臺灣二 OO 四年國際科學展覽會

科　　別：生物化學科

作品名稱：八分鐘快速免疫呈色法檢測市售牛乳中有無摻雜粉

得獎獎項：生物化學科第一名
美國第五十五屆國際科技展覽會正選代表

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作 者 簡 介

我的姓名是彭資貽，目前就讀於新竹女中數理資優班二年級，家庭成員有一位妹妹、父親及母親。從小我就非常喜歡生物、生命科學、化學及數學，也非常喜歡做實驗。高一時，我考上國科會高中生人才培育計畫，進入交大生科研究所毛仁淡教授實驗室，開始我三年的實驗學習及完成我這次的國際科展實驗；高二的寒假，我幸運地爭取到代表學校出席中部教育局主辦的 2004 年全國高中生生物聯合盟。在我進入了生物領域後，從中我學習到了冷靜、判斷、思考、邏輯及不怕挫折的心和自信，更懂得對自己及實驗永不放棄，希望有一天自己能成爲一偉大的科學家。

中文摘要

台灣過去 40 年來，許多牛乳廠商會因牛乳供應量不足摻雜奶粉以增加利潤，爲了解決這問題，本實驗提供一種快速且準確的免疫呈色法檢測牛乳中有無摻雜奶粉。因奶粉的加工過程中加熱是必須的，所以本實驗是利用單株抗體只與牛乳中因加熱而變性的蛋白質反應。實驗步驟非常簡單，首先，用一支玻棒沾附待測鮮乳，經過簡短的清洗及化學處理後，玻棒尖端會與經特殊製備的單株抗體反應。最後，將玻棒放入已製備好的溶劑中呈色。當溶液呈現綠色，表示待測乳中摻有奶粉。整個實驗流程只需短短八分鐘。據我所知，該方法是相當具有新穎性且從來未被應用過。這是個令人興奮的發現，足以解決多年來酪農業中牛乳摻雜奶粉的問題。

英文摘要

For the last 40-years, owing to the limited supply of commercial raw milk, the industry sometimes would mix the dry milk into the raw milk to increase their profit. To detect whether or not the milk on the market contains the poor quality’s dry milk, I invented a rapid and sensitive colorimetric immunoassay. The assay essentially utilizes a monoclonal antibody that only reacted with the thermal denatured protein presented in the dry milk. First, a glass tip-stick was dipped in to the milk to be tested. Second, following a brief wash and chemical treatment, the glass tip was reacted with the monoclonal antibody that has been specifically produced. Finally, the glass tip was dipped into a reagent containing developer. When the solution develops a color in green, it indicates the milk definitely contains dry milk. The entire procedure only takes 8 minutes to finish. To the best of my knowledge, this method is novel and has never been shown before. It represents an exciting discovery that solves the malpractice of mixing dry and raw milk in our dairy industry.

壹、前言:

一、 研究動機:

在現在經濟發達，社會繁榮的時代裡，人類的營養源可說是既齊全又豐富，其中人們常飲用的鮮奶，更是現代人攝取蛋白質不可或缺的飲品，但牛乳的生產量會受到季節、溫度等因素的影響而供不應求。以台灣來說，每逢夏季，市場上牛乳就有此情況，造成有些商家爲了應付大量消費者需求，利用奶粉沖泡成還原乳，販售於市面，使消費者的權益受損。在世界各地許多國家，對於鮮奶中摻雜奶粉的問題皆無法徹底解決。於是本實驗欲利用抗體專一性，研發快速且簡便的免疫檢測法，來解決這惱人的問題。長久以來無法解決此問題的主因爲鮮乳及奶粉中的蛋白質組成極爲類似，兩者的差異僅在於加工過程中不同的熱處理方式，如此增加尋找此乳蛋白變異的困難度。所以本實驗主要採用的方式爲製作出奶粉的專一性抗體，再利用鮮乳及奶粉的交互篩選，挑選出只會與奶粉結合的單株抗體，再利用此專一抗體爲試劑，開發出抗體結合玻璃棒的快速免疫檢測法，用以檢驗國內牛乳品質。期待藉由這個實驗可以保護消費者權益，使消費者買得品質優良之鮮乳。

二、 研究目的:

1. 篩選出奶粉的專一性抗體。
2. 研發快速又準確的方法來檢測鮮奶中有無摻雜奶粉。
3. 利用單株抗體分析牛乳經不同溫度加熱不同時間的影響。

貳、研究方法與過程:

一、實驗器材

1. 玻璃棒：一端被摩擦成粗糙面
2. 還原乳 (250 g 即溶奶粉/200 g 去離子水)
3. 牛乳 (已離心去脂肪)
4. 清洗液 (0.1％ gelatin + 0.05％ Tween 20/PBS)：除去玻璃棒未沾附的物質。
5. 填充液(1％ gelatin/PBS)：將玻璃棒未沾附蛋白處填滿(blocking)。
6. 單株抗體(具有 HRP)
7. 呈色液 (ABTS + 0.01％ H_2O_2)
8. 電泳下片膠配方 (15％ 5 mL):
   (1) ddH_2O 2.6 mL
   (2) 30％ acrylamide mix 1.0 mL
   (3) 1.5 M Tris-HCl, pH 8.8, 1.3 mL
   (4) 10％ SDS 0.05 mL
   (5) 10％ ammonium persulfate
   (6) TEMED 0.004 mL
9. 電泳上片膠配方 (5% 1 mL)
   (1) ddH_2O 0.68 mL
   (2) 30％ acrylamide mix 0.17 mL
   (3) 1.0M Tris-HCl, pH 6.8, 0.13 mL
   (4) 10％ ammonium persulfate 0.01 mL
   (5) TEMED 0.001 mL
10. 5x SDS loading buffer 配方:
   (1) ddH_2O 0.9 mL
   (2) 1 M Tris-HCl, pH6.8, 0.6 mL
   (3) 50％ Glycerol 5 mL

(4) 10％ (w/v) SDS　2 mL

(5) β-mercaptoethanol　0.5 mL

(6)1％ (w/v) bromophenol blue 1 mL

11.電泳樣品:

