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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紅麴菌中citrinin之免疫化學分析法的建立以及毒性安全限量之探討

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執行期間：　90 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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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

紅麴菌中 citrinin 之免疫化學分析法的建立以及毒性安全限量之探討

Immunochemical analysis and cytotoxicity test of citrinin on *Monascus spp.*

計畫編號：NSC 90-2316-B-040-001

執行期限：90 年 8 月 1 日至 91 年 7 月 31 日

主持人：余豐益　　中山醫學大學生命科學系

共同主持人：劉秉慧　中山醫學大學生命科學系

一、中文摘要

本研究的目的在於建立一套適用於紅麴中內生毒素橘黴素(citrinin)之酵素免疫化學分析法以求有效定量紅麴食品 citrinin 的含量, 並且初步探討 citrinin 的細胞毒性。實驗以紐西蘭大白兔來生產對於 citrinin 具有專一性的多株抗體，首先將 citrinin 以化學接合方法分別接到牛血清蛋白(bovine serum albumin,BSA)與血藍蛋白(keyhole limpet hemocyanin,KLH)上使其成為良好的免疫原，然後將這些接合物免疫到兔子體內並收集血清以進行抗體效價測試，由非直接酵素免疫分析法的結果顯示，兩種不同接合物所得到的抗體效價均隨著週次增加而升高，但是競爭型酵素免疫分析法的分析結果則顯示外加毒素 並無法有效取代毒素酵素標記鍵結到抗體上。另一方面, 利用人類(293)及犬類腎臟細胞株(MDCK)來探討 citrinin 對標的細胞株的生化毒性時，發現經由不同濃度之 citrinin 處理 72 小時後，與對照組相比之 50%致死濃度約為 80 μM，人類及犬類腎臟細胞具有相似的敏感度。實驗結果顯示 citrinin 的細胞毒性應非經由細胞凋亡(apoptosis)的途徑。

關鍵詞：橘黴素、酵素免疫分析法、細胞凋亡

Abstract

The objectives of this research were to establish an enzyme-linked immunosorbent assay for determination of citrinin in *Monascus. Spp.* and cytotoxicity test of citrinin. Polyclonal antibodies for citrinin were generated from rabbits after immunizing the animals with citrinin conjugated with BSA and KLH, respectively. A competitive indirect enzyme-linked immunosorbent assay (ciELISA) was used for the characterization of the antibodies. The antibody titers increased progressively following the week. A competitive direct enzyme-linked immunosorbent assay (cdELISA) was established for analysis of the toxin. However, the marker antigen (CTN-HRP conjugates) could not displace the free toxin in the cd ELISA. The Human cell line H293 and dog kidney cell line (MDCK) were used to test the biochemical toxicities. The results showed that the LD_{50} for citrinin on these two cell lines was about 80 μM after treatment of 72 hr. The results indicated that the cytotoxicity of citrinin is not through the apoptosis pathway.

Keywords: citrinin,enzyme-linked immunosorbent assay, ELISA, antibodies, apoptosis

二、緣由與目的

紅麴菌(*Monascus spp*)產生具有醫療用途的 monacolin K，所以可以生產作為預防心臟血管疾病方面的多功能保健食品。但是培養紅麴菌(*Monascus spp*)的過程中時常會伴隨著黴菌毒素 citrinin 的產生。citrinin 是一種小分子毒素(分子量為 250)；此類毒素已知會造成多種動物在肝臟與腎臟方面的傷害，甚至可能會導致人類的 ndemica Bal-Kan nephropathy，針對雄性的F344大白鼠長期餵食低量的citrinin會導致 70%以上的實驗鼠產生鼠類罕見的腎臟腫瘤。目前 citrinin 含量的分析方法一般以高效液相層析法(HPLC)配合螢光偵測

法是最常被使用來偵測樣品的污染，但是該法非常耗時，而且靈敏感度不足，最低偵測限制僅達到 0.1ppm，因此不利於快速分析篩選樣品，為了確保紅麴產品的安全，針對 citrinin 開發一快速、簡單而且敏感的酵素免疫分析法(ELISA)配合高效液相層析法來分析 citrinin 在紅麴中的含量是非常迫切而且需要的。此外由於 citrinin 可能具有之生化毒性和基因毒性，為了探討 citrinin 在紅麴中的安全限量，以 citrinin 對標的腎臟細胞株的細胞毒性將做初步研究

