行政院國家科學委員會專題研究計畫 成果報告

建立定性及定量分析系統進行人類血漿中肝球蛋白亞型及幾丁質分解酵素之研究
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 96-2314-B-040-031-
執 行 期 間 ：96 年 08 月 01 日至 97 年 10 月 31 日
執 行 單 位 ：中山醫學大學醫學系

計 畫 主 持 人 ：林中生

計畫參與人員： 博士班研究生-兼任助理人員：廖純沂

處 理 方 式 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 98 年 05 月 04 日

96 年度 【 建立定性及定量分析系統進行人類血漿中肝球蛋白亞型及幾丁質分解酵素之研究 】成果報告

執行機構：中山醫學大學

主持人：林中生教授(醫學系)

目錄

報告內容

前言

肝球蛋白質分子 (haptoglobin, Hp) 的表型 (phenotype) 和幾丁質分解酵素 (chitinolytic enzyme) 比活性與人類疾病有無關聯性的流行病學截至目前已知的證據很少。除了與血紅素有強的結合能力外，近年來的研究證實肝球蛋白質分子和促使發炎作用的激素、細胞和蛋白質的相關性，以及它在調節 T 淋巴球細胞群發展的角色，因而開始了一連串突破性的發現及流行病學相關研究的啟蒙；相對地，剛起步的人類幾丁質分解酵素在研究資料上就顯得單薄許多。但無論分子研究的進展如何，如何有效、快速及省時又省錢的的鑑定及分析技術應該是加速臨床流行病學研究的主要動力。

研究目的

本研究除了著重於快速鑑定分析人類血漿中的肝球蛋白質表型和快速分析幾丁質分解酵素比活性等技術平台的建立外，更進一步利用已經建立的技術平台分析一般群體 (general population) 的血漿樣本和臨床群體 (clinical population) 的血漿樣本中肝球蛋白質表型和幾丁質分解酵素比活性的表現，透過流行病學尋找此兩種蛋白質和疾病間是否有相關性。

研究方法

利用新開發的 mCBB-R250 染色法和西方墨點法進行肝球蛋白質表型鑑定。Hp 1-1、Hp 2-1 和 Hp 2-2 等三種肝球蛋白質表型在收集的血漿樣本中出現頻率百分比 (percentage of frequence) 和比例 (proportion) 則用敘述性統計法、單一樣本 t 檢定和 Chi-square 檢定進行分析。

利用乙二醇幾丁聚醣 (glycol chitosan) 做為研究人血漿幾丁質分解酵素反應的基質，建立使用鐵氰化鉀試劑 (K3Fe(CN)6) 溶液做為酵素反應產物呈色反應試劑的活性分析方法，用以標定人血漿中可以分解乙二醇幾丁聚醣的幾丁質分解酵素；利用已經開發的 mCBB-G250 活性染色方式直接鑑定電泳分離膠上幾丁質分解酵素的活性及分子量。利用常態檢定和獨立樣本母數或無母數檢定等方法進行幾丁質分解酵素比活性值的檢定分析。

結果與討論

利用新開發的 mCBB-R250 染色法成功地分析了 1148 個血漿樣本中肝球蛋白質表型，提供臨床上有效且快速分析肝球蛋白質表型的一新方向。1148 個血漿樣本中，肝球蛋白質表型為 Hp 1-1 者佔 11.67%；肝球蛋白質表型為 Hp 2-1 者佔 42.60%；肝球蛋白質表型為 Hp 2-2 者佔 45.73%。對照 151 個罹患心血管疾病的血漿樣本，其肝球蛋白質表型為 Hp 1-1 者佔 11.26%；肝球蛋白質表型為 Hp 2-1 者佔 41.06%；肝

球蛋白質表型為 Hp 2-2 者佔 47.68%。經統計檢定分析，罹患心血管疾病患者肝球蛋白質表型的頻率百分比與總樣本的頻率百分比相似。然，151 個罹患心血管疾病的血漿樣本中，有 101 位罹患穩定性心絞痛 (stable angina, CAD) 患者和 50 位罹患急性冠狀動脈綜合症 (acute coronary syndrome, ACS) 患者，兩者間 Hp 2-2 表型的頻率百分比的比例經統計檢定發現沒有差異，但是 Hp 1-1 和 Hp 2-1 表型的頻率百分比的比例是有明顯差異的，其中 CAD 患者 Hp 2-1 表型的頻率百分比較高，ACS 患者 Hp 1-1 表型的頻率百分比較高。

利用乙二醇幾丁聚醣做為基質和使用鐵氰化鉀試劑溶液做為酵素反應呈色試劑的方法，成功地分析 899 個血漿樣本中幾丁質分解酵素比活性值。經統計檢定分析，899 個血漿樣本中幾丁質分解酵素比活性值分佈為一常態分佈。其中，幾丁質分解酵素比活性平均值 (95%信賴) 為 92.00 U/mg (90.584，93.416)，對照 151 個心血管疾病患者的幾丁質分解酵素比活性值，發現 CAD 患者血漿中幾丁質分解酵素比活性值 (102.724 U/mg) 高於 ACS 患者 (90.513 U/mg)。另外，利用 mCBB-G250 活性染色法直接鑑定電泳分離膠片中幾丁質分解酵素活性，在部分分離純化血漿幾丁質分解酵素的過程中，已經先行鑑定一新的幾丁質分解酵素，其分子量約為 30 kDa。本研究已經完全確立有效、快速及省時又省錢的肝球蛋白質表型鑑定法、幾丁質分解酵素比活性分析法和幾丁質分解酵素活性鑑定法的技術平台。發展的技術平台應用於 1178 個收集的血漿樣本後，已獲得初步臨床數據。後續研究目標將進行相關流行病學的研究設計和分析臨床疾病的血漿樣本，增加檢定的可信度，釐清肝球蛋白質表型和幾丁質分解酵素比活性與疾病間的相關程度。