(1) 12.5％ 牛奶稀釋 1:50

(2) 牛乳稀釋 1:50

12. Maker (prestain):

(1) β-galactosidase　118 kDa

(2) bovine serum albumin　86 kDa

(3) Ovalbumin　47 kDa

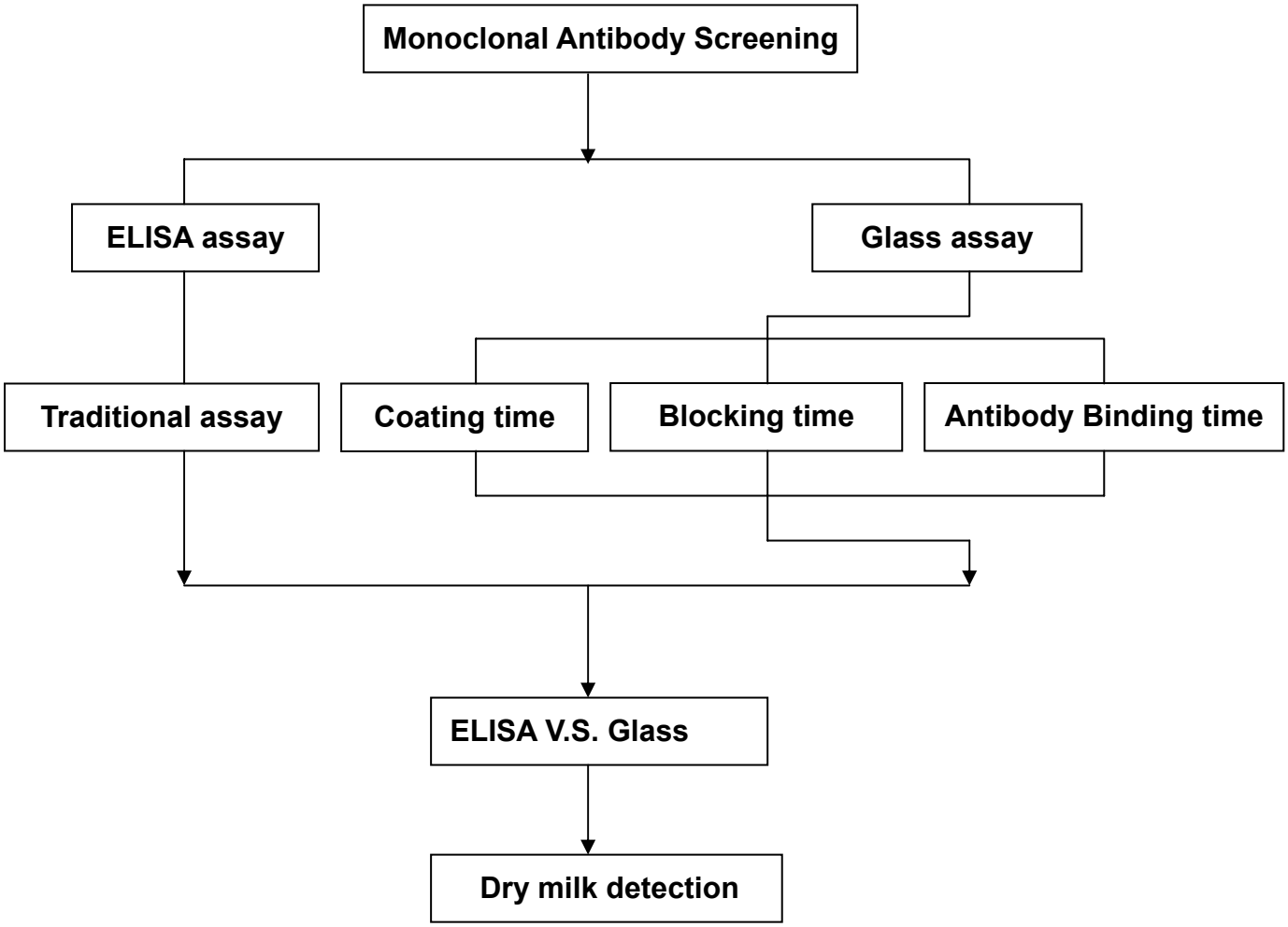
(4) Cartonic anhydrase　34 kDa

(5) β-lactoglobulin　26 kDa

(6) Lysozyme　19 kDa

13.Coomassie Blue staining solution (50％甲醇，10％醋酸，0.125％ [w/v] Coomassie Brilliant Blue R250)

二、實驗步驟流程圖，如圖一：



圖一: 實驗流程圖

1.過氧化氫酵素 (horseradish peroxides, HRP) 之單株抗體 (Monoclonal Antibody , mAb) 的製作方法:

(1)免疫小鼠 (Immunization)

a. 第零天，注射第一劑 (將 antigen (dry milk) 及佐劑等體積混合均勻，取出 100 uL 注射老鼠的腹腔及皮下)。

（圖二）

b. 第七天，打入第二劑。 (第二劑佐劑爲 incomplete 佐劑)

c. 第十四天，打入第三劑。 (第三劑佐劑爲 incomplete 佐劑)

d. 第十七天，測 titer。

(2)單株抗體的製作 (Monoclonal antibody production) ：

a. 解凍 FO 細胞，且大量繁殖。

b. 製作前 1~2 天將 FO 細胞以 1: 2 繼代培養。

c. 取老鼠脾臟與 FO 細胞以 5: 1 混合，做細胞融合。

d. 加入 1ml PEG/OMSO 在 1 分鐘內加完，並邊加邊混合均勻。

e. 放入 37℃培養一分鐘。

f. 緩慢加入 10 ml 的細胞培養液 (DMEM) ，2 分鐘內加完。

g. 用細胞培養液清洗 3 次 (將 PEG/OMSO 清洗掉)。

h. 加入含有 HAT 及 20% FBS 的 HT 培養液 50 ml。

i. 將細胞以 10^4~10^5的數目分別放入 96well 的細胞培養盤。

j. 培養 14 天。

k. 細胞數佔孔洞約 50~90% ，培養液轉黃取 50 1，做 ELISA 篩選。

l. 將陽性細胞群落單株化。

m. 以 ELISA 篩選陽性單株細胞群落。

n. 大量繁殖陽性單株細胞，收集培養液，做後續實驗用。

(3) HRP 接合的步驟 (原理如圖三)：

a. 將 2mg HRP 溶解在 50ml　0.1M 檸檬酸鈉鹽溶液（sodium citrate buffer）中，調整至 pH5，加熱至 37℃。

b. 將 HRP 上的葡萄糖（glucose group）氧化。將 Sodium meta-period ate（NaIO430 ml /ml in ddH2O）和 HRP 依 40:1 的莫耳濃度混和，計時 5 分鐘。

c. 停止反應。 將 Ethylene glycol（FW：62 Density：1.113　16M）稀釋至 0.01M

d. 準備 Antibody：

（a） 將 Antibody 和 1.0M NaHCO3 buffer（pH10）依 10：1 混合。

（b） 準備 0.001M 的 NaBH3CN（10mg/ml）。將 HRP 和 Antibody 依質量 6:1 混合，反應必須在 4℃下持續 24 小時。

（c） 用 NaH2PO4 調整 2 步驟的產物至 pH6.0。用相同體積的 NaBH3CN 和步驟三的產物混合，放置在室溫下 30 分鐘。

e. 將未接上的 HRP 去除：

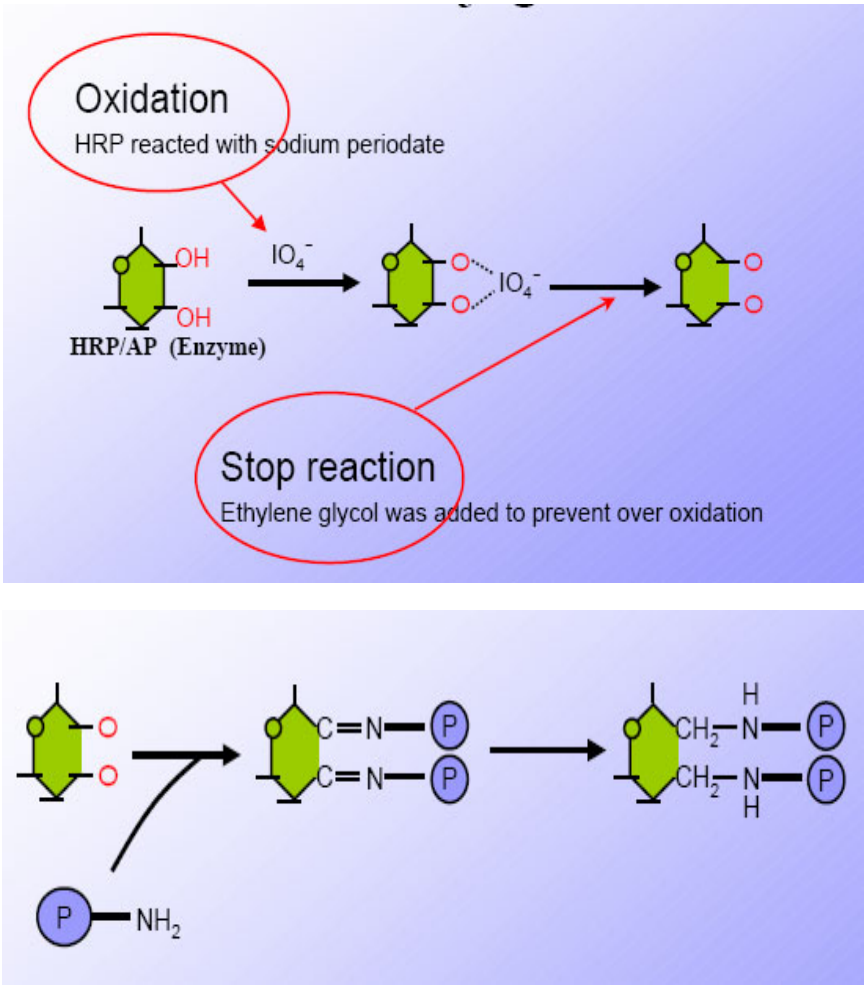
（a）取相同體積 100% （NH_4）$_2SO_4$和步驟 4.的產物混合好，放在冰上 30 分鐘。

（b）離心 10000 x g，20 分鐘。

（c）去上清液，加 5 cc PBS 溶 ppt，加 100% （NH4）2SO4 和上述步驟二的產物混合好，放置冰上 30 分鐘，再離心 10000 x g，20 分鐘。

（d）最後將沉澱溶解在 2ml 的 PBS 加 50% 甘油。

f. 完成後，利用 ELISA assay 測定抗體與酵素結合後之力價（titer）。



圖三:抗體及 HRP 結合原理:將 HRP 之醣基的氧化，成兩個醛基，使抗體上的胺基與醛基能結合因此酵素及抗體能結合成功。

2.利用 SDS-PAGE 及 Western Blot 確認抗體所認之蛋白質：

(1) SDS-PAGE

（a） 將製膠器具架好，注入 5mL 下片膠配方於玻璃之間的溝槽，以去離子水覆蓋其表面，靜至 30 分鐘待其完全凝結。

（b） 去除去離子水，注入適當體積的上片膠配方，立即斜插入 comb，避免產生氣泡，靜置 30 分鐘。待其凝結後小心移去 comb。

（c） 將凝結完成的膠片連同玻璃片，架設於電泳槽上，倒入 SDS Tris-glycine electrophoresis buffer (25mM Tris、250mM glycine [pH8.3] 及 0.1% SDS 於 ddH2O)，將樣品與 5x SDS loading buffer 混合後，連同 maker 取適當量注入孔中，以 14mA 進行電泳。