三、材料與方法

Citrinin 抗體的生產所使用的動物為紐西蘭大白兔, 免疫原分別為 citrinin-牛血清蛋白(BSA)與 citrinin-血藍蛋白(keyhole limpet hemo- cyanin,KLH)藉由 carboidiimide or mannrich reaction 加以共價接合在一起。Citrinin 的細胞毒性測試細胞株計有 MDCK,Human 293,and Hela cell. MTT assay: 先將待測細胞以定量(10^4/well 種植在 96 well 微孔盤中，待細胞貼附於盤底，以 citrinin 及相關溶液處理 72 hours 後，加入已被細胞培養液溶解的 MTT 粉末至最後濃度為為 0.5 mg/ml，接著在 37oC 培養箱再培養三到四個小時，最後以酸性異丙醇將經 MTT 分解後所產生的紫色結晶溶解，於波長 570 nm 下以微孔盤測定儀定量分析。

四、完成的結果

Production of Polyclonal Antibodies.
Sera collected from rabbits immunized with citrnin-BSA or with citrinin-KLH were subjected to the indirect ELISA. Typical titration curves of antibody titers obtained from a citrinin-KLH immunized rabbit over a period of 18 weeks are shown in Figure 1. Antibodies against citrinin 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. A competitive direct enzyme-linked immunosorbent assay (cdELISA) was established for analysis of the toxin. However, the marker antigen (CTN-HRP conjugates) could not displace the free toxin in the cd ELISA. The antibody titers of the rabbits immunized with citrinin--BSA were found to be considerably lower than those immunized with citrinin-KLH (data omitted).

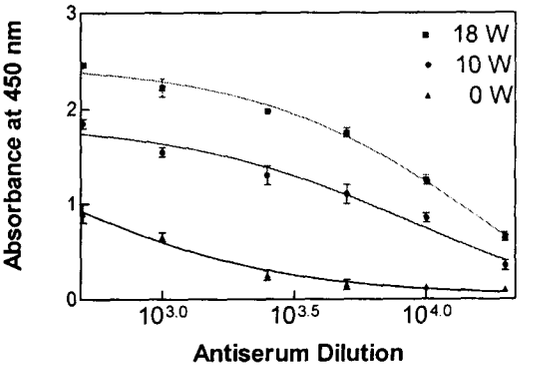

Figure 1. Determinant of antibody titers for a representative rabbit after immunization with citrinin-KLH by an citrinin-PLL-based indirect ELISA. The antiserum were obtained 0 (▲), 10(●),and 18(■) weeks after immunization.

Cytotoxic effects of citrinin on various cell lines

Human cervix epitheloid carcinoma cells (Hela), Human 293, and MDCK were treated with various concentrations of citrinin for 72 hours. The results of cytotoxic effects of citrinin on various cell lines were showed in the Figure 2. The results showed that Hela cell is the most sensitive to citrinin cytotoxicity; the H293 and MDCK showed the similar cytotoxicity to citrinin. The lethal dose 50% for citrinin on Hela cell, H293, and MDCK were 60, 80, and 80 μM, respectively.

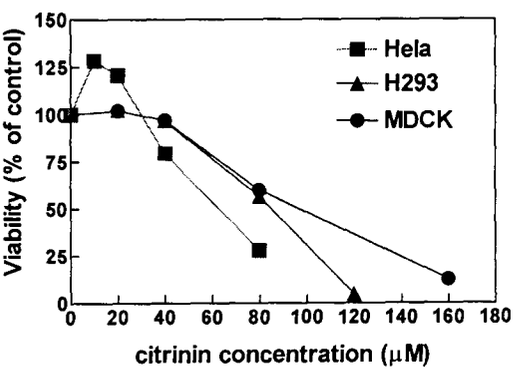

Figure 2. Comparison of the cytotoxic effects of citrinin on various cell lines. Human cervix epitheloid carcinoma cells (Hela),Human 293, and MDCK were treated withvarious concentrations of citrinin for 72 hours.Cell viability was expressed as a percentage of control.