計畫成果自評

本研究成果豐碩，目前已發表 SCI 學術期刊 2 篇，另有一篇已獲得接受。

1. C. Y. Liau, K.- C. Ueng, and C.- S. Lin*, 2009, Classifying human haptoglobin phenotypes on native-PAGE using a modified Coomassie Brilliant Blue R 250 staining, *Journal of the Chinese Chemical Society* (SCI), *Accepted*.

2. C. Y. Liau and C.- S. Lin*, 2008, A Modified Coomassie Brilliant Blue G 250 Staining Method for the Detection of Chitinase Activity and Molecular Weight after Polyacrylamide Gel Electrophoresis, *Journal of Bioscience and Bioengineering* (SCI), Vol. 106, No. 1, p111-113. (2008年7月)

3. C. Y. Liau* and C.- S. Lin, 2008, Detection of Chitinolytic Enzymes in Ipomoea batatas Leaf Extract by Activity Staining after Gel Electrophoresis, *Journal of the Chinese Chemical Society* (SCI), Vol.55, No.3, p678-681. (2008年6月)

SCI 期刊論文成果

Detection of Chitinolytic Enzymes in *Ipomoea batatas* Leaf Extract by Activity Staining after Gel Electrophoresis

Chun-Yi Liau[a,b]* (廖純沂) and Chung-Sheng Lin[a] (林中生)
[a]*Institute of Medicine, Chung Shan Medical University, Taichung 402, Taiwan, R.O.C.*
[b]*Department of Bioengineering, Tatung University, Taipei 104, Taiwan, R.O.C.*

A non-denatured SDS-PAGE followed by in-gel activity staining using embedded glycol chitin as a substrate was used to identify the proteins with chitinolyitc activities from sweet potato leaf extract. At least two chitinase activity zones can be clearly identified on the gel at positions with estimated molecular weights of 54.1~55.6 kDa and 39.6 kDa. Furthermore, our data also indicate that the activity of the larger one can withstand the standard SDS-PAGE sample preparation. Both of these chitinases, however, are different from that of the previously identified chitinase in sweet potato leaves, which has a molecular weight of 16 kDa. By using an embedded substrate, our method has superior sensitivity in detecting chitinases with higher molecular weights. It is a simple, affordable way and may aid in the future discovery of new chitinases.

Keywords: Chitinase; Glycol chitin; Activity staining; Sweet potato.

INTRODUCTION

Chitinases (EC 3.2.1.14) belong to family 18 and family 19 *O*-glycoside hydrolases, which hydrolyze the glycosidic bonds between two or more carbohydrates. Chitinases catalyze the hydrolysis of chitin, a β-1,4-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc), which is the main structural component of fungal cell walls and arthropod integuments. By means of endochitinase activity, chitinolytic enzymes hydrolyze chitin to produce chitooligosaccharides with 2 to 6 GlcNAc units. Chitinases are classified into various classes, such as class I, II, III, or IV chitinases, based on their amino acid sequences.[1,2]

To date, chitinolytic activities have been reported in microorganisms,[3] plants,[4] and humans.[5,6] It is reasonable that all microorganisms that make chitin also make use of chitinases to sustain their morphogenesis, growth and nutrition.[3,7-8] Intriguingly, chitinases have also been identified in some organisms that do not synthesize chitin, such as plants and humans. The discovery of chitinases and chitinolytic enzymes in these organisms therefore suggests other functions of these enzymes. It has been proposed that chitinases may play a role in the defense against pathogens.[1-2,9-10] For example, in plants, chitinolytic enzymes are secreted in response to the attack of microorganisms and insects.[11-14] Therefore, these enzymes are also known as pathogenesis-related protein (PR-protein) families. Furthermore, families 3, 4, and 8 of the PR-proteins are known to be associated with food allergies.[15]

Chitinases are useful in the production of many biomedical and biotech products. They can be used in the production of chitooligosaccharides, glucosamines and GlcNAc, which have an immense pharmaceutical potential.[16-17] Other applications such as mosquito control and plant pathogenic fungi control have also been investigated.[18-21] The applications have stimulated the discovery and research of new chitinases from many organisms.

The sweet potato is one of the major vegetable crops worldwide. In Taiwan, the storage roots, young leaves and shoots are consumed. Many of the properties and physiological functions such as trypsin inhibitors, proteolytic systems, and specific proteases have been extensively studied.[22-26] However, it was not until 1998 that chitinase activity was found in sweet potatoes.[27] Here we report the identification of novel chitinases in sweet potato leaves by electrophoresis using glycol chitin-embedded SDS-polyacrylamide gel.