（d） 電泳結束後，將膠片移至染色劑中約 30 分鐘，去除染色劑並加褪染劑一(50%甲醇及 10%)褪染。

（e） 加褪染劑二(5%甲醇及 7%醋酸)褪染。

（f） 乾燥膠片。

(2) 西方轉漬法 (Western blot):

（a） 以與膠片同樣面積之硝化纖維膜 (Amersham Pharmacia Biotech)和 6 片 3MM paper (Whatman) 放置於 transfer buffer (48 mM Trisbase、39mM glycine、0.037%SDS、20% methanl) 浸泡 15 分鐘。

（b） 當 SDS-PAGE 電泳完成後，將膠片用二次水略爲沖洗，浸泡於 transfer buffer 中。

（c） 使用 Semi-dry transfer machine(Bio-rad)，將 3 片 MMpaper 平鋪，上爲硝化纖維膜，將膠覆蓋於硝化纖維膜上，最後覆蓋另外 3 片 3MM paper，步驟中皆避免氣泡產生。

（d） 以 90mA 電流 50 分鐘進行 transfer。

（e） 取出硝化纖維膜，以 PBS 略爲沖洗後，浸泡於填充液中並放至於緩慢運轉的 shaker 上 1 小時。

（f） 取出硝化纖維膜，以 PBS 清洗未沾黏的填充液，放置震盪器上 5 分鐘，重複三次。

（g） 取出硝化纖維膜，浸泡於內含 0.1% gelatin 及 0.05%tween-20 的 PBS 溶液以 1:1000 稀釋的初級抗體溶液取出硝化纖維膜，並放至於緩慢運轉的 shaker 上 1 小時。

（h） 取出硝化纖維膜，以清洗液清洗未沾黏的初級抗體，放置震盪器上 5 分鐘，重複三次。

（i） 取出硝化纖維膜，浸泡於內含 0.1% gelatin 及 0.05%tween-20 的 PBS 溶液以 1:2500 稀釋的二級抗體溶液並放至於緩慢運轉的 shaker 上 1 小時。

（j） 取出硝化纖維膜，以清洗液未沾黏的二級抗體，放置震盪器上 5 分鐘，重複三次。

再用 PBS 略爲清洗。

（k） 放入 DAB 呈色溶液 (12.5 mg DAB 溶於 500 µl DMSO、600 µl 1%$CoCl_2$、50 µl 30% H_2O_2、29 mL PBS 混合均勻)

（1） 以二次水終止反應，壓乾且護貝。

3. ELISA assay

(1) 取測定乳加至 96 孔 ELISA 分析盤(96 well ELISA plate)中，每一孔洞 50 µl(50 µl / well)，靜置 30 分鐘。

(2) 清洗 3~4 次。

(3) 取填充液加至 ELISA 分析盤中，每一孔洞 350 µl ，靜置 30 分鐘。

(4) 清洗 3~4 次。

(5) 取辨識奶粉的單株抗體液加至 ELISA 分析盤中，每一孔洞 50 µl ，靜置 30 分鐘。

(6) 清洗 3~4 次。

(7) 取標示有酵素之抗小鼠免疫球蛋白抗體(Anti-mouse IgG conjugate with HRP)加至 ELISA 分析盤中，每一孔洞 50 µl ，靜置 30 分鐘。

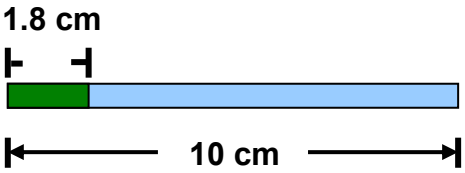
(8) 清洗 3~4 次。

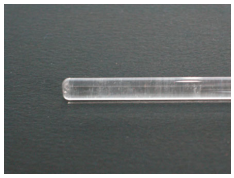
(9) 利用 ABTS 呈色。

4. 玻璃棒實驗 (Glasses assay)：如圖四所示

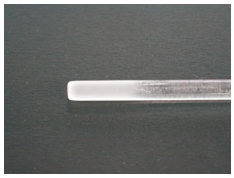
(1) 摩擦玻璃棒：

將玻璃棒用砂紙來回摩擦 8 次，粗糙面長度 1.8cm。





光滑玻璃棒



粗糙玻璃棒

(2) 測定沾附時間(coating time)

a. 將玻璃棒沾附待測乳時間分別爲 15 秒,30 秒,1.5,2.5,5,10,20,30 分。

b. 清洗 5~8 次。

c. 將玻璃棒沾附填充液 30 分鐘.

d. 清洗 5~8 次。

e. 玻璃棒沾附 mAb 液 30 分鐘.

f. 清洗 5~8 次。

g. 利用 ABTS 呈色。

(3) 測定沾附填充液時間(blocking time)

a. 將玻璃棒沾附待測乳 30 分鐘。

b. 清洗 5~8 次。

c. 將玻璃棒沾附填充液時間分別爲 15 秒,30 秒,1.5,2.5,5,10,20,30 分鐘。

d. 清洗 5~8 次。

e. 玻璃棒沾附 mAb 液 30 分鐘.