The cytotoxic effect of citrinin on human embryonic kidney cell line (293)

Human 293 cultures were treated with various concentrations of citrinin for 72 hrs. Cell viability was determined as metabolic integrity using MTT assays and expressed as a percentage of control. The result of cytotoxic effect of citrinin on human embryonic kidney cell line (293) was showed in the Figure 3. The cell viability is about 50 fter using 80 μ M citrinin for 72hrs. Moreover, the cell viability is about 10 after using 120 μ M citrinin for 72hrs.

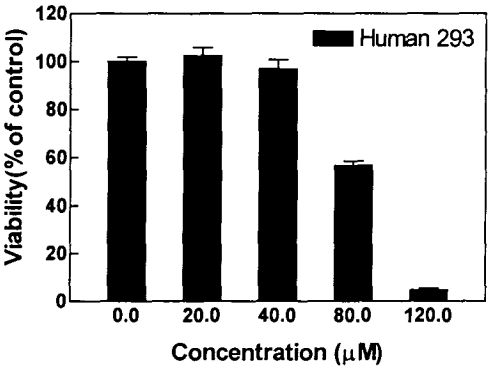

Figure 3. The cytotoxic effect of citrinin on human embryonic kidney cell line (293). Human 293 cultures were treated with various concentrations of citrinin for 72 hours. Cell viability was determined as metabolic integrity using MTT assays and expressed as a percentage of control, which was treated with vehicle only. Control values were taken as 100%. Data are given as themean±SEM (n=5)

The cytotoxic effect of citrinin on dog kidney cell line (MDCK)

The dog kidney cell line (MDCK)cultures were treated with various concentrations of citrinin for 72 hrs. Cell viability was determined as metabolic integrity using MTT assays and expressed as a percentage of control. The result of cytotoxic effect of citrinin on MDCKey cell line was showed in the Figure 4. The cell viability is about 50 fter using 80 μ M citrinin for 72hrs. Moreover, the cell viability is about 10 after using 160 μ M citrinin for 72hrs. The results indicated that the MDCK cell lineis more tolerant on citrinin cytotoxicity than H293 cell line.

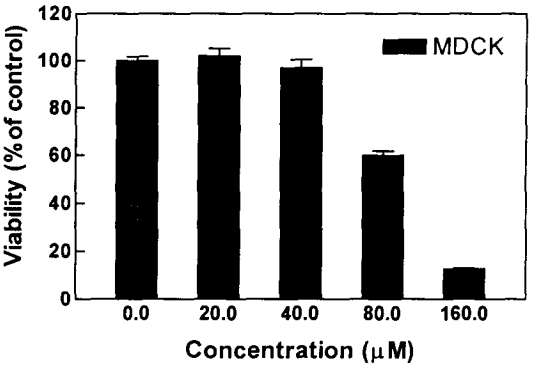

Figure 4. The cytotoxic effect of citrinin on dog kidney cell line (MDCK). MDCK cultures were treated with various concentrations of citrinin for 72 hours. Cell viability wasdetermined as metabolic integrity using MTT assays and expressed as a percentage of control, which were treated with vehicle only. Control values were taken as 100%. Data are given as the mean±SEM (n=5).

四、計畫成果自評

本研究的主要目的是針對紅麴中內生毒素橘黴素(citrinin)建立一套適用之酵素免疫化學分析法以求有效定量紅麴食品 citrinin 的含量，並且進一步探討 citrinin 的細胞毒性，由本研究所得到之多株抗體雖然隨週數之增加，效價亦隨之增高，但是利用此一抗體並無法建立一套有效之橘黴素酵素免疫分析法，這也說明了為什麼截至目前為止，世界上只有一個德國的研究團隊發表過有效的 citrinin 的酵素免疫化學分析法，大部分原因乃是由於 citrinin 本身結構共振穩定性之特性，使得許多官能基無法衍生以接到載體蛋白質上來免疫動物以獲取專一性之抗體，來建立有效之 citrinin 競爭型酵素免疫分析法。至於在 citrinin 的細胞毒性方面本研究已經獲得相當不錯有關 citrinin 對許多不同細胞株之細

胞毒性方面之研究，並且想進一步探討其生化毒性與細胞毒性方面之致毒機制，並且研究有關其細胞體內一些酵素之變化。但是由於本計畫僅補助一年經費，而且延續性計畫亦遭腰砍，使得本研究計畫無法繼續執行，實在覺得有點遺憾。

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