RESULTS AND DISCUSSION

The crude protein extracts from the sweet potato were separated by SDS-PAGE. (Fig. 1(A)) In lanes 1 and 2, pro-

* Corresponding author. Tel: +886-2-25925252 ext. 3306 ext. 31; Fax: +886-2-25854735; E-mail: chunyi@ttu.edu.tw

tein staining revealed that, by heating the sample with β-mercaptoethanol, the protein bands in the lower molecular weight region appeared to be relatively noticeable, although the major protein bands were still located near 54-55 kDa. It seems that, by breaking the disulfide linkage between the subunits of a larger protein complex, the proteins with lower molecular weights were released and thus resulted in the enhanced detection in the lower molecular weight region on the gel.

While using the SDS-glycol chitin polyacrylamide gel, we are able to detect the chitinase activities by silver staining (Fig. 1(B)). The chitinolytic activity in the gel can be visualized by detecting the clearance of embedded glycol-chitin, which resulted in bright zones after the silver staining. As shown in lane 3, several light zones were detected, in which the two with higher molecular weights apparently have higher chitinolytic activity. In lane 4, the sample was heated with β-mercaptoethanol before electrophoresis. It appeared that only the activity at 55 kDa survived the treatment of β-mercaptoethanol and heat. The activities detected at the lower molecular weight range seemed to be abolished by the treatment.

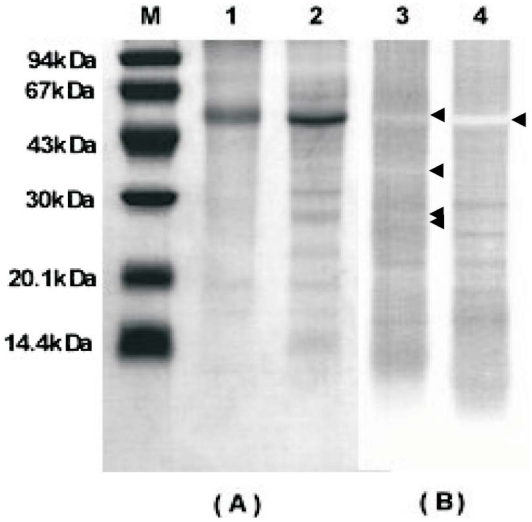

Fig. 1. Protein staining (A) on 15% SDS-PAGE by Coomassie Brilliant Blue R250 and activity staining (B) on 15% SDS-glycol chitin PAGE using the silver staining. Lane M, Commercial SDS-PAGE standards (Phamarcia, Amersham Biosciences Ltd.): phosphorylase b (94,000 Da), albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da), and α-lactalbumin (14,400 Da); lanes 1 and 3, crude extract of sweet potato leaves loaded with sample loading buffer without β-mercaptoethanol and are not heat-treated; lanes 2 and 4, crude extract of sweet potato leaves, heat-treated with sample loading buffer containing 100 mM β-mercaptoethanol. Arrows indicate the positions with chitinolytic activity in lanes 3 and 4.

The molecular weights of the major activity zones detected in lane 3 were preliminarily determined at 55.6 kDa and 39.6 kDa according to the calibration curve of the mobility of standards on SDS-PAGE (Fig. 2). The thermo stable chitinolytic activity in lane 4 was determined to be 54.1 kDa. The discrepancy of the estimated molecular weights of the largest chitinase detected in lanes 3 and 4 were almost indistinguishable. It is reasonable that different sample treatment may have slightly changed the migration pattern of the PAGE, as the slight difference seen between lanes 1 and 2. However, since it is unlikely that heating a protein sample with β-mercaptoethanol can possibly shift the 39.6 kDa band in lane 3 to 54 or 55 kDa, we conclude that the two chitinolytic zones in lane 3 were the results of two enzymes. On the other hand, the activities detected at 55.6 kDa and 54.1 kDa, respectively, in lanes 3 and 4 were the results of the same enzyme.

Both of the 39.6 and 54/55 kDa activities were apparently different in molecular weights from the previously identified chitinase in sweet potato, which has a molecular

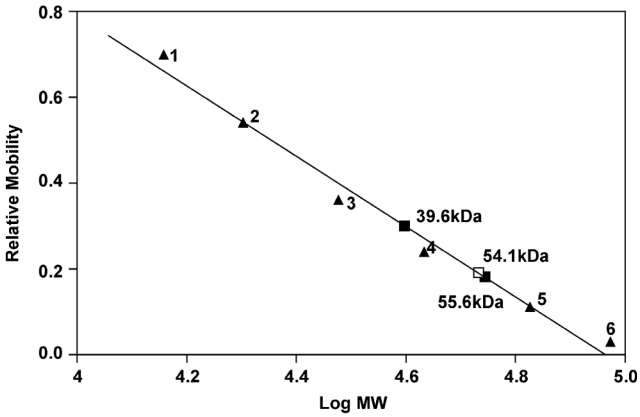

Fig. 2. Molecular weight determination of chitinases on SDS-glycol chitin PAGE. (▲) Commercial standards: 1, α-lactalbumin (14,400 Da), 2, trypsin inhibitor (20,100 Da), 3, carbonic anhydrase (30,000 Da), 4, ovalbumin (43,000 Da), 5, albumin (67,000 Da), 6, phosphorylase b (94,000 Da); (—) The calibration curve of the mobility of commercial standards; (■) Non-denatured extracts on SDS-glycol chitin PAGE; (□) Denatured extracts on SDS-glycol chitin PAGE.

weight of 16 kDa as reported by Hou et al.[27] In their study, the chitinase activity in the crude extract of sweet potato leaves was detected by overlaying another gel with incorporated glycol chitin onto the resolving gel. It is worth mentioning that several lesser clear zones were also detected in our SDS-glycol chitin PAGE analysis at lower molecular weight regions (Fig. 1(B), lane 3). It is plausible that the former studies fail to detect chitinases with higher molecular weights simply because the substrate they used in the activity staining was overlaid on the resolving gel rather than castled inside the gel. In our study, by embedding the glycol chitin in the resolving gel, direct interaction between the substrate and enzymes was assured. This allowed better detection of chitinolytic enzymes with higher molecular weights, which may have poorer mobility in the gel. Silver staining also allowed the direct visualization of clear zones with higher resolution than the previous methods.[30]

In conclusion, our analyses have identified new chitinolytic enzymes in sweet potato leaves. Further investigations on these chitinases are valuable in the understanding of their biochemical properties as well as their roles in host defense. The SDS-glycol chitin PAGE combined with silver stain is an excellent method in the detection of chitinolytic enzymes. The method is a simple and affordable way in the future discovery of new chitinases from different organisms.

EXPERIMENTAL SECTION

Plant materials

Fresh storage roots of the sweet potato (*Ipomoea batatas* [L.] Lam var. Tainong 57) were purchased from a local market in Taipei. The roots were placed in a thermostated (30 °C) growth chamber in the dark and water was sprayed twice a day. Sprouted roots of the sweet potato were planted in the open air and watered once every day. Leaves were collected and weighed immediately, put in an envelope and frozen in liquid nitrogen immediately. The frozen samples were kept at -70 °C for further use.

Crude extract from sweet potato leaves

Sweet potato leaves were milled to extremely fine powder in liquid nitrogen with a mortar and pestle. Powders were suspended in extraction buffer (20 mM sodium acetate buffer, pH 5.0, containing 0.7 mM β-mercaptoethanol) with a volume of 4 times the leaf weights and then homogenized by Polytron homogenizer (Kinematic, Switzerland) at 7,000 rpm for 3 min. The homogenate was centrifuged at 12,000 rpm for 20 min (Sigma 2K15, Rotor Nr. 12139). The supernatant was collected and designated as crude extract.

Electrophoresis

A discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate sample proteins under denatured conditions. 15% polyacrylamide gel was prepared in a vertical mini-gel system (Bio-Rad Laboratories, Richmond, CA) with a thickness of 1.0 mm. The gel, buffers and solutions were prepared as described by Weber and Osborn.[28] Samples (typically containing 10-15 μg total protein) for SDS-PAGE were preheated at 100 °C for 15 min in the SDS sample loading buffer (50 mM Tris-HCl, 2% (w/v) SDS, 100 mM β-mercaptoethanol, pH 6.8), run for 1 h at 120 V and then stained by Coomassie Brilliant Blue R-250. For molecular mass calibration, a subset of the following standards was included: phosphorylase b (94,000 Da), albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da), and α-lactalbumin (14,400 Da).

SDS-glycol chitin polyacrylamide gel electrophoresis and activity staining of chitinase

The SDS-glycol chitin polyacrylamide gel was used for the in-gel detection of chitinase activity. The electrophoresis was performed in a 15% discontinuous SDS-glycol chitin PAGE containing 0.01% (w/v) glycol chitin. The sample loading buffer is the same as that used in SDS-PAGE but β-mercaptoethanol was absent. After electrophoresis, gels were first immersed in a 0.1 M sodium acetate buffer, pH 5.0, containing 1% (v/v) deionized Triton X-100, placed on a shaker, and incubated for 30 min. The gels were then transferred to a fresh 0.1 M sodium acetate buffer, pH 5.0, and incubated in a 37 °C thermostated chamber for 12 h. After incubation, the activity staining was executed according to the method adopted from Marek et al.[29] Fixation was performed in a liquid solution containing 50% (v/v) methanol, 12% (v/v) acetic acid, and 0.0185% (v/v) formaldehyde. Gels were then incubated on a shaker for 10 min in 40% (v/v) ethanol and for 10 min in 30% (v/v) ethanol. Pretreatment, rinsing, and silver impregnation were performed as reported by Marek et al.[29] The developer solution diluted at 1:4 with water was used to prevent staining of proteins within the clear zone and to provide better contrast over development. The development time, which usually takes 1 to 2 min, could be slightly varied upon visual inspection. The time that the gel was rinsed by

pure water between the developer and stop solutions was shortened to 10 s. After the development was stopped, gels were washed in 30% (v/v) methanol for 20 min and 10% (v/v) methanol for 20 min and were then stored in 10% (v/v) methanol at 4 °C before drying. Silver-stained gels were dried with a gel dryer (Bio-Rad Laboratories, Richmond, CA) and preserved at room temperature.

ACKNOWLEDGMENT

This study was supported by grants NSC 96-2314-B-040-031 from the National Science Council, CSMU 96-OM-A-033 from Chung Shan Medical University, Taichung, and B95-S08-070 from Tatung University, Taipei, Taiwan.

Received July 11, 2007.

REFERENCES

1. Stintzi, A.; Heitz, T.; Prasad, V.; Wiedemann-Merdinoglu, S.; Kauffmann, S.; Geoffroy, P.; Legrand, M.; Fritig, B. *Biochimie* **1993**, *75*, 687.
2. Theis, T.; Stahl, U. *Cell Mol. Life Sci.* **2004**, *61*, 437.
3. Watanabe, T.; Kanai, R.; Kawase, T.; Tanabe, T.; Mitsutomi, M.; Sakuda, S.; Miyashita, K. *Microbiology* **1999**, *145*, 3353.
4. Graham, L. S.; Sticklen, M. B. *Can. J. Bot.* **1994**, *72*, 1057.
5. Renkema, G. H.; Boot, R. G.; Muijsers, A. O.; Donker-Koopman, W. E.; Aerts, J. M. *J. Biol. Chem.* **1995**, *270*, 2198.
6. Boot, R. G.; Blommaart, E. F. C.; Swart, E.; Ghauharali-van der Vlugt, K.; Bijl, N.; Moe, C.; Place, A.; Aerts, J. M. *J. Biol. Chem.* **2001**, *276*, 6770.
7. Rast, D. M.; Horsch, M.; Furter, R.; Gooday, G. W. *J. Gen. Microbiol.* **1991**, *137*, 2797.
8. Wang, S. L.; Shih, I. L.; Liang, T. W.; Wang, C. H. *J. Agric. Food Chem.* **2002**, *50*, 2241.
9. Hollak, C. E.; van Weely, S.; van Oers, M. H.; Aerts, J. M. *J. Clin. Invest.* **1994**, *93*, 1288.
10. Zhu, Z.; Zheng, T.; Homer, R. J.; Kim, Y. K.; Chen, N. Y.; Cohn, L.; Hamid, Q.; Elias, J. A. *Science* **2004**, *304*, 1678.
11. Büchter, R.; Strömber, A.; Schmelzer, E.; Kombrink, E. *Plant Mol. Bio.* **1997**, *35*, 749.
12. Takemoto, D.; Furuse, K.; Doke, N.; Kawakita, K. *Plant Cell Physiol.* **1997**, *38*, 441.
13. Molano, J.; Polacheck, I.; Duran, A.; Cabib, E. *J. Biol. Chem.* **1979**, *254*, 4901.
14. Sela-Buurlage, M. B.; Ponstein, A. S.; Bres-Vloemans, S. A.; Melchers, L. S.; Van Den Elzen, P.; Cornelissen, B. *Plant Physiol.* **1993**, *101*, 857.
15. Hoffmann-Sommergruber, K. *Biochem. Soc. Trans.* **2002**, *30*, 930.
16. Dahiya, N.; Tewari, R.; Hoondal, G. S. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 773.
17. Donnelly, L. E.; Barnes, P. J. *Trends Pharmacol. Sci.* **2004**, *25*, 509.
18. Shirazi, F.; Kulkarni, M.; Deshpande, M. V. *Lett. Appl. Microbiol*. **2007**, *44*, 660.
19. Li, F.; Patra, K. P.; Vinetz, J. M. *J. Infect Dis.* **2005**, *192*, 878.
20. Chang, W. T.; Chen, C. S.; Wang, S. L. *Curr. Microbiol.* **2003**, *47*, 102.
21. Lima, L. H.; Ulhoa, C. J.; Fernandes, A. P.; Felix, C. R. *J. Gen. Appl. Microbiol.* **1997**, *43*, 31.
22. Lin, Y. H. *J. Amer. Hort. Sci.* **1989**, *114*, 814.
23. Lin, Y. H.; Chu, H. H. *J. Chin. Biochem. Soc.* **1989**, *18*, 18.
24. Lin, Y. H.; Chan, H. Y. *Bot. Bull. Acad. Sin.* **1990**, *31*, 19.
25. Lin, Y. H.; Tsai, M. G. *Bot. Bull. Acad. Sin.* **1991**, *32*, 79.
26. Yan, T. R.; Liau, C. Y.; Lin, Y. H. *J. Agric. Food Chem.* **1995**, *43*, 2035.
27. Hou, W. C.; Chen, Y. C.; Lin, Y. H. *Bot. Bull. Acad. Sin.* **1998**, *39*, 93.
28. Weber, K.; Osborn, M. *J. Biol. Chem.* **1969**, *244*, 4406.
29. Marek, S. M.; Robert, C. A.; Beuselinck, P. R.; Karr, A. L. *Anal. Biochem.* **1995**, *230*, 184.
30. Trudel, J.; Asselin, A. *Anal. Biochem.* **1989**, *178*, 362.

JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 106, No. 1, 111–113. 2008
DOI: 10.1263/jbb.106.111

A Modified Coomassie Brilliant Blue G 250 Staining Method for the Detection of Chitinase Activity and Molecular Weight after Polyacrylamide Gel Electrophoresis

Chun Yi Liau[1,2] and Chung-Sheng Lin[1]*

Institute of Medicine, Chung Shan Medical University, Taichung 402, Taiwan, R.O.C.[1]
and Department of Bioengineering, Tatung University, Taipei 104, Taiwan, R.O.C.[2]

Received 4 February 2008/Accepted 4 April 2008

A modified Coomassie Brilliant Blue G 250 staining method for detecting chitinolytic enzymes in chitin-containing polyacrylamide gel electrophoresis (PAGE) is presented. The staining formed achromatic zones at the locations of the migrated enzyme. Using *Streptomyces griseus* chitinase, we have demonstrated that our method is more sensitive and less complicated than the conventional Calcofluor white M2R staining.

[**Key words:** chitinase, SDS–PAGE, Coomassie Brilliant Blue G 250]

Chitinases (EC 3.2.1.14) catalyze the hydrolysis of chitin, a β-1,4-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc), and is the main structural component of fungal cell walls and arthropod integuments. By means of endochitinase activity, chitinolytic enzymes hydrolyze chitin to produce chitooligosaccharides with two to six GlcNAc units (1). The chitinolytic enzymes have been discovered in a variety of organisms ranging from bacteria and fungi to plants and humans (2–8). It is plausible that chitin-producing organisms also produce chitinases for physiological functions such as modulation of the formation of cell walls and exoskeletons (2, 3). Intriguingly, chitinases have also been identified in plants (4–6) and humans (7, 8), organisms that do not synthesize chitin. In case of plants, several of these chitinases were believed to be associated with the anti-pathogen abilities of certain plants (4), while in case of humans, they were believed to associated with food allergies for edible plants (5, 6). In humans, chitotriosidase is expressed by macrophages and can also be detected in atherosclerotic plaques. Moreover, it is known that chitotriosidase levels are elevated in the serum of patients with Gaucher's disease, the most common lysosomal storage disease (7). Till date, the presence and function of chitinases in non-chitin producers, such as plants and humans, have not been investigated thoroughly because of procedural limitations. Therefore, we believe that the development of a faster and more convenient method of discovery of chitinases and chitinolytic enzymes will allow better future investigation and understanding of their functions.

Methods of visible detection of chitinase activity have been available since 1989, when Trudel and Asselin presented their method for detecting chitinolytic activity (9). This method utilized denaturing polyacrylamide gel electrophoresis for the separation of samples. By casting the glycol chitin into the resolving gel, dark zones can be visualized in the gel at the position of the chitinase activity using Calcofluor white M2R (Cw-M2R) staining and a transilluminator. Since simultaneous visualization of multiple chitinases in a complex mixture is difficult using this method, a silver stain was subsequently introduced in a modified method in 1995, which successfully overcame the narrow dynamic range problem of the Cw-M2R stain (10). However, although the modified silver stain method is relatively simple in comparison to the Cw-M2R method, the procedure is still time-consuming and complex.

In the present study, we report a simple and rapid detection technique of *in situ* glycol chitin activity staining. As a demonstration, we subjected *Streptomyces griseus* chitinase (Sigma Chemical, St. Louis, MO, USA) with defined activity units to electrophoresis, utilizing 12% SDS–PAGE prepared according to the Laemmli's method (11), but containing 0.01% (w/v) glycol chitin (Sigma Chemical). The chitinase samples were mixed with the β-mercaptoethanol-free SDS sample-loading buffer (50 mM Tris–HCl, 2% [w/v] SDS, pH 6.8) and subjected to electrophoresis at 140 V for 1 h using a protein vertical mini-gel electrophoresis system (Hoefer Mighty Small S250 apparatus; Amersham Biosciences, Piscataway, NJ, USA). After electrophoresis, we removed the separation gels from the glass plates and immersed them in 0.1 M sodium acetate buffer (SAB) (pH 5.0), containing 1% (v/v) deionized Triton X-100, and then incubated them with gentle agitation for 15 min at room temperature for removal of SDS. The gels were then transferred to fresh SAB and incubated in a 37°C thermostatic chamber for the *in-gel* chitinolytic reaction. After incubation, gels were transferred into staining solution, which was modified from the Coomassie Brilliant Blue G 250 (CBB-G250) staining (12) and contained of 0.025% (w/v) CBB-G250 (Merck, Darmstadt, Germany), 25% isopropanol, and 10% acetic acid. After staining for

* Corresponding author. e-mail: lcs@csmu.edu.tw
phone: +886-4-24739595 ext. 34711 fax: +886-4-24739220

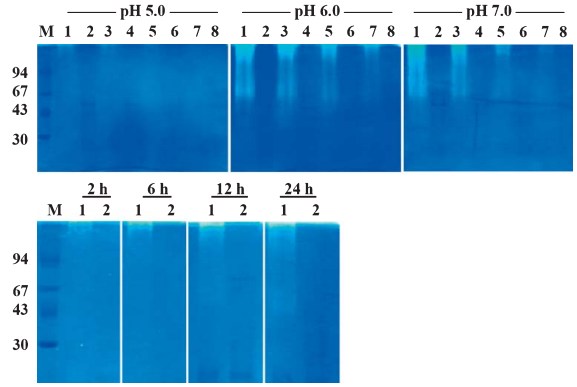

FIG. 1. (A) Chitinase activity staining performed under different pH values. The resolving gels were immersed and incubated in 0.1 M sodium acetate buffer (pH 5.0) and 0.1 M sodium phosphate buffer (pH 6.0 and 7.0) at 37°C for 24 h and then stained using CBB-G250. Chitinase activity units loaded in lanes 1and 2, 3 and 4, 5 and 6, and 7 and 8 were 5.0×10^{-4}, 2.5×10^{-4}, 1.3×10^{-4}, and 6.5×10^{-5}, respectively. Lanes 2, 4, 6, and 8 were heat-treated with SDS sample-loading buffer. (B) CBB-G250 staining after prolonged chitinolytic incubation. Chitinase activity units loaded in each lane were 6.5×10^{-5}. The samples loaded in the lane 2 of each gel were heat-treated in SDS sample-loading buffer prior to electrophoresis. The resolving gels were immersed and incubated in 0.1 M sodium phosphate buffer, pH 6.0, at 37°C for 2 h, 6 h, 12 h, and 24 h, respectively.

10 min, the gels were destained to remove the background using destaining solution (15% methanol and 10% acetic acid). Finally, we dried the gels on chromatography paper (3MM Chr; Whatman International, Maidstone, UK) using a vacuum gel dryer (model 583; Bio-Rad, Hercules, CA, USA).

Enzymatic reactions at different pH conditions can be performed by immersing the gels in buffers of different pH values (pH 5.0, 0.1 M sodium acetate buffer; pH 6.0 and pH 7.0, 0.1 M sodium phosphate buffer). As a demonstration, triplicates of the chitin-containing SDS–PAGE were carried out with different amount of *S. griseus* chitinase loaded in the wells (Fig. 1A). Prior to electrophoresis, the enzymes mixed with SDS sample-loading buffer (50 mM Tris–HCl, 2% (w/v) SDS, 100 mM β-mercaptoethanol, pH 6.8) were placed in boiling water for 15 min then loaded in lanes 2, 4, 6, and 8. Non-heat-treated enzyme samples mixed with β-mercaptoethanol-free SDS sample-loading buffer (50 mM Tris–HCl, 2% [w/v] SDS, pH 6.8) were loaded in lanes 1, 3, 5, and 7 (in the same quantities as those in lanes 2, 4, 6, and 8, respectively). For the *S. griseus* chitinase, the optimal reaction pH is between 6 and 7. As demonstrated in Fig. 1A, we carried out the reactions at pH 5.0, 6.0, and 7.0, respectively, for the *S. griseus* chitinase sample. The results suggested an optimal reaction pH in the predicted range. We also tested the in-gel reaction for prolonged incubation at pH 6.0 at 37°C. In Fig. 1B, the visible achromatic zones showed an increase after 12 h and were even more clear and readable after 24 h.

We performed a side-by-side comparison for evaluating the detection sensitivity of our CBB-G250 staining method against the Cw-M2R method. We separated different amounts of *S. griseus* chitinase in chitin-containing 12% SDS–PAGE by electrophoresis in a duplicated set up as aforementioned.

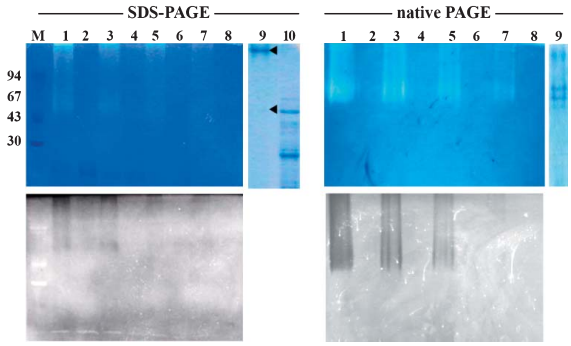

FIG. 2. The detection sensitivities of chitinase activities on chitin-containing SDS- and native PAGE. In all the gels for activity staining, *S. griseus* chitinase with activity units of 1.3×10^{-4}, 6.5×10^{-5}, 3.0×10^{-5}, and 1.5×10^{-5} were loaded respectively in lanes 1 and 2, 3 and 4, 5 and 6, and 7 and 8. Lanes 2, 4, 6, and 8 were preheated at 100°C for 15 min in the sample-loading buffer to inactivate the enzyme. (A) The two chitin-containing SDS–PAGE gels stained with the CBB-G250 (above) and the Cw-M2R (below) staining methods are shown. After electrophoresis, both SDS gels were placed in a 0.1 M sodium phosphate buffer, pH 6.0, containing 1% (v/v) deionized Triton X-100 to remove SDS. The gels were then transferred to a fresh buffer and incubated at 37°C for 24 h. After incubation, the gels were either stained with CBB-G250 staining solution or Cw-M2R solution (0.01% (w/v) Calcofluor white M2R [Sigma Chemical, St. Louis, MO, USA] in 500 mM Tris–HCl, pH 8.9) as described by Trudel *et al.* (9). (B) The two chitin-containing native PAGE gels stained with the CBB-G250 (above) and the Cw-M2R (below) methods are shown. The procedures are basically identical to that of the chitin-containing SDS–PAGE, but the SDS removal step is skipped. The result of 2.0 μg *S. griseus* chitinase on conventional SDS–PAGE and native PAGE stained by Coomassie Brilliant Blue R 250 using the standard protocols are shown beside the results of CBB-G250 staining to indicate the corresponding location of the migrated protein. Lane 9 of both the SDS–PAGE and native PAGE contained non-heat-treated samples. The arrows in lane 9 indicate the two protein bands formed by non-heated chitinase. Lane 10 of the SDS–PAGE was loaded with heat-treated chitinase sample.

The samples loaded in lanes 2, 4, 6, and 8 were same in quantity as those loaded in lanes 1, 3, 5, and 7, respectively, but were heat-inactivated as described above. After electrophoresis, we stained the two gels using CBB-G250 and Cw-M2R, respectively (Fig. 2A). The chitinase activity units from lanes 1 and 2, 3 and 4, 5 and 6, and 7 and 8 were 5.0×10^{-4}, 2.5×10^{-4}, 1.3×10^{-4}, and 6.5×10^{-5}, respectively. Moreover, we performed another side-by-side comparison for the staining methods using 10% native PAGE with chitin added to the gel. The results were shown in Fig. 2B. With the result of a conventional SDS–PAGE and native PAGE indicating the location of the migrated chitinase (see the black arrows in lane 9 in Fig. 2A), our results showed that chitinase activity, indicated by clear achromatic zones after the CBB-G250 staining, is clearly visible in all of the lanes loaded with different amounts of active chitinases (Fig. 2A, B). As for the Cw-M2R method, the dark zones representing the chitinase activity are barely visible in the lanes loaded with 6.5×10^{-5} chitinase activity units in the gel without SDS and are not detectable for less than 2.5×10^{-4} chitinase activity units loaded in SDS-containing gels. Inevitably, to preserve the enzymatic activity, the samples must not be heat-treated prior to electrophoresis in either method. However, lack of heating might have limited the separation power of

electrophoresis, and thus, resulted in broad activity zones (see Figs. 1 and 2). It appears that the detection of chitinase activity using CBB-G250 is at least comparable, if not superior, to the Cw-M2R method in chitin-containing SDS–PAGE (Fig. 2A).

Since their introduction in 1963, the Coomassie brilliant blue dyes have been conventionally used for protein staining (12). The CBB-G250 dye is less soluble than the CBB-R250 and produces a somewhat brighter blue coloration. As a result of its lower solubility, CBB-G250 preferentially binds to the protein bands and not to the polyacrylamide gel (12). Binding of CBB-G250 to the proteins is facilitated by electrostatic attraction of its $-SO_3^-$ groups and is enhanced by hydrophobic interactions (13, 14). Glycol chitin is cationic and can be preferentially stained with CBB-G250.

In the chitinolysis incubation step, the incorporated glycol chitin was hydrolyzed by chitinase to monomers or oligomers of GlcNAc, which were subsequently washed away from the gel. The resulting achromatic activity-zones were visible at the location of the migrated chitinase band. The CBB-G250 method described in this report is an alternative to the well-established Cw-M2R staining and silver staining methods for the detection of chitinase activity in polyacrylamide gel. The CBB-G250 staining method is less expensive, less complicated, and less toxic. Moreover, quantitative analysis on a silver stained gel is difficult; therefore, Coomassie brilliant blue has another advantage over silver staining.

In conclusion, the modified CBB-G250 staining technique is an appealing alternative to the current methods used for detecting chitinolytic enzymes in chitin-containing PAGE. The method applies to both SDS- and native chitin-containing PAGE and possesses comparable detection sensitivity to that of other methods.

This study was supported by grants NSC 96-2314-B-040-031 from the National Science Council, CSMU 96-OM-A-033 from Chung Shan Medical University, Taichung, and B94-S03-064 from Tatung University, Taipei, Taiwan.

REFERENCES

1. **Koga, D., Mistutomi, M., Kono, M., and Matsumiya, M.:** Biochemistry of chitinases. EXS, **87**, 111–123 (1999).
2. **Theis, T. and Stahl, U.:** Antifungal proteins: targets, mechanisms and prospective applications. Cell Mol. Life Sci., **61**, 437–455 (2004).
3. **Wang, S. L., Shih, I. L., Liang, T. W., and Wang, C. H.:** Purification and characterization of two antifungal chitinases extracellularly produced by *Bacillus amyloliquefaciens* V656 in a shrimp and crab shell powder medium. J. Agric. Food Chem., **50**, 2241–2248 (2002).
4. **Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M., and Fritig, B.:** Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie, **75**, 687–706 (1993).
5. **Hoffmann-Sommergruber, K.:** Pathogenesis-related (PR)-proteins identified as allergens. Biochem. Soc. Trans., **30**, 930–935 (2002).
6. **Sanchez-Monge, R., Blanco, C., Diaz-Perales, A., Collada, C., Carrillo, T., Aragoncillo, C., and Salcedo, G.:** Isolation and characterization of major banana allergens: identification as fruit class I chitinases. Clin. Exp. Allergy, **29**, 673–680 (1999).
7. **Hollak, C. E., van Weely, S., van Oers, M. H., and Aerts, J. M.:** Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease. J. Clin. Invest., **93**, 1288–1292 (1994).
8. **Zhu, Z., Zheng, T., Homer, R. J., Kim, Y. K., Chen, N. Y., Cohn, L., Hamid, Q., and Elias, J. A.:** Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. Science, **304**, 1678–1682 (2004).
9. **Trudel, J. and Asselin, A.:** Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal. Biochem., **178**, 362–366 (1989).
10. **Marek, S. M., Robert, C. A., Beuselinck, P. R., and Karr, A. L.:** Silver stain detection of chitinolytic enzymes after polyacrylamide gel electrophoresis. Anal. Biochem., **230**, 184–185 (1995).
11. **Laemmli, U. K.:** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**, 680–685 (1970).
12. **DeSilva, T. M., Ursitti, J. A., and Speicher, D. W.:** Protein detection in gels using fixation, p. 10.5.1–10.5.12. *In* Coligan, J. E., Dunn, B. M., Pleogh, H. L., Speicher, D. W., and Wingfield, P. T. (ed.), Current protocol of protein science, vol. 1. John Wiley & Sons, NY (1995).
13. **Tal, M., Silberstein, A., and Nusser, E.:** Why does Coomassie Brilliant Blue R interact differently with different proteins? A partial answer. J. Biol. Chem., **260**, 9976–9980 (1980).
14. **Wilson, C.:** Staining of proteins on gels: comparison of dyes and procedures. Methods Enzymol., **91**, 236–247 (1983).