f. 清洗 5~8 次。

g. 利用 ABTS 呈色。

(4) 測定玻璃棒沾附 mAb 液時間

a. 將玻璃棒沾附待測乳 15 秒。

b. 清洗 5~8 次。

c. 將玻璃棒沾附填充液 2.5 分鐘。

d. 清洗 5~8 次。

e. 玻璃棒沾附 mAb 液時間分別爲 15 秒,30 秒,1.5,2.5,5,10,20,30 分鐘。

f. 清洗 5~8 次。

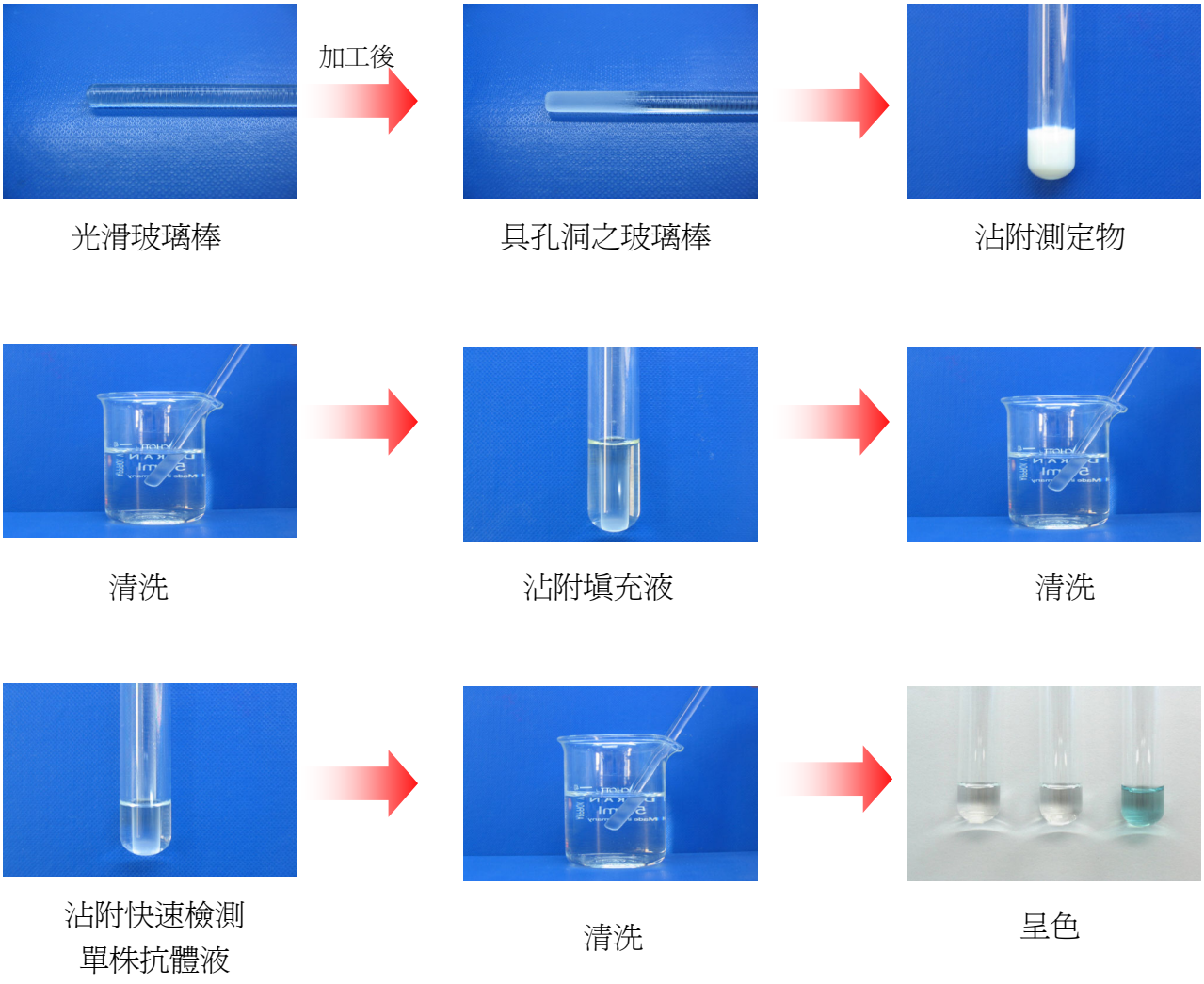
g. 利用 ABTS 呈色。

5.利用單株抗體分析牛乳經不同溫度加熱不同時間的影響：

(1) 將加熱器加熱至不同的溫度，分別爲 50,60,70,80,90,100℃，在各溫度分別加熱 15、30 秒 1.5,2.5,5,10,20,30 分鐘。

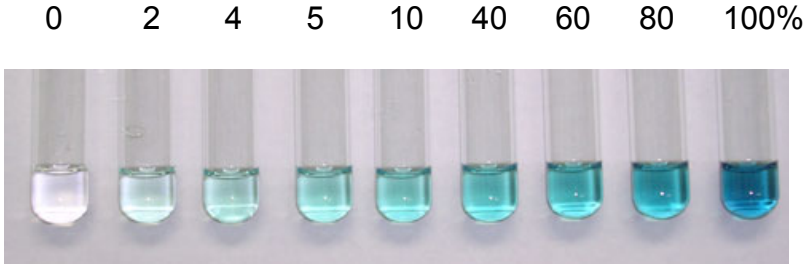
(2) 進行 ELISA 呈色。

檢測步驟



呈色（本實驗在 37℃時呈色效果最佳）

奶粉摻雜濃度百分比

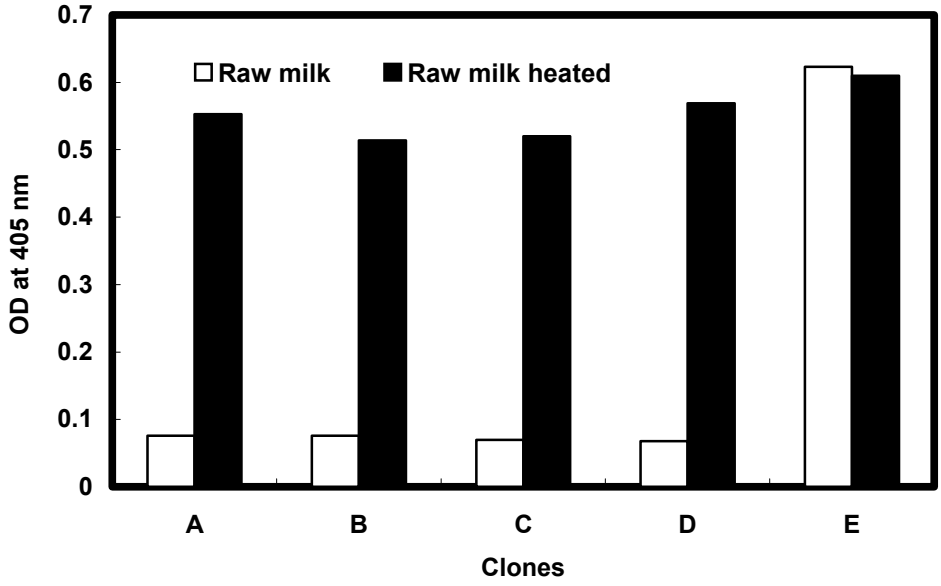


圖四：玻璃棒流程圖

參、實驗結果與討論：

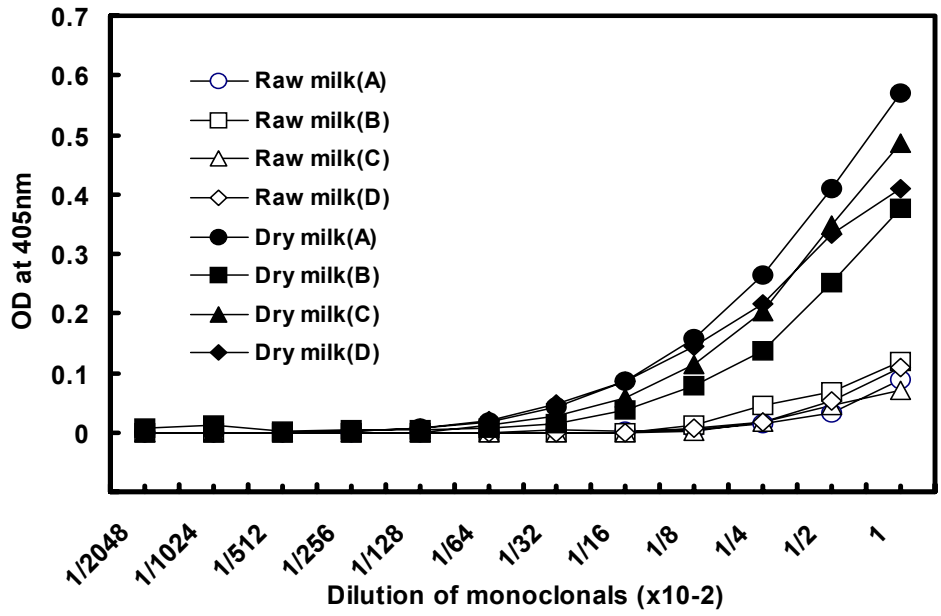
一、實驗結果

1. 單株抗體的篩選：



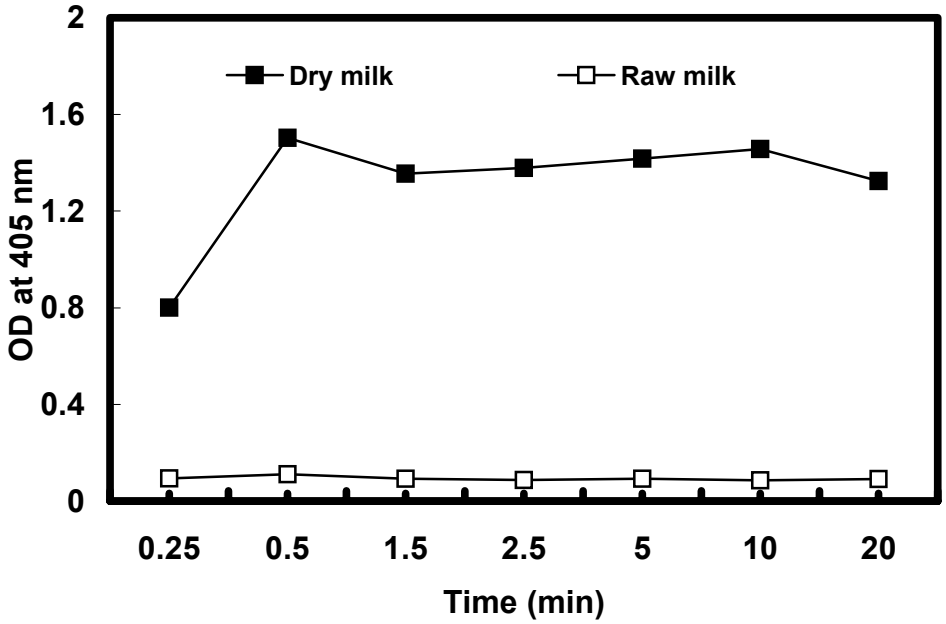
圖五:利用 ELISA 成功篩選出四株單株抗體 (A.B.C.D) 。此單株抗體，可專一性的辨識奶粉，另外 E 爲其他可同時辨認牛乳與奶粉的單株抗體。

2. ELISA 的分析法：



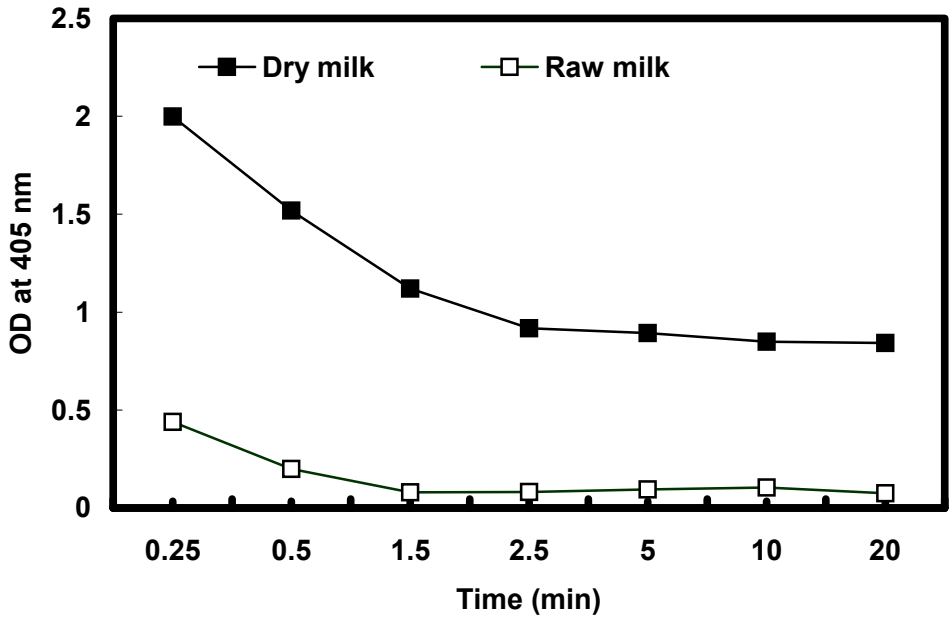
圖六:利用 ELISA 分析單株抗體在牛乳及奶粉之結合力。其結果爲隨著抗體的濃度越高，所得之吸光讀值越高，表示此單株抗體對奶粉具有專一性的結合能力。

3.測定物沾附時間 (coating time)：



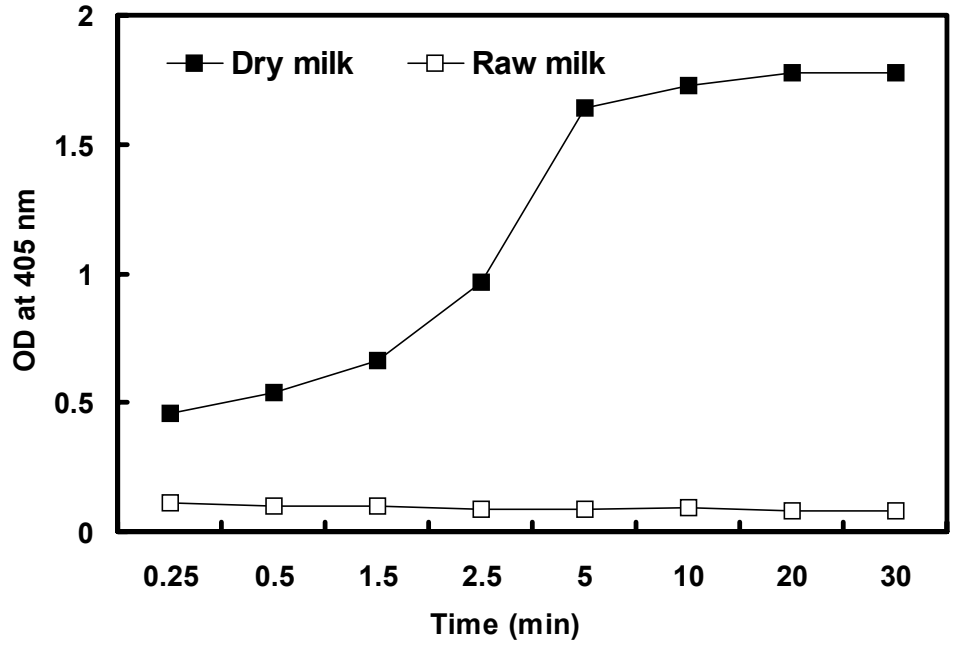
圖七:　玻璃棒沾附時間之分析。其結果可得沾附測定物時間只需 15 秒，其 OD 值就已達 0.801，超過 15 秒以上之讀值大致相同，因此得到沾附時間只需 15 秒。

4.填充液沾附時間 (blocking)：



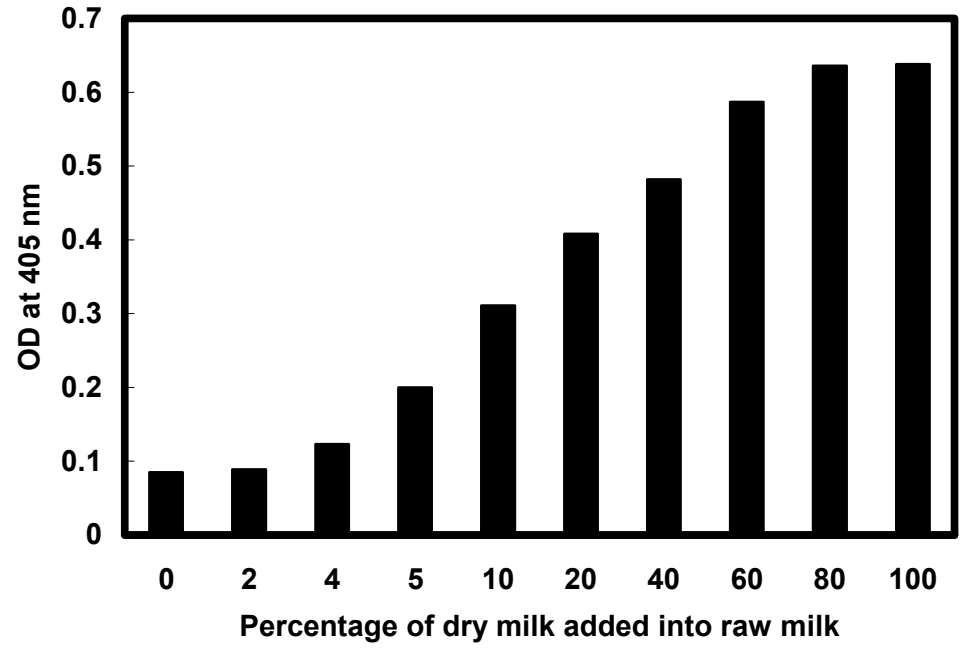
圖八:　玻璃棒沾附填充液之時間分析。填充液主要目的在於降低非專一性的結合 (如抗體直接沾附於玻璃棒上)，由圖得知在少於 1.5 分鐘時抗體會產生非專一性的結合，而在 2.5 分鐘後所測得的讀值趨於穩定，此表示抗體無產生非專一性之結合。因此選取 2.5 分鐘當最少沾附之時間。

5.測定玻棒沾附 mAb 液的恰當時間



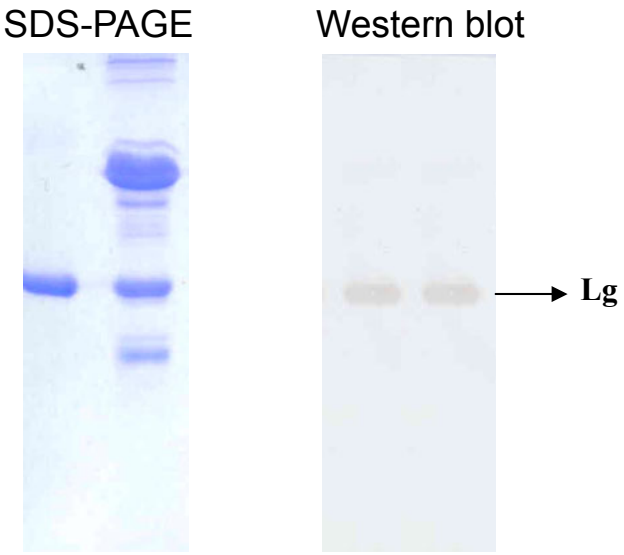
圖九: 分析抗體在玻璃棒中所需的結合時間。由圖得知 10 分鐘時 OD 值爲抗體結合最佳時所需的最短時間，在實驗結果顯示，沾附 5 分鐘時 OD 值和沾附 10 分鐘時差異並不大，因此爲求快速，決定沾附時間爲 5 分鐘。

6.奶粉摻雜不同百分比所得結果



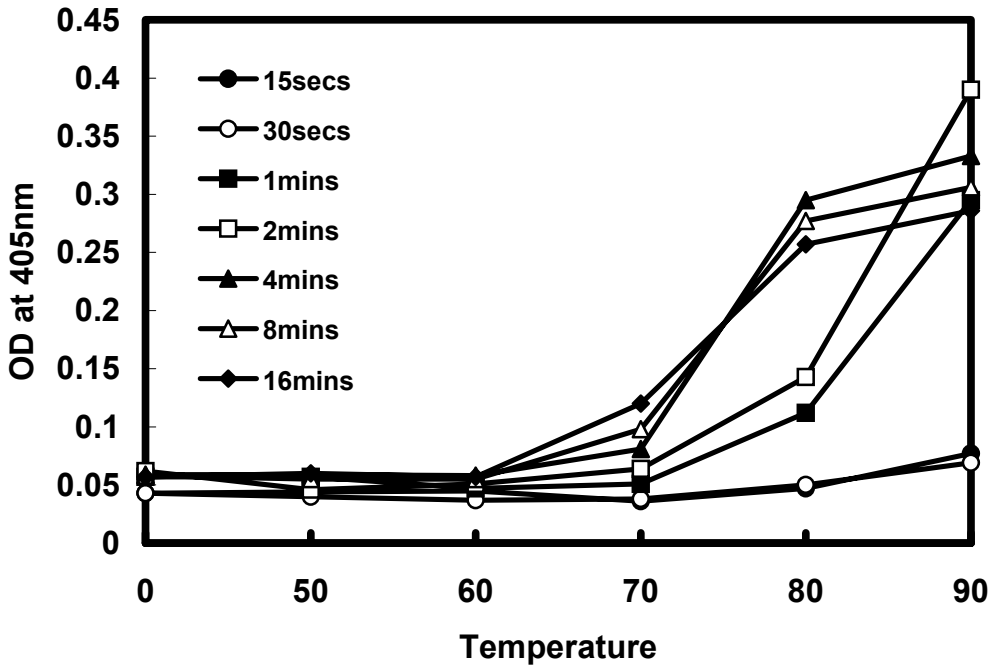
圖十: 利用玻璃棒檢測奶粉的摻雜度。由圖得知，隨著添加奶粉的比例增加時，其讀值也隨著上升，由此可知當奶粉添加至 10% 以上時，則可利用此單株抗體偵測出。

7.單株抗體對應之蛋白質：



圖十一: 利用 SDS-PAGE 及 Western blot 分析單株抗體專一性結合之乳蛋白質。A ： LG protein 。B ： Raw milk 。 此單株抗體所辨識的乳蛋白爲 Lg 蛋白質（Lactoglobulin protein）。

8.利用單株抗體分析牛乳經不同溫度加熱不同時間的影響：



圖十二： 利用 ELISA 分析辨認還原乳單株抗體對於牛乳經不同時間不同溫度加熱後的免疫結合力。將牛乳用 50, 60, 70, 80 及 90℃加熱 15’’, 30’’, 1’, 2’, 4’, 8’ 及 16’。結果當牛乳加熱 80℃以上 1 分鐘即可被此抗體辨認。

二、實驗討論

1. 單株抗體的選取：

本實驗成功的製作出四株只會辨認奶粉而不辨識牛乳的單株抗體如圖五，利用 ELISA 分析單株抗體的專一性測試中，隨著抗體的濃度越高其所測得的讀值越高如圖六，而本實驗能成功的篩選出此單株抗體，主要的因素是利用乳中蛋白因加熱所造成結構上的改變，而此特殊的結構引發小鼠所產生抗體，即可專一性的辨識奶粉。另一項因素爲牛乳中的蛋白質含量相當多，不易尋找出因受熱所導致結構改變的蛋白質，因此本實驗在篩選單株抗體時設計出不同的方式篩選。本實驗利用奶粉免疫小鼠，使小鼠產生抗奶粉蛋白的多株抗體，此多株抗體並不能運用於區分乳中部分蛋白因受熱導致的結構改變，但其多株抗體中含有可區分的抗體，因此製作單株抗體以辨識受熱改變之結構，而篩選的方法是利用製作出的單株抗體交互篩選牛乳及奶粉，淘汰兩者皆會辨識的抗體，剩下的單株抗體即可得到只會辨識牛乳或奶粉。在本實驗中只得到辨識奶粉的單株抗體。因此運用此單株抗體可專一性的檢測出牛乳中有無摻雜奶粉。其所辨認的爲乳球蛋白（Lactoglobulin protein），在牛乳中含量 15% ，具有抗癌、降血脂、提供胺基酸養分的功能，可抗胃酸及胃中酵素分解，直接由小腸吸收至血液，但加工過後，會因加熱而變性變質，失去原有的功能及效果。

2. 摩擦玻璃棒：

本實驗利用砂紙摩擦玻璃棒使之表面粗糙。玻璃棒表面粗糙度越大，表面所吸附的蛋白質越多。因此經砂紙摩擦後之玻璃棒用於免疫檢測，比光滑玻璃棒檢測效果佳。此外，表面粗糙增加除增大玻璃棒之表面積外，對於蛋白質之吸附能力也相對提高。因此本實驗研發利用玻璃棒檢測時，特將玻璃棒表面利用砂紙摩擦，以增加粗糙度，提高檢測的效果。

3. 測定物的沾附時間 (coating time)：

本實驗的第一步驟爲沾附測定物。當玻棒沾附測定物時，測定物會進入玻棒的孔洞內，達到沾附的目的。理論上，沾附時間越久，測定物填滿孔洞的時間越多，實驗結果讀值也越高如圖七。但單一玻棒其表面的孔洞數有限，如測定物完全將孔洞填滿，則再加長沾附時間也無法使更多的測定物沾附於玻璃棒上，如圖中 0.5 分鐘時，玻棒上的孔洞已完全填滿，所

以即使時間加長至 1 分鐘，所測出的讀值仍一樣。因此本實驗測定物的沾附時間爲 0.5 分鐘。

4. 填充液的沾附時間 (blocking time)：

本實驗的第二步驟爲沾附填充液。根據實驗結果如圖八，當玻棒沾附測定物 15 秒後，測定物並無法將玻棒上的孔洞完全填滿，如果此時直接進行沾附單株抗體的步驟，但單株抗體除了吸附測定物中改變的結構外，也會沾附在未填滿的孔洞中，因此所得的結果讀值就會增加，產生實驗誤差。所以，在沾附完測定物後須進行填充步驟。一般來說，沾附時間越短測定物所能填滿之剩餘孔洞就越少，也就越增加單株抗體直接沾附於孔洞的機會，實驗結果的讀值越高如圖八，造成的實驗誤差也相對增加，此結果稱之爲非專一性接合。但是，當填充物質把剩餘孔洞填滿後，單株抗體就不可能直接沾附於玻璃棒，而是與測定物摻生專一性結合，此時所測出的讀值最爲精確。因此當填充物質把剩餘孔洞填滿後之後，就算再加長沾附填充物時間，其所能達到的效果和已填滿全部剩餘孔洞的效果一致，所以實驗結果讀值不再降低，所以不須再增加填充液沾附時間，如圖八超過 2.5 分鐘讀值不再降低，因此填充液沾附時間爲 2.5 分鐘，因此。另一方面須注意的是準備填充液時，所選取的填充物質必須是不會和本實驗採用的單株抗體產生反應的物質，才可避免實驗誤差。本實驗選取 gelatin。

5. 單株抗體沾附時間 (Antibody binding time)：

本實驗的第三步驟爲單株抗體的沾附。利用測定物所含的物質與本實驗之單株抗體產生的專一性辨識來測定沾附的時間。本實驗的單株抗體可辨識牛乳在加工過程中改變的結構，其上的 HRP(山葵過氧化氫酵素)可和呈色液中的 H_2O_2 結合產生反應，呈現綠色。根據實驗結果，沾附單株抗體所需時間比沾附測定物及填充液的時間長如圖九，原因爲單株抗體並不是直接進入孔洞，而是和結構改變之蛋白質結合，因此須有充裕的時間。結合時間越長，期所測得的讀值就越高。當單株抗體完全與結構改變之蛋白質結合後，即使再增加沾附的時間，所測得的讀值不會改變。如圖九再超過 5 分鐘其所測讀值不再改變，因此單株抗體沾附 5 分鐘爲最短所需時間。

6. 清洗 (wash)：

在本實驗中，分別在每一個步驟後清洗 3 次。分別爲(1)沾附後，(2)填充後，(3)沾 mAb

液後。清洗(1)，目的爲去除未沾附好的測定物（牛乳蛋白）。清洗(2)，目的爲去除遮蓋住測定物或填充不完全的填充物質（gelatin）。清洗(3)，目的爲去除未結合之單株抗體。理論上，如果爲求得最快速時間，清洗(1)和清洗(2)可省略，因下一步驟（將玻璃棒放入下一試管）的過程，可取代清洗。根據實驗結果未發現如果不清洗，會造成很大的實驗誤差。

7. ELISA V.S. Glass assay：

利用製作出的單株抗體開發另一種快速檢測方式，本實驗開發出利用玻璃棒快速檢測牛乳中有添加奶粉，本實驗之實驗步驟與傳統的 ELISA 大致相同，但傳統的 ELISA 須費時 2~3 小時及利用特定的 ELISA 分析盤才可實驗。本實驗則利用以磨砂過之玻璃棒，在短短的 7.5 分鐘即可得出結果，在實際操作上方便及快速，產業利用價值高。玻璃棒與傳統 ELISA 必較如表一。

表一、傳統 ELISA 及本實驗之比較

	實驗器材	沾附測定乳時間	沾附填充液時間	實驗總耗費時間	應用處
ELISA assay	須特定的 ELISA plate	30 分鐘以上	30 分鐘以上	2~3 小時	多侷限在實驗室
Glass assay	玻璃棒	15 秒	2.5 分鐘	7~8 分鐘	消費者、政府相關單位、乳品加工廠

8. 奶粉的摻雜：

本實驗利用玻璃棒及單株抗體直接檢測牛乳摻雜奶紛其結果顯示如圖十，奶粉摻雜比例越大，所檢測出結果讀值越高。其中摻雜奶粉的含量高於 10%則可利用此檢測方式檢測。一般乳品加工廠或酪農戶爲增加自己的利益會添加奶粉還原成牛乳，而此添加絕對大於 10%，因此本實驗所研發出的方式，必定可用於奶粉的檢測。

9. 單株抗體對應之蛋白質：

篩選到只認得奶粉的單株抗體後，爲找尋對應之蛋白質，進行電泳把蛋白質分離，對照 Mark，知道其分子量，再進行胺基酸序列分析，最後就可以確定其蛋白質爲乳球蛋白（Lg protein），也就是說，本實驗經由 Lg 蛋白質在牛乳加工過程中改變的結構，檢測市售鮮乳中有無摻雜奶粉。

10. 利用單株抗體分析牛乳經不同溫度加熱不同時間的影響：

由圖十二可知，當牛乳加熱至 80℃、一分鐘後，因爲牛乳中的蛋白質受熱結構改變，就可被 Lg 單株抗體辨認出。一般牛乳滅菌法有三種，保持滅菌法（62-65℃30 分鐘）、高溫瞬間殺菌法（72-75℃2-3 秒）、超高溫瞬間滅菌（120-135℃2-3 秒），本實驗可經由政府制訂法律後作爲政府檢測市售鮮乳之方法。

肆、實驗結論與應用：

一、實驗結論

1. 本實驗成功的篩選出只辨識奶粉的單株抗體。
2. 測定物沾附時間 15 秒就可達到實驗目的
3. 測定物沾附量和玻璃棒粗糙度有關。
4. 沾附填充液 2.5 分鐘就可達到實驗目的。
5. 單株抗體的沾附時間 5 分鐘即可達到實驗目的。
6. 玻璃棒實驗，只需費時 7.5 分鐘。
7. 奶粉摻雜比例越高，實驗結果的讀值越高。
8. 玻璃棒的檢測可取代一般傳統的 ELISA，且更爲快速。
9. 本實驗可成功檢測出牛乳中有無摻雜奶粉，解決台灣四十年來奶粉摻雜問題。
10. 此單株抗體所辨認的蛋白質爲乳球蛋白（Lg protein）。
11. 單株抗體所辨認處爲加熱後結構改變的蛋白質。
12. 牛乳經 80℃加熱一分鐘後，可被單株抗體辨認出。
13. 若政府規定加工過程中，採取保持滅菌法、高溫瞬間滅菌法，則本實驗可做爲國家牛乳檢測之方法。

二、應用

1. 消費者在家就可檢測鮮乳，維護自己的消費權益。
2. 政府單位可在短時間內完成鮮乳品質檢測，維護市場上銷售鮮乳的品質。
3. 乳品加工廠向酪農戶收購牛乳時，可快速完成檢測，防止酪農摻假。
4. 本實驗所製作出之單株抗體具有極大之應用性，如可用於研究蛋白質加熱後結構上的改變或進一步研發更快速的檢測。

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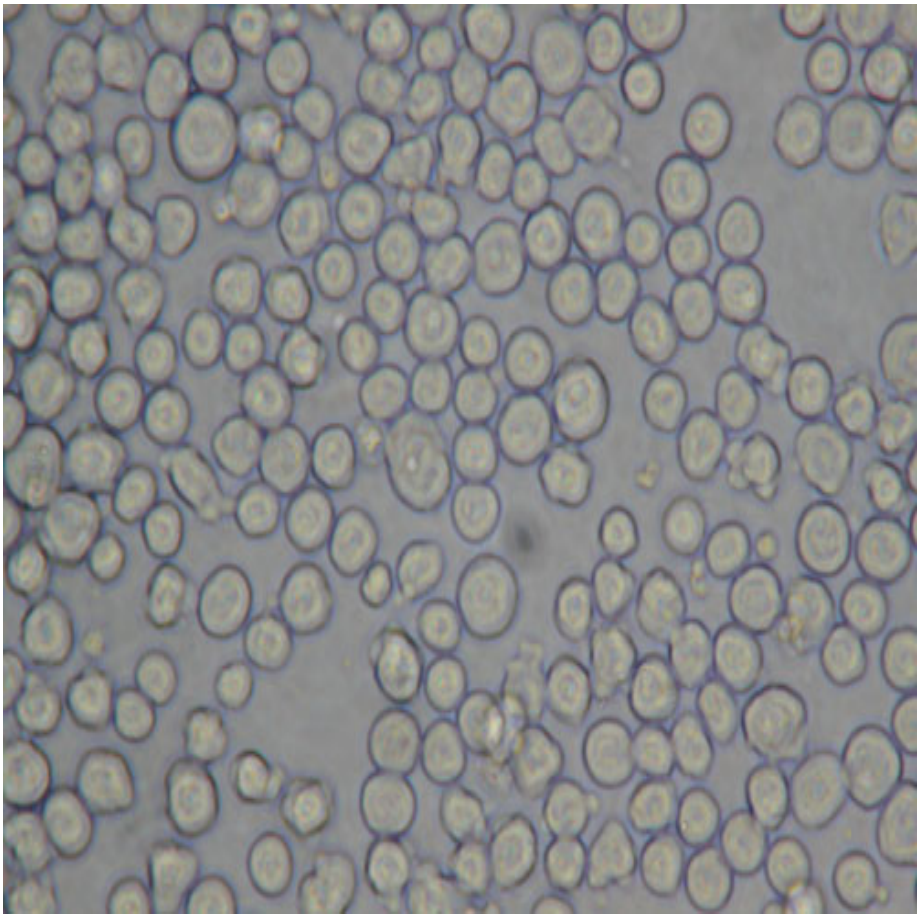
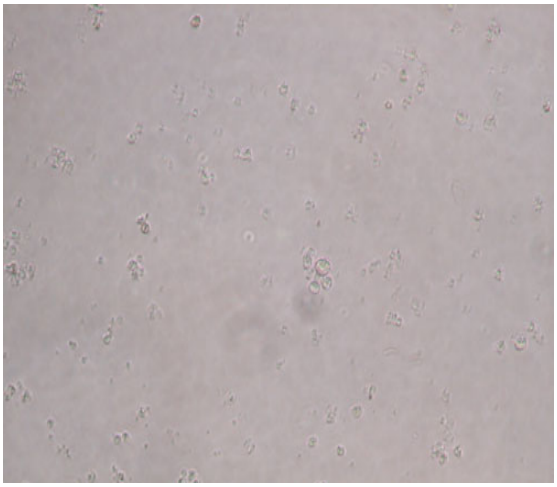
陸、附件

1. 骨髓瘤細胞 (FO cell)



放大倍率爲 10x40 倍，第十代細胞。細胞狀況良好。

2.細胞融合後之融合瘤細胞 (hybridoma cell)



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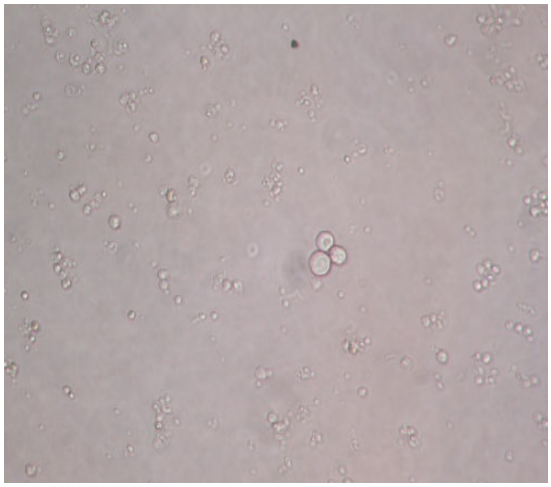

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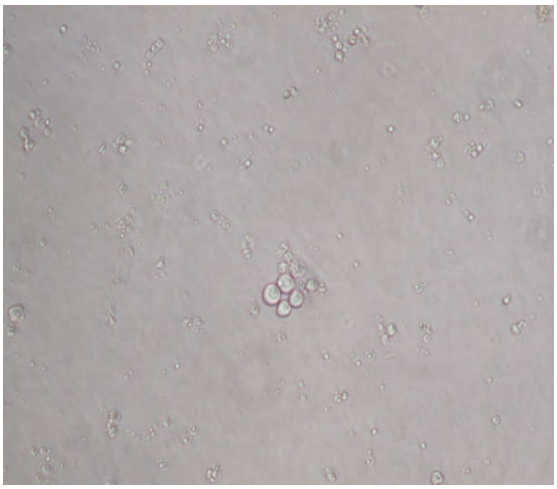

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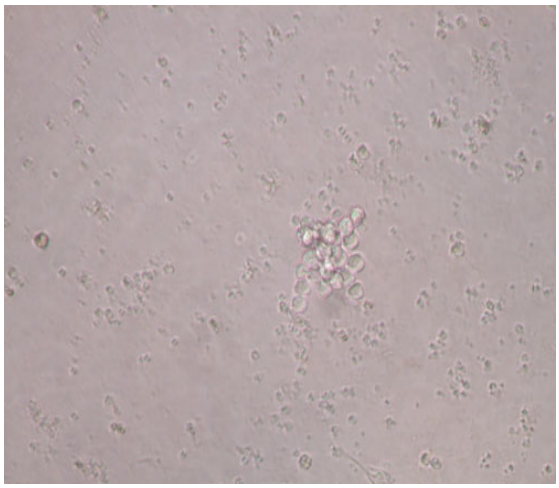

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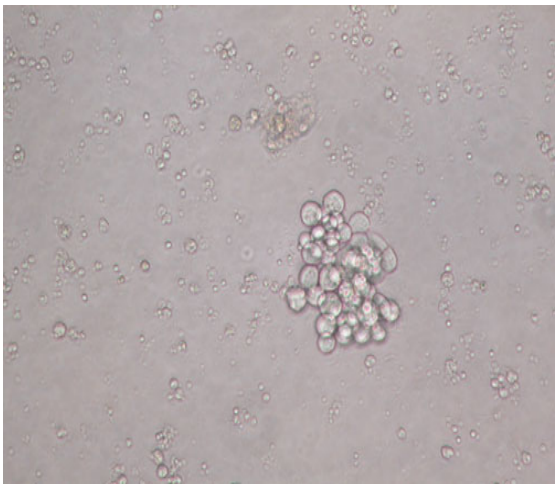

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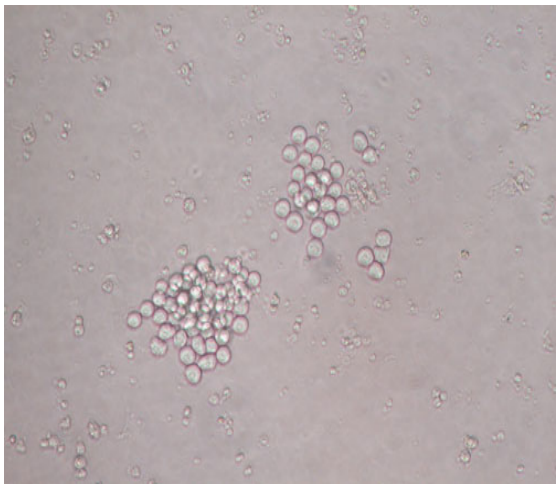

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ABSTRACT

It is always questionable whether there is a nutritional difference between raw milk and processed milk. Recent findings indicate that β-lactoglobulin, one of the major milk proteins that can be directly absorbed in the gastro-intestinal system, may function as an agent for lowering serum cholesterol and as a free radical scavenger. In this study, I found that lactoglobulin was substantially denatured in processed milk and then developed a rapid method for distinguishing raw milk and all other processed milks by using a monoclonal antibody specific to denatured lactoglobulin. The method is utilizing a sanded-tip of glass rods with procedures involves binding of milk proteins, blocking, reaction with a peroxidase-conjugated monoclonal antibody, and color development. The procedure can be completed within eight min and is highly specific. It enables us to monitor the denaturing process of β-lactoglobulin in raw milk heated at different temperature for different durations. Moreover, I found that the level of denatured β-lactoglobulin was inversely correlated with the antioxidant activity in market-available milk products, including fresh, ultra-heated, and dry milks. In summary, I developed a rapid and simple monoclonal antibody-based method for identifying denatured β-lactoglobulin, which was inversely correlated with the antioxidant activity that may be deemed to be one of the indicators on the overall quality of various milk products.

(Key words: denatured β-lactoglobulin, colorimetric immunoassay, antioxidant)

Abbreviations: ABTS = 2, 2-Azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) **ELISA** = enzyme linked immunosorbent assay, **HRP** = horse-radish peroxidase, **LG** = β-lactoglobulin, **LDL** = low density lipoprotein, **mAb** = monoclonal antibody, **PAGE** =

polyacrylamide gel electrophoresis, **PBS** = phosphate buffered saline, **SDS-PAGE** = Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

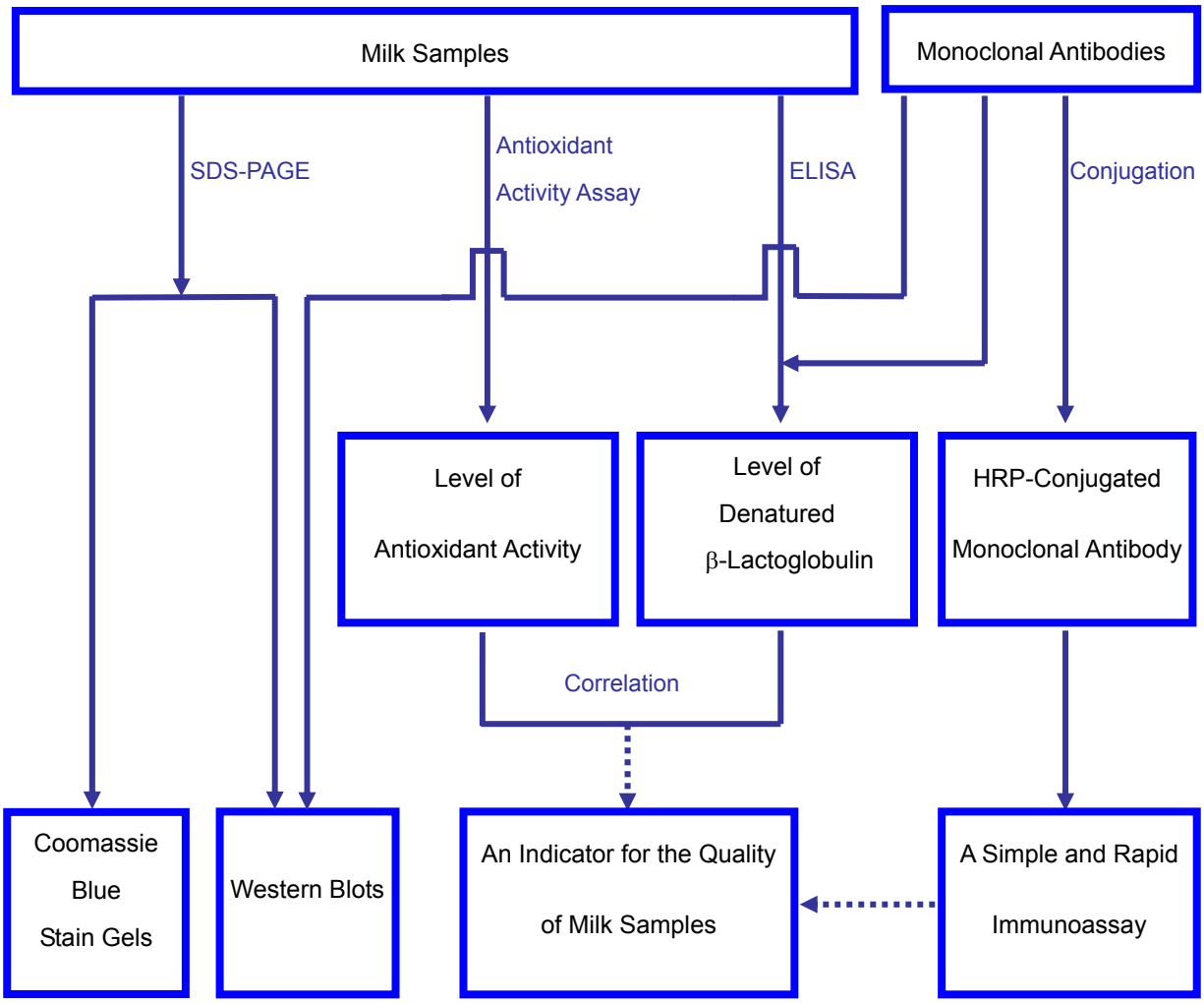
INTRODUCTION

The nutritional difference between raw and processed milk remains to be controversial in the past decades. Since heating procedures are necessary in producing processed milk, it is conceivable that heat-labile components may contribute to the overall quality of milk. In my early experiments, β-lactoglobulin, one of the major milk proteins, was found to be substantially denatured in processed milk. With a monoclonal antibody that was shown to be able to distinguish denatured β-lactoglobulin from its native form, I develop a simple and rapid immunoassay for the monitoring of the levels of denatured β-lactoglobulin in milk samples. Additionally, I show that the level of denatured β-lactoglobulin and antioxidant activity are inversely correlated.

Specific aim

1. To search a reagent that can detect the denatured β-lactoglobulin
2. To develop a fast and simple method monitoring the denatured β-lactoglobulin in min
3. To demonstrate the poor physiologic role in milks underwent overheating

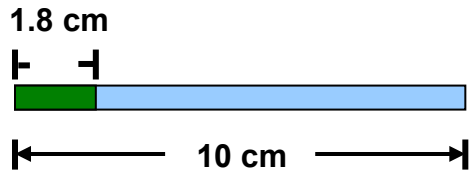
Experimental scheