) with 2.5 ml buffer, while the substrate solutions were prepared by dissolving 250 mM *p*-NPP in the same

buffer.

Each concentration of sample extracts (**Sam.**) including the control (**Con.**) that contained no *Microcystis* extract but methanol of the same ratio in buffer occupied a row of 6 wells separately on a plate. Each well in the row was filled with 100 μl buffer and 50 μl sample solutions. Among the 6 wells in each row, three were added individually with 50 μl enzyme solution and 50 μl buffer as an experiment (**Exp.**) group in triplication, while the other three were added 100 μl buffer separately as the blank (**Blk.**) group. Sample solutions were allowed to incubate with the enzyme solutions for 10 min before the adding of 50 μl 250 mM *p*-NPP substrate solutions to each well to initiate the enzyme reaction. The absorbance at 405 nm was measured for each well at time zero and time 60 min, and the absorbance difference between these two measurements was then recorded. Average of the absorbance difference of the triplicates in each group was applied in the enzyme activity calculation for each sample groups and control. The term 'percentage activity of the control' here is used to describe the remaining enzyme activity after the sample inhibition and its calculation as followed:

1. *Activity of Control* = average absorbance difference of **Exp.** (in **Con.**) – average absorbance difference of **Blk.** (in **Con.**)

2. *Activity of Sample* = average absorbance difference in each well of **Exp.** (in **Sam.**) – average absorbance difference of **Blk.** (in **Sam.**)

3. *Percentage activity of control* = *Activity of Sample* / *Activity of Control*

Microcystin-LR solutions were also prepared in a successive dilution and they were 1280, 640, 320, 160, 80, 40, 20, 10 and 5 pg per well in the same enzyme activity assay. The same percentage activity of control was plotted against the toxin concentrations to reveal the 50% inhibition (IC_{50}) of the toxin standard to the protein phosphatase activity. The correlation between the toxin concentration and the percentage activity of control remained was analyzed by several regression methods provided by Microsoft Excel®, and the best-fitted regression formula was applied for the calculation of IC_{50}. Protein phosphatase inhibition activities of the methanolic extracts of various *Microcystis* strains were also judged by their IC_{50} through the same correlation analysis. Microcystin contents in cell masses of different *Microcystis* strains thus can be estimated by converting their IC_{50} in the protein phosphatase inhibition assay to the amount of MCYST-LR equivalent. The conversion used the ratio of IC_{50} of MCYST-LR and the IC_{50} of *Microcystis* extract, which is multiplied by a factor of 1000 to represent a value equivalent to MCYST-LR (in mg) per gram of dried *Microcystis* cell mass.

An alternative procedure in protein phosphatase inhibition assay was also developed in this study for direct measurement of MCYST-LR equivalent in *Microcystis* cell mass. The same protein phosphatase assay was done by diluting the cell extract to a concentration equivalent to 200 ng dried cell per well instead of a series of dilution of the extract as mentioned above and the percentage activity determined. Toxin content of the strain, represented by Microcystin-LR equivalent, was then determined directly from the percentage activity-concentration curve of MCYST-LR. The data of toxin content in different strains from these two methods were

compared and their correlation was judged by the linear regression.

2.4. Correlations among mouse toxicity, Artemia toxicity, and PP1 inhibition assays

To determine whether the toxicities of mice or brine shrimps caused by the methanolic extract of various *Microcystis* strains were due to their microcystin contents, we first converted the LD_{50} and LC_{50} in different toxicity assays into toxin contents in these strains and compare the toxin contents obtained by different assays. The conversion of to the toxin contents of MCYST-LR equivalent followed the same method as described in protein phosphatase inhibition assay, using the LD_{50} and LC_{50} of toxin standard, MCYST-LR in their respective assays.

2.5. HPLC analysis of different microcystins in Microcystis

An isocratic HPLC with UV absorption at 238 nm was applied for microcystin analysis. A Luna phenyl-hexyl 5 μ column (4.6 x 250 mm, Phenomenex, Torrance, CA, USA) and a mobile phase of 0.01 M ammonium acetate/acetonitrile = 75/25, flow rate 1 ml/min were used for a complete separation of different microcystins. From the mentioned methanolic extracts of *Microcystis*, 2.4 ml were brought to dryness by N_2 stream and re-dissolved in 0.3 ml ethyl acetate/isopropanol = 4/3 (v/v). These solutions went through an SPE sample preparation procedure as described in the *Artemia* toxicity assay. The collected toxin fractions were dried and re-dissolved in 100 μl MeOH before injection of 5 μl for each HPLC analysis.

Ten microcystin standards, including MCYST-LR, MCYST-RR, MCYST-FR, MCYST-WR, MCYST-RA, $[Dha^7]$MCYST-LR, $[Dha^7]$MCYST-RR, $[D\text{-}Asp^3]$MCYST-RR,

[D-Asp^3]MCYST-FR, and [D-Asp^3]MCYST-WR (Fig. 1), previously purified and identified by Lee et al. (1998) were used to identify the different microcystins in *Microcystis*. Quantitative determination of microcystins was based on the calibration curve of MCYST-LR within the range of 0.9 μg and 1 ng.

3. Results

3.1. Mouse toxicity assay

Table 1 shows the results of mouse toxicity assay of MCYST-LR and extracts of various *Microcystis* strains. Probit analysis revealed the LD_{50} of MCYST-LR to be 46.9 μg/kg with the 95% confidence interval of 42.4-51.9 μg/kg. Methanolic extracts of various *Microcystis* strains also showed different toxicities against mice, and their LD_{50} were within the range of 9.8-102.2 mg dry cells/kg of mouse, except strains of M.TN-1 and M.KS-1. These two strains were regarded as nontoxic due to the zero death of mice even after intra-peritoneal injections of extract equivalent to 400 mg cell mass, the highest dose in this experiment. Ranking of the toxic strains based on their potency of toxicity against mice was listed as follows, M.TY-1>M.TN-4>M.TN-3>M.TY-2>M.CY-1>M.TN-2.

3.2. Artemia toxicity assay

Mortalities of brine shrimp in solutions of MCYST-LR and extract of *Microcystis* strains are shown in Table 2. Five dilutions of MCYST-LR, from 20 μg/ml to 1 μg/ml have been tried

on *Artemia* larvae. Probit analysis showed the LC_{50} of MCYST-LR at 5.68 μg/ml with 95% confidence interval between 5.17-6.23 μg/ml. Extract of various *Microcystis* strains also showed different toxicities from 0.6 mg to 4.5 mg cells equivalent per ml. Although strain M.KS-1 showed some toxicity to brine shrimp larvae at concentration above 12.5 mg cell mass equivalent/ml, it is regarded as non-toxic as strain M.TN-1. Further purification by solid phase extraction on the de-fatted methanolic extracts of *Microcystis* showed a reduction of the toxicity of the extracts when they were applied for the same *Artemia* toxicity assay (Table 2). Solid phase extraction was designed to remove the non-polar component and save the microcystins. It is apparent that the methanolic extract of *Microcystis* contains components other than microcystins that may kill brine shrimp larvae or reinforce the toxicity of microcystins on brine shrimp. Ranking of the toxic strains of *Microcystis* according to their toxicities against brine shrimp larvae was listed as follows, M.TY-1>M.TN-4>M.TY-2>M.TN-3>M.TN-2>M.CY-1. The ranking may be slightly different without SPE treatment (Table 2). However, if we divided these six strains into 3 groups of the toxic, less toxic and least toxic, two strains in one group, we could find the consistency in the ranking of groups, in both *Artemia* and mouse toxicity assay (Table 3).

3.3. PP1 inhibition assay

Nonlinear regression curves showing the inhibition of protein phosphatase activity by MCYST-LR and extracts of various *Microcystis* strains were presented in Fig. 2. A formula, $y = -0.1374 \text{Ln}\, x + 0.2196$ (where y = % activity of control, x = concentration of MCYST-LR, ng/ml),

using the natural logarithm function that best fit the curve of the toxin standard (R^2=0.97) was then established. From this curve IC_{50} of MCYST-LR was determined to be 0.13 ng/ml. Similar calculations have been applied for the corresponding curves of various *Microcystis* extracts and their IC_{50} were determined as 25.0 ng/ml (M.TY-1), 198.5 ng/ml (M.TY-2), 241.0 ng/ml (M.CY-1), 457.5 ng/ml (M.TN-2), 196.9 ng/ml (M.TN-3), and 129.5 ng/ml (M.TN-4) (Fig. 2).

3.4. Correlations among mouse toxicity, Artemia toxicity, and PP1 inhibition assays

Toxin contents of various *Microcystis* strains, in a form of MCYST-LR equivalent, those were converted from LD_{50} and LC_{50} in different assay methods were listed in Table 3. Correlation charts showing the toxin contents obtained from different assays were presented in Fig. 3. It is obvious that the toxin content in the methanolic extracts without SPE treatment is much higher from brine shrimp toxicity assay than those from mouse toxicity, and the regression line diverges from the diagonal line a lot (Fig. 3A). However, with the SPE sample preparation the toxin contents in various *Microcystis* strains obtained from *Artemia* toxicity assay were close to those from mouse toxicity assay with slight overestimation (Fig. 3B). Toxin contents converted from IC_{50} of PP1 inhibition assay showed much agreement with those converted from LD_{50} of mouse toxicity assay (Fig. 3C), but a direct conversion from the percentage of activity of PP1 inhibition assay showed less agreement (Fig. 3D). It showed that the sensitive method like PP1 assay might overestimate at lower toxin content, especially using the direct conversion from percentage activity. From the results of collateral correlation among protein phosphatase inhibition, *Artemia* toxicity and mouse toxicity assay, there should be no argument to say that

both mouse and brine shrimp toxicity were related to their protein phosphatase 1 inhibition caused by the microcystins in *Microcystis*. However some other toxic effects from other components in *Microcystis* may be exist, especially in the death of brine shrimp larvae.

3.5. HPLC analysis of microcystins in various strains

From HPLC analysis, a calibration line, y = 830.9x + 4608.3 (R^2=0.9939, where y: peak area, x: MCYST-LR in ng) of MCYST-LR within the range of 0.9 μg and 1 ng was obtained from HPLC analysis, and the HPLC chromatograms of microcystins of various *Microcystis* strains were presented in Fig. 4. There was no identifiable microcystins could be found in the same profile of nontoxic M.TN-1 and M.KS-1, therefore their chromatograms were excluded here. Nevertheless, those strains showed toxicity to mice and brine shrimp larvae were found to contain various microcystins (Fig. 4). It was found that strain M.TY-1 contained MCYST-LR mainly, which was more than 85% of its microcystin composition. Quantitative analysis from the calibration curve of MCYST-LR (Fig. 4) revealed the content of MCYST-LR in M.TY-1 was 4.75 ppt. Since there existed other minor microcystins, such as [Dha^7]MCYST-LR and [D-Asp^3]MCYST-LR (unpublished data) that were not clearly distinguishable in this experiment, the actual MCYST-LR equivalent of M.TY-1 considering the conversion from the toxicity of the extract should thus be a little more than 4.75 ppt. Quantitative analysis of MCYST-LR in other *Microcystis* strains also showed agreement with their MCYST-LR equivalent obtained from either mouse toxicity assay or *Artemia* toxicity assay, except the result from extracts without SPE treatment in *Artemia* assay. Since the specific toxicities of different microcystins are not

available at this time, it is not possible to calculate the total toxicity in different strains from HPLC analysis.

4. Discussion

There were two major objectives of this research, one is to determine whether the toxicities of *Microcystis* against mice or brine shrimp larvae are due to the protein phosphatase inhibition activity of microcystins, and another one is to evaluate the sensitivity and accuracy of these two toxicity assays and protein phosphatase inhibition assay. It has been reported that the LC_{50} of MCYST-LR on *Artemia salina* is 5±0.2 μg/ml (48 hr treatment) (Vezie et al., 1996). We also reported an LC_{50} of 22.3 μg/ml of MCYST-LR (Lee, et al., 1999). In this experiment we obtained a value of 5.68 μg/ml for the LC_{50} of MCYST-LR in a 24 hr treatment. It was found the brine shrimp eggs used for hatching in both experiments were of the same batch and the hatching rate of this durable egg package had decreased from 90% in 1998 to 30% in 2002. It was speculated the older eggs have a poor viability, so do the larvae from aged eggs. Hence a less LC_{50} of MCYST-LR or in other word, more sensitive while using larvae from aged eggs in the *Artemia* toxicity assay. The age of the eggs should be controlled while comparing the LC_{50} of microcystins or extract of *Microcystis* on brine shrimp larvae.

To prevent the alteration of aged brine shrimp on LC_{50} and to make the LC_{50} comparable and more useful, we designed the conversion of LC_{50} of unknown samples to the toxin content of MCYST-LR equivalent based on the LC_{50} of MCYST-LR analyzed at the same time. It was a rather simple way to present the toxicity of *Microcystis* extracts. We also found a sample

preparation procedure of SPE for HPLC analysis was very useful for the removal of interfering substances in the methanolic extract of *Microcystis*. With the same conversion of LD_{50} to the toxin content of MCYST-LR equivalent in mouse toxicity assay, it was found that the toxin content obtained from mouse assay had a better correlation with the toxin content obtained from *Artemia* assay with SPE sample preparation than the toxin content obtained from *Artemia* assay without SPE sample preparation (Fig. 3a, b).

Similar conversion was also applied for IC_{50} of different *Microcystis* extracts in PP1 inhibition assay, and a very good correlation between the toxin contents obtained from mouse toxicity assay and PP1 inhibition was observed. This result indicated that the mouse toxicity caused by microcystins was directly related to their inhibitions on the protein phosphatase 1 without any other toxic effects from microcystins or other unknown components. It has been reported that the death of mouse is due to the massive hepatic haemorrhage as a result of the liver cytoskeleton disruption caused by the protein phosphorylation imbalance from inhibition of serine/threonine protein phosphatase 1 and 2A by accumulated microcystin in liver (Falconer et al., 1981; Eriksson et al., 1990; Honkanen et al., 1990). However, in the *Artemia* toxicity assay, the death of brine shrimp larvae may be also from protein phosphatase 1 inhibition of microcystins (a slight correlation between the *Artemia* and mouse toxicity assay of the sample extracts having SPE treatments), but definitely it is not a case of massive hepatic haemorrhage as in mouse toxicity. Although the pathological effect of microcystins on brine shrimps could not be identified in this research, components other than microcystins in the *Microcystis* extracts

were observed to exert toxic effect on brine shrimps. It has been reported that long-chain unsaturated fatty acids are Na^+/K^+-ATPase inhibitors in brine shrimps (Morohashi et al., 1991). Bury et al. (1998) also reported that long chain fatty acids in *Microcystis aeruginosa* were the cause of fish death due to their potent inhibitory effects on fish gill Na^+/K^+-ATPase. We speculate similar components that may kill brine shrimp larvae or intensify the toxicity of microcystins are in our *Microcystis* strains when brine shrimps were immersed in the solutions of *Microcystis* extract, and these components can be removed by SPE sample treatment, especially in the strains of M.TN-4 and M.TN-3 (Table 3). Hence, there would be a better correlation between the *Artemia* and mouse toxicity, if the *Microcystis* extracts went through the SPE procedure before adding to the assay media.

Our practice of PP1 inhibition assay has proven itself a fast and reliable method for quantitative analysis of microcystin when comparing the results from PP1 inhibition assay with the results from other methods (Table 3). Toxin contents obtained as MCYST-LR equivalent were quite different from the data obtained by our previous ELISA analysis (Yu et al., 2002). It was realized that strains of *Microcystis* might not maintain their toxin content in different batches of culture. We found in this experiment that most of the strains produce more toxins than before, especially the strain of M.TY-1, but not M.TN-2. Causes of the change of toxin contents were not studied, but the PP1 inhibition assay was found very practicable in monitoring the microcystin fluctuations in *Microcystis* cultures or even in the field samples. We have determined in this experiment the detection limit of this PP1 assay system to be 5 pg MCYST-LR

per well. So, an alternate methodology has been designed and tested. Instead of testing the sample in series concentrations to obtain the IC_{50} for toxin content conversion, we applied the quantity of extract equivalent to 200 ng dry cells per well in this PP1 assay. From its percentage activity of control MCYST-LR equivalent of the sample was converted based on the regression curve showing the correlation of percentage activity of control and the dose of pure MCYST-LR standard (Fig 2). It was found that such a conversion might give accurate data when the sample concentration was adjusted to have a percentage activity of control falling within the range of 40% to 80%.

Although the detection limit of the PP1 assay applied in the study can reach 5 pg/well for pure MCYST-LR, same power is only equivalent to 25 ppm when 200 ng dry cell/well was applied. The sensitivity is beyond the action limit of 1.0 μg MCLR/g AFA regulated by the State of Oregon Department of Agriculture (MCLR: microcystin-LR, AFA: *Aphanizomenon flos-aquae* a cyanobacteria food supplement) (Schaeffer et al., 1999). Increasing cell amount or the related algal food supplement has been tried in PP1 assay in order to reach the needed 1 ppm toxin level for positive detection. However, components other than MCYST in samples might interfere with the detection or nonspecifically deactivate PP1 activity that gave false positive results. It was observed when extract of 60 μg or higher dry cell equivalent was applied in one well in PP1 assay, it showed 100% inhibition of PP1 activity, even without any microcystin. To solve the problem, a pretreatment with the mentioned SPE procedure has been proven to be very effective in removing the interfering components (determined by MALDI-TOF and HPLC

analysis, unpublished data), an amount up to 250 μg dry cell equivalent per well was proven to be free from the non-MCYST inhibition. The detection sensitivity then can reach to 20 ppb, equal or higher than the sensitivity of ELISA (Yu et al., 2002).

Another phenomenon is also observed while comparing the non-toxic samples with the control, the absorbance of 405 nm from the product of PP1 reaction was slightly enhanced in the tests of nontoxic *Microcystis* extract or algal food supplement. Similar observation was also reported by Honkanen et al. (1996), in which PP-2A was applied on the detection of okadaic acid contained in mussels. The increase of absorbance was speculated to be from some unknown phosphatase activators indigenous to samples or trace components that gave absorption at 405 nm. Basically the inherited phosphatases should have been excluded from the list of causing agents, since the samples were extracted with methanol and then cleaned by solid phase extraction. These types of activation may influence the sensitivity and accuracy of PP1 inhibition assay, and need to be eliminated by some clean-up procedures.

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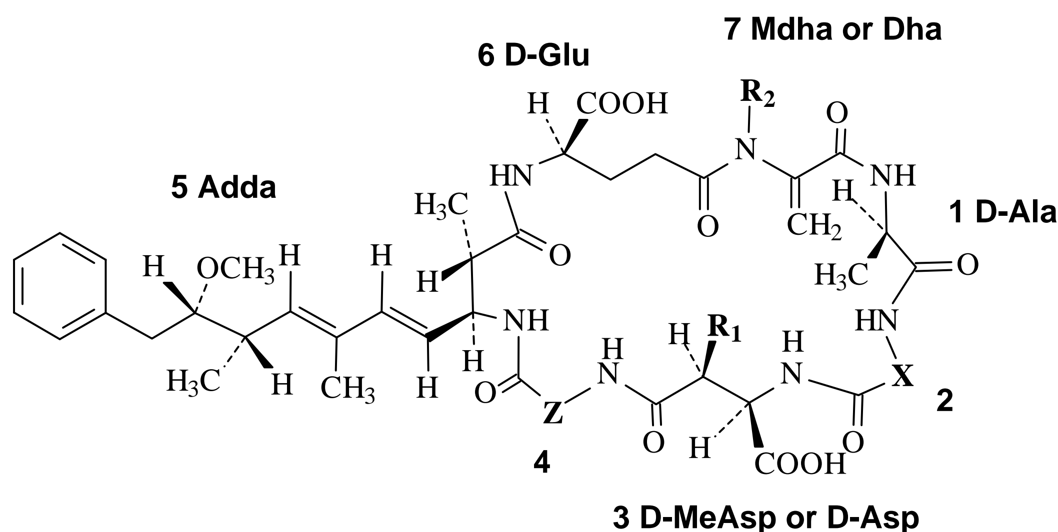

microcystin	X	Z	R_1	R_2
MCYST-LR	Leu	Arg	CH_3	CH_3
MCYST-RR	Arg	Arg	CH_3	CH_3
MCYST-FR	Phe	Arg	CH_3	CH_3
MCYST-WR	Trp	Arg	CH_3	CH_3
MCYST-RA	Arg	Ala	CH_3	CH_3
[Dha^7]MCYST-LR	Leu	Arg	CH_3	H
[Dha^7]MCYST-RR	Arg	Arg	CH_3	H
[D-Asp^3]MCYST-RR	Arg	Arg	H	CH_3
[D-Asp^3]MCYST-FR	Phe	Arg	H	CH_3

[D-Asp^3]MCYST-WR	Trp	Arg	H	CH_3

Fig. 1. Structures of microcystins studied in this research.

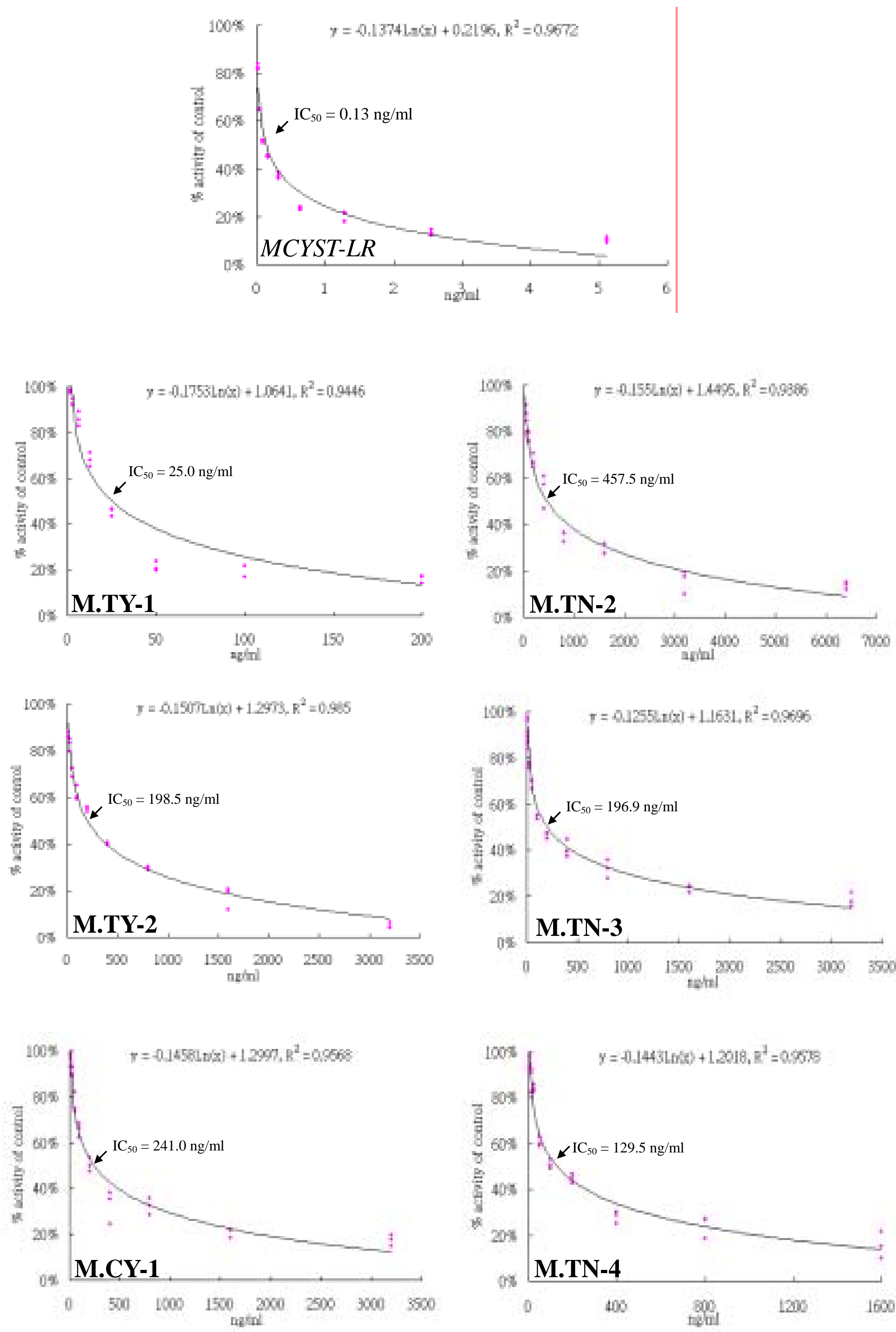

Fig. 2. Regression curves showing the protein phosphatase activity inhibited by various concentrations of MCYST-LR and extracts of *Microcystis* strains.

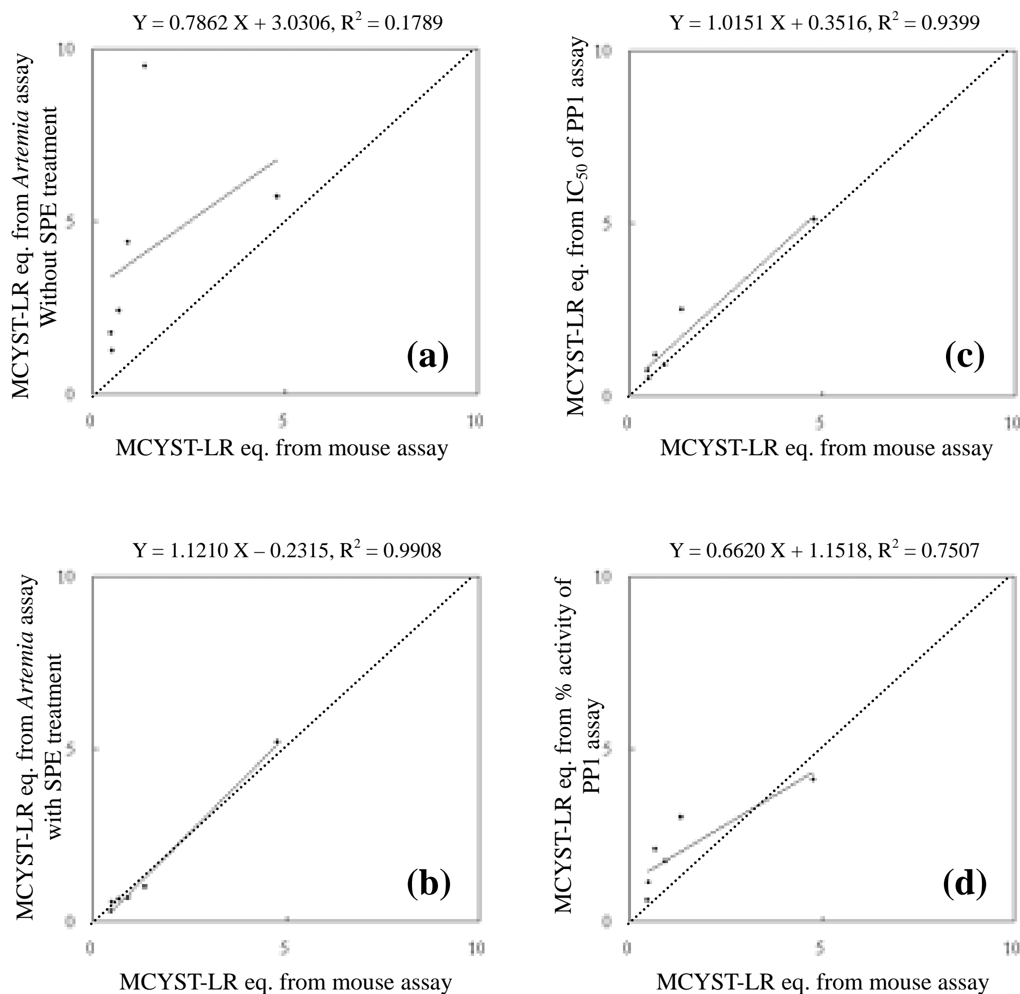

Fig. 3. Correlations between mouse toxicity assay and other assays using the toxin content of MCYST-LR equivalent converted from the LD_{50} in mouse assay, LC_{50} in *Artemia* assay and IC_{50} and percentage activity of control in protein phosphatase 1 inhibition assay: (a) *Artemia* toxicity assay without solid phase extraction procedure, (b) *Artemia* toxicity assay without solid phase extraction procedure, (c) protein phosphatase 1 inhibition assay using conversions from IC_{50}, (d) protein phosphatase 1 inhibition assay using conversions from direct measurement of percentage activity of control.

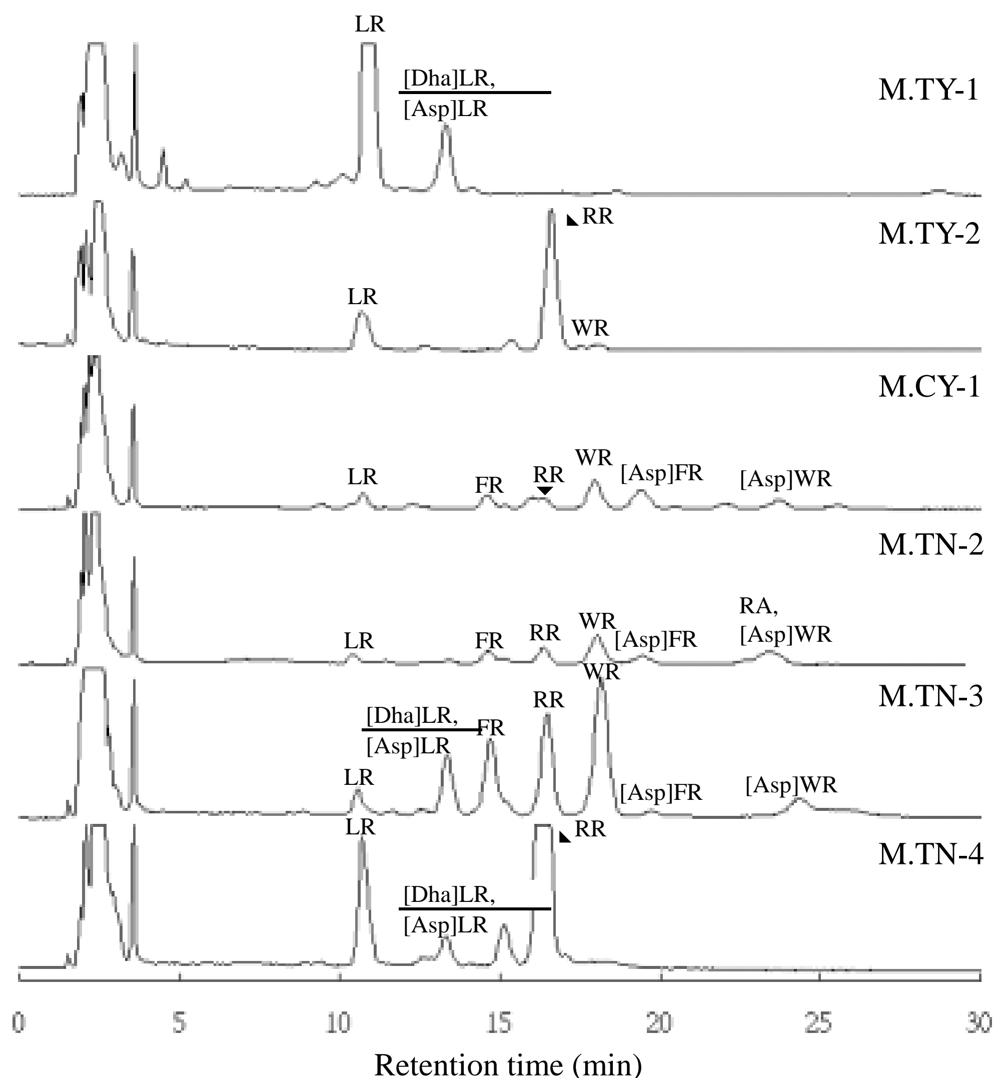

Fig. 4. HPLC analysis of microcystins in toxic *Microcystis aeruginosa* strains, M.TY-1, M.TY-2, M.CY-1, M.TN-2, M.TN-3, and M.TN-4, revealing MCYST-LR (LR), MCYST-FR (FR), MCYST-RR (RR), MCYST-WR (WR), MCYST-RA (RA), [D-Asp^{3}]MCYST-LR ([Asp]LR), [D-Asp^{3}]MCYST-FR ([Asp]FR), [D-Asp^{3}]MCYST-WR ([Asp]WR), and [Dha^{7}]MCYST-LR ([Dha]LR) in these strains. Column, Luna phenyl-hexyl 5 μ, 4.6 x 250 mm; Mobile phase, 0.01M ammonium acetate/acetonitrile=3/1 (v/v), flow rate 1 ml/min; Detector, UV 238 nm.

Table 1. Mouse toxicity assays of (a) MCYST-LR, and (b) *Microcystis* strains.

(a) MCYST-LR

Dose (μg/kg)	**200**	**100**	**50**	**37.5**	**25**	**12.5**	**LD$_{50}$ (μg/kg)**	**95% confidence interval (μg/kg)**
*Mortality**	6/6	6/6	6/6	0/6	0/6	0/6	46.9	42.4 - 51.9

(b) *Microcystis* strains

	Mortality*	**Dose (mg dry cells/kg)**							**LD$_{50}$ (mg/kg)**	**95% confidence interval (mg/kg)**
		6.25	**12.5**	**25**	**50**	**100**	**200**	**400**		
***Microcystis* strains**	**M.TY-1**	0/3	2/3	3/3	-	-	-	-	9.8	8.6 - 11.2
	M.TY-2	-	-	-	0/3	3/3	-	-	70.4	65.0 - 76.2
	M.CY-1	-	-	-	0/3	2/3	3/3	-	96.5	84.0 - 110.8
	M.TN-1	-	-	-	-	0/1	0/1	0/3	>400	-
	M.TN-2	-	-	-	0/3	1/3	3/3	-	102.2	88.7 - 117.9
	M.TN-3	-	0/3	1/3	3/3	-	-	-	51.1	44.4 - 59.0
	M.TN-4			0/3	3/3				35.1	32.5 - 38.1
	M.KS-1					0/1	0/1	0/3	>400	-

* Survival rate is represented by the ratio: number of the death/number of the mouse i.p. injected.

Table 2. ***Artemia*** **toxicity assays of (a) MCYST-LR, (b)** ***Microcystis*** **extracts, (c) Solid phase extraction treated** ***Microcystis*** **extracts.**

(a) MCYST-LR

Dose (μg/ml)	20	10	5	2	1	LC_{50} (μg/ml)	95% confidence interval (μg/ml)
Mortality*	150/150	56/122	5/99	4/98	4/88	5.68	5.17 - 6.23

(b) Microcystis extracts

		Dose (mg dry cells/ml)								LC_{50} (mg/ml)	95% confidence interval (mg/ml)
	Mortality*	50	25	12.5	6.25	3.125	1.5625	0.7812	0.3906		
Microcystis strains	M.TY-1	47/47	45/45	43/43	27/47	36/50	18/40	20/42	15/35	1	0.79 - 1.26
	M.TY-2	42/42	40/41	36/45	25/46	15/33	16/43	10/40	11/44	2.37	1.80 - 3.11
	M.CY-1	39/39	40/42	40/48	33/44	21/50	9/45	1/39	0/40	4.56	3.92 - 5.30
	M.TN-1	0/42	0/38	0/43	0/44	1/41	0/40	0/44	0/42	>>50	-
	M.TN-2	39/39	43/43	36/44	23/46	15/50	8/42	2/39	2/41	3.24	2.78 - 3.78
	M.TN-3	44/44	38/38	41/45	34/43	26/36	25/40	19/42	6/41	1.3	1.05 - 1.61
	M.TN-4	39/39	41/41	45/45	36/38	36/45	23/41	26/40	29/50	0.6	0.47 - 0.78
	M.KS-1	12/39	9/42	3/40	0/45	0/39	0/37	0/44	0/41	55.1	44.2 - 72.1

(c) Solid phase extraction treated Microcystis extracts

		Dose (mg dry cells/ml)								LC_{50} (mg/ml)	95% confidence interval (mg/ml)
	Mortality*	40	20	10	5	2. 5	1.25	0.625	0.3125		
Microcystis strains	M.TY-1	41/41	32/32	41/41	35/37	29/41	18/46	10/36	4/40	1.12	0.97 - 1.31
	M.TY-2	39/39	33/33	26/41	8/39	3/41	3/38	1/39	0/41	4.86	3.83 - 4.97
	M.CY-1	36/36	27/50	4/36	2/40	2/41	0/31	0/32	0/32	10.92	9.81 - 12.2
	M.TN-2	49/49	33/36	20/51	8/46	4/40	0/36	0/35	0/39	7.78	7.13 - 8.47
	M.TN-3	44/44	34/35	17/37	7/39	3/46	1/40	0/32	0/33	6.3	5.65 - 7.02
	M.TN-4	42/42	39/39	30/30	38/48	20/46	2/42	1/30	0/40	2.28	2.07 - 2.50

* Survival rate is represented by the ratio: number of the death/number of the brine shrimp in one well.

Table 3. Toxin contents, in MCYST-LR equivalent, of various *Microcystis* strains converted from LD_{50}, LC_{50}, and IC_{50} in mouse toxicity assay, *Artemia* toxicity assay, and protein phosphatase 1 (PP1) inhibition assay respectively.

Microcystis Strains	Toxin content (mg MCYST-LR equivalent/g dry cells of *Microcystis*, or ppt)*				
	Mouse toxicity assay (from LD_{50})	*Artemia* toxicity assay (from LC_{50})		PP1 inhibition assay	
		Crude extract	SPE treated	from IC_{50}	converted from % activity
M.TY-1	4.79 (1)	5.70 (2)	5.09 (1)	5.20 (1)	4.11 (1)
M.TN-4	1.34 (2)	9.50 (1)	2.50 (2)	1.00 (2)	3.00 (2)
M.TN-3	0.92 (3)	4.38 (3)	0.90 (4)	0.66 (3)	1.73 (4)
M.TY-2	0.67 (4)	2.41 (4)	1.17 (3)	0.65 (4)	2.07 (3)
M.CY-1	0.49 (5)	1.25 (6)	0.52 (6)	0.54 (5)	1.13 (5)
M.TN-2	0.46 (6)	1.76 (5)	0.73 (5)	0.28 (6)	0.61 (6)

* Numbers in the parentheses showing the ranking in the row.

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

93 年 7 月 13 日

報告人姓名	周 宏 農	服務機構及職稱	國立臺灣大學漁業科學研究所 教授兼所長
會議 時間 地點	93 年 6 月 19 日至 93 年 6 月 29 日 挪威 卑爾根	本會核定補助文號	臺會合字第 0930024596 號
會議名稱	(中文) 第六屆有毒藍綠菌國際研討會 (英文) 6th International Conference on Toxic Cyanobacteria		
發表論文題目	(中文) 1. 比較不同微囊藻毒的蛋白質磷酸酵素抑制活性與及性毒性之差異 2. 微囊藻毒之蛋白質磷酸酵素抑制活性與動物毒性 (英文) 1. Comparison of protein phosphatase inhibitory activities and acute toxicity of microcystins. 2. Protein phosphatase inhibitory activities and animal toxicities of microcystins		

報告內容應包括下列各項：

一、參加會議經過

二、與會心得

三、考察參觀活動（無是項活動者省略）

四、建議

五、攜回資料名稱及內容

六、其他

一、參加會議經過

第 6 屆的 ICTC 由挪威卑爾根大學 (the University of Bergen) 主辦, 時間訂於 93 年 6 月 21　27 日。與會期間有來自世界各地 35 個國家的 205 位研究人員的參與，外加 103 個口頭報告以及 100 篇海報的發表。由於今年的第十八屆國際海藻研討會 International Seaweed Symposium 亦在卑爾根是舉行，時間為 6 月 20　25 日，加以轉機航班問題，故不得不提早一天出發，十九日晚上十時到達後，日光尚存，一切夜間活動才開始。第二天下午方為有毒藍綠菌之會議的報到，早上就赴海藻研討會的開幕式與主要演講，同時也會見些在海藻領域的國際友人，趁機看了些學術海報論文，爾後因毒藍綠菌議程精彩緊湊，竟再也無暇參加海藻會議的學術討論。

毒藍綠菌會議於週一晚上的接待酒會前，邀請 Prof. Skulberg 作了一個藍綠菌與藍綠菌毒研究的回顧與未來發展的報告，雖是走馬看花，但也瞭解到人類文明發展所帶來水體優養化，造成藍綠菌的水華問題，加以化學、基因體學的發展，套用在藍綠菌毒素與產毒機制上的研究，另一項則是如何應用所知於水華管理與保護水源，特別是飲用水的安全。

會議時程安排緊湊，原安排在週四的單日旅遊，因發表論文太多而另安排報告討論，讓大部分與會者仍然堅持於會場。主辦單位所安排的海報講解於午餐與晚餐時間，除延長午、晚餐時間外，也提供簡單餐飲，而能夠整天足不出戶的留守在校園內建築的會議廳，會議廳外為圖書館區的大廊，配備多台電腦連上網路，供與會者隨時可與家中工作聯繫。由於週四部分與會者的觀光遊覽取消，故將週六半天的演講往前挪移至週二至週五的討論議程中，空出週六自由活動。另外在週三、週五晚上安排了大會接峰與惜別晚宴，享受挪威豐富的海鮮水產品。

整整四天會議的主軸包括演講或海報內容，分成八大議題，茲分別摘述如下：

議題一、一個世界觀的毒藍綠菌 Toxic Cyanobacteria -A World View

微囊藻毒是所有已知藍綠藻毒素中，對於飲用水安全危害最為嚴重的一種。其主要的毒源生物 – 銅綠微囊藻 (*Microcystis aeruginosa*) 在全球各地包括美洲、亞洲、非洲、歐洲及紐澳等地的地表水體內均十分常見，甚至形成藻華，其中中國可能為微囊藻毒危害最為嚴重的地區。目前已知其境內有超過 80 %的淡水水域有嚴重優養化的情形，而包括著名的巢湖、太湖與滇池等諸多水域內均已有銅綠微囊藻藻華的經年性分佈，對於飲用水安全及旅遊業所造成的衝擊難以估計。在包括西班牙、挪威、丹麥、瑞典、芬蘭、波蘭以及法國在內的歐陸諸國中，微囊藻毒的危害同樣是十分嚴重。挪威的產毒藻種 *Planktothrix* 即使在結冰的湖面下亦同樣能形成藻華，不但問題嚴重，研究人力多、研究標的與內容均深入，由此瞭解這次會議在挪威舉辦的理由。此外由其他的報告中，得知

非洲著名的維多利亞湖以及北美五大湖中伊利湖 (Lake Erie) 亦曾出現嚴重的微囊藻水華，對於當地的供水造成極大影響。除了微囊藻毒以外，另一個備受關注的焦點是 cylindrospermosin (CYN) 毒素及其產毒來源 *Cylindrospermopsis*。CYN 曾於 80 年代中期於澳洲昆士蘭地區造成嚴重的水源污染，在當時共有 140 名幼童及 10 名成年人出現明顯的肝、腎功能受損。近年來 *Cylindrospermopsis* 的研究，發現有毒藻珠的全球性分佈相當廣泛，詳細內容在下一個議題內有詳盡的討論。

議題二、入侵種類與水華 Invasive Species and Water Blooms

繼前一議題，以 *Cylindrospermopsis* 為例，探討有毒藍綠藻在全球擴散的趨勢。報告中利用 rDNA 親緣關係的鑑定，認定最早發現於澳洲昆士蘭造成危害的熱帶藍綠菌株 *Cylindrospermopsis* 品種，正逐漸向其他大陸熱帶甚至溫帶的區域擴散；現除鄰近的紐西蘭、美洲的美國、亞洲的日本、泰國及以色列均有發現外，在澳洲北部、美國佛羅里達州以及以色列等地也已有水源污染的問題出現。

議題三、毒源性藍綠菌與生態影響 Toxigenic Cyanobacteria – Ecological Effects

有毒藍綠菌對於生態系中其他生物亦造成嚴重的危害。研究報告中指出微囊藻毒除能造成水域中其他生物，諸如甲殼類浮游動物及蝦、蟹類，以及水生植物的死亡，進而改變原本的浮游動物及水生植物相之外，尚能造成多種貝類的蓄毒，間接危害高階的消費者。近年來節球藻在波羅的海 (Baltic Sea) 及紐、澳等地的半淡鹹水水域形成藻華的情況很普遍。報告中也指出伴隨節球藻水華所產生的毒素也能透過生物累積的方式造成魚、貝類的蓄毒，對於當地水產品的食用安全造成影響，同時考量部分魚類在長期暴露於含毒水體下可能出現肝、腎的損傷甚至生殖能力降低等負面影響，可以想見其對於漁業資源的危害。

除了水生生物之外，美國公園管理處的研究提出一種流行於美國的鳥類傳染疾病 – avian vacuolar myelinopathy，引發鳥類腦部白質的病變，造成雁鴨類及鷹類的死亡的原因，乃來自於附著於水生植物上屬 Stigonematales 目類的藍綠菌所產生的毒素。雁鴨吃食水生植物時直接造成毒害，間接的也造成食鴨類的鷹受害。

議題四、藍綠菌毒代謝與可能的作用機制 Cyanotoxin Metabolism and Putative Functions

部分藍綠菌為何要產毒，至今仍無法提出合理的解釋。以 microcystins 為例，過去僅能以其對於部分浮游動、植物的生長抑制活性，而以化學防禦機制的推理來解釋，然並無法圓滿說明為何無毒微囊藻株也未在演化淘汰過程中被消失，以及其對於許多種類的攝食者並未有明顯的毒性等。反倒是有學者提出藍綠菌所產生其他短鏈的 peptide 具有抑制攝食動物體內蛋白質分解酵素，如 trypsin 及 chymotrypsin 等活性的能力，可能和藍綠菌化學防禦機制的相關還較明顯。另有論文指出微囊藻毒有類似 pheromone 的功

能，認為干擾部分野生動物的正常代謝。在過去，微囊藻毒一向被視為是一種內毒素 endotoxin，亦即只有在細胞破裂的時候才被釋出的毒素。然最近發現藻細胞在特定時間能釋放毒素至水體內，造成細胞間族群聚落型態的改變，進而改變細胞所處的水深位置，認為是藍綠菌細胞間訊息傳遞的分子，此生活形態調整可使細胞接受到最佳的光照，既不會光照不足，又不會因光照過量而產生光抑制，認為藍綠菌產毒是經過長久的演化過程所演繹出的應變機制。

議題五、人類健康顧慮與問題管理 Human Health Aspects and Problem Management

以各種藍綠菌毒對於人類健康的影響為討論議題，同時提出解決其危害的管理辦法。前者包括各種毒素毒理機制的探討、污染（暴露）的途徑，乃至於完整的危險性評估等，後者則包括法規的制訂、水處理法去毒的效能，乃至各國執行毒素檢測與管制等問題，內容包羅萬象，為本次會議中發表與討論最多的一個議題。

毒理機制部分，CYN 已知會造成肝、腎的病變，報告指出利用「慧星分析法 Comet assay」證實 CYN 具有很強的基因毒性。而另一位知名的澳洲先進 Falconer 更利用流行病學的調查，證明在澳洲昆士蘭曾經爆發嚴重 CYN 中毒事件的區域，其民眾腸胃道癌症的罹患率有明顯偏高的情形，顯示 CYN 對於飲用水安全危害的嚴重性。筆者在本次的論文發表中，藉由不同藻毒純毒的活性作用，提出微囊藻毒對於哺乳類的肝臟毒性和其對 PP-2A 抑制活性表現間的關係，大於其對 PP-1 抑制活性表現間關係之實驗結果，被接受為口頭發表。美國奧勒岡州利用天然採收的 *Aphanizomenon* 作為健康食品進行販售，歐洲各地對這些輸入產品進行檢測時，發現其微囊藻毒含毒量時有超出 1 ppm 以上的情形，擔心其在天然水域中受相同習性之毒藻污染而含毒，因此建議對於 *Aphanizomenon*，甚至同為藍綠菌的 *Spirulina* 產品，制訂出其可含毒素的限量標準，並進行商品監測。

藍綠菌毒對於人類最大的威脅還是在於其對於飲用水的污染。聯合國除已針對飲用的微囊藻毒訂出 1 ppb 的禁制含量之外，目前還積極推動跨國性的「水安全計畫 Water Safety Plain」，想將其他藻毒或藍綠菌毒一併納入管制考量。少數包括波蘭、捷克及中國在內的國家，已依據聯合國的標準來執行其國內飲用水的監控管理；其他國家亦有類似計畫工作之推動。紐西蘭教育部在目前推動的飲用水水質監測管理計畫中，即有將藍綠菌毒的威脅列入評估；丹麥因飲用水多來自地下水，較少受藻毒或藍綠菌毒的威脅，但對於水域遭受藍綠菌水華影響，因此針對沐浴及遊憩用水也制訂有藍綠菌細胞數及毒素含量的安全用水管理規範，澳洲亦然。有鑑於歐洲諸國藍綠菌水華問題的嚴重性，歐盟提出「水管理架構法 Water Framework Directive」的指導方針，期許在 2015 年以前，將歐洲所有的河川、湖泊以及沿岸地區均合乎好的生態條件 good ecological condition。

2002 年聯合國頒佈的反恐怖主義協定(Antiterrorist Act)中，將奶油蚌毒 STX、河豚

毒 TTX、熱帶珊瑚礁魚毒 ciguatoxin 以及藍綠菌所產微囊藻毒 MCYST 與念珠藻毒 anatoxin 等均被列為具有發展成生物武器潛力的生物毒素，相關於毒素的生產與運送等，將因此受到嚴格管制。此對於未來管理程序中的毒素分析所需藻毒標準品的生產與流通可能造成阻礙，影響水質、食品的監測極大，如何在此兩者間取得平衡，在會議中亦對研究與管理者提出警訊。

議題六、分子生物學與基因關係 Molecular Biology and Genetic Relationships

由於分子生物學研究方法的長足進展，也延伸到探討藍綠菌的產毒基因及藻株間親源關係與產毒基因在不同藻種中的演化。歐盟並針對會產毒的兩株藍綠菌 *Anabaena* 90 與 *Microcystis aeruginosa* 7806 展開跨國的全基因體研究計畫。微囊藻毒由 7 個氨基酸所組成的環狀 peptide 結構，目前已知和一般蛋白質經由 DNA 序列轉譯合成的方法大不相同，屬次級代謝物。而負責微囊藻毒各特殊氨基酸合成及串接成環狀連結的基因統稱為 *mcy* genes，包括 polyketide synthase 及 non-ribosomal peptide synthetase 等，這些基因存在於相近的群集 cluster。目前研究進展已披露出約十一種以上不同 *mcy* 基因序列，在利用分子生物學的工具比較各種不同種類藍綠菌的 *mcy* 基因時，發現微囊藻毒產毒基因已演化存在有相當長的一段時間。除利用 *mcy* 基因的差異之外，部分學者利用 rDNA 的分析，試圖歸納出同屬內有、無毒藻株的親源關係，結果發現有毒藻株間的親源關係的確較為接近，並利用分析藻株、菌株 *mcy* 基因的有無或是 rDNA 序列上的差異，發展出用來分辨有毒、無毒藻菌細胞的方法。

議題七、藍綠菌毒素和相關化合物 Toxin and related compounds

除了微囊藻毒及節球藻毒之外，藍綠菌還能生產各式各樣具活性的短鏈 peptides。截至目前為止已有超過 400 種以上不同的 peptide 化合物，存在於 *Microcystis, Anabaena, Nostoc, Planktothrix, Lyngbya* 等種類中。主要的報告是針對這些化合物的種類及命名加以系統化歸納整理，因二十多年來的密集研究，化合物先後被發表，個別採用了不同的稱法，產生了同物異名，或僅僅為已命名之化合物的衍生，卻又冠上不同之名，極易在學術界中引起混淆。同時在不同種藻、菌株中產生相同毒素，這其間又牽涉到演化與親源關係。

議題八、田間與實驗室研究法 Field and Laboratory Methods

藻毒或藍綠菌毒分析方法的發展，最常使用的方法有適合現場快速偵檢的酵素連結免疫法(ELISA)，而在實驗室研究使用則以高效液相層析(HPLC)為主。ELISA 的優點在於分析速度快、靈敏度高、以及輕巧可攜等，然亦有容易出現基質效應 (Matrix effects) 以及同一系列結構近似但毒性不一的不同藻毒產生不同程度的 cross-reactivity，難以反映出活性。就微囊藻毒的分析而言，通常是以最常見的 MCYST-LR 來製造對應之抗體，

對於其他諸多種類的微囊藻毒的鑑識能力而言，自然會隨結構近似程度之不同而有差異，然 ELISA 的設計理念在於能把所有藻毒全數偵檢出來，因此在本議題中，較新的發展為利用 *in vitro* affinity mutation 的方法，來增加基因重組抗體對於各種 MCYSTs 間的 cross-reactivity。另有利用相同理論與技術，於開發醫藥用能即時解毒的微囊藻毒抗體。而 HPLC 偵檢部分，仍受限於不同毒素標準品的可獲性，及層析管柱系統的解析能力，本次會議提出有 ISRP 及 Monolithic silica C18 的逆相層析管柱可在 15 分鐘之內區隔十餘種的微囊藻毒。另配合質譜的 LC-MS 的技術亦已普遍應用於藻毒的分析中，被視為現今能同時分析各種藻毒成分效能最佳的方法。另亦可同時分析多種藻毒成分的生化晶片，亦正在研發測試的階段。以產毒細胞 DNA 序列的研究為基礎，可針對產毒相關代謝之基因或其他特殊的 DNA 序列進行 DNA probe 的設計，進而應用於 *in situ* hybridization 及 quantitative real time PCR 等，可用以定量分析水樣中有毒無毒藻族群細胞比例，MALDI-TOF 質譜配合固相萃取 SPE 的樣品前處理操作也漸次被應用於例行微囊藻毒組成的分析。目前這類方法已廣泛使用於國外水體中毒藻數目的調查，其對於有無毒藻細胞的鑑識力仍持續透過研究不斷的改進中。

二、與會心得

我國西南部沿海水產養殖池雜生著許多不知名的微細藻、菌，是否也可能含毒，而在水華時透過食物鏈轉積存于魚體中，而讓人、畜或禽鳥類中毒，甚或海洋哺乳類？七股地區曾發生黑面琵鷺的不知原因大量死亡，沿岸經常性的鯨豚類的迷失而擱淺上陸事件，是否可能和毒藻或毒藍綠菌有關？從以往不知藻類有毒，以致於現今知道在臺灣也無可免除於外的存在毒藻藻株，由於未曾對臺灣環境徹底體檢，難保繼各國之後，這些問題也會在本地發生。

臺灣水庫優養化嚴重，但因特殊的環境體質，相較於報告中所提出特殊案例尚稱慶倖，六月初國內水資源研討會披露出南部兩個水庫因微囊藻水華偵測出含有微囊藻毒的新聞，媒體一陣報導又一陣官方澄清。其實澄清湖早在二十年前，是經常性的微囊藻華，許多淡水魚蝦養殖池塘，也都經常性的存在。十多年前筆者實驗室開始分離這些水域中水華藻株，並予培養，在當時無毒素標準品可獲的環境下，只有經由培養與生物毒性分析來針對毒素加以提純，多年來陸續分離不同毒素並與結構解析，從培養所需生長條件來探討水華成因，針對毒素的化學檢測、ELISA 抗體的開發、毒素在蛋白質磷酸酵素活性抑制性分析、MALDI-TOF 在毒素的檢測及應用於毒素與蛋白質磷酸酵素共價鍵形成的動力學也都有所涉獵，目前則藉由已知不同 *mcy* 基因序列，探討有毒、無毒微囊藻株間毒素生合成酵素蛋白質與基因組成上的差異，並從基因體學上的技術，規劃水庫毒藻即時診斷的方法，來對國內水庫作一健康總體檢。另外在過去的研究中，對於毒素代謝與在水產生物間殘留的實驗結果，仍存在一些不解的問題，也需要在及時的未來規劃研

究。

藍綠菌藉演化發展出這一系列活性次級代謝物，對於菌種本身存在著些什麼樣的作用？這和其容易產生水華的作用間存在些什麼關係？是否具備生態演化上的優勢？毒素本身在人體或哺乳類體中的影響？毒素的代謝、分解及其在環境中的消長？等等問題在會前、會中、會後仍然存在於與會人的談話中，這也是筆者在一連串微藻活性天然物提純研究後所產生的疑問與深入探討的原動力。

三、考察參觀活動（無是項活動者省略）

四、建議

在本次會議中另闢有一段特殊之會議，由 WHO 在德國辦公室主辦，主要為各國藍綠菌危害與管理法規現況的交流，最後結論與建議摘要如下，應可為我國之借鏡。本第六屆有毒藍綠菌國際研討會，來自 35 國 200 多位專家的出席，許多的報告均指出環境中危害到人類健康的藍綠菌毒素 cyanotoxin 濃度高頻的發生，除了已為人所熟知的多種不同 microcystins 與 cylindrospermopsin 外，尚有許多有待鑑定的毒素，這些觀察均強調水資源的管理需要監測與法規的管理，而已有許多國家已建立起管理機制，並結論出以下五點的建議：

1. 對於飲用水中漸增的藍綠菌毒素，為強化發生機率的風險管理與訊息聯繫，標準毒素的建立是必要的。
2. WHO 已建立有限量的準則，但僅針對 microcystin-LR 一項是不夠的，所有相關 microcystins 的七十多種衍生毒素也都伴隨著普遍存在，其限量準則與標準品也都應被重視。
3. 認證毒素標準品與含有這些毒素之參考品物質與分析方法的開發也是必要。
4. 技術訓練研習於毒素分析有待加強以促進國際間技術轉移、競爭力與相互對照。
5. 各國間仍存在對藍綠菌細胞與毒素的危害與風險上的認知與瞭解，需要強化教育與訊息傳遞。

五、攜回資料名稱及內容

研討會論文摘要集。網頁：http://www.im.uib.no/nyheter/cyano04/index.html

六、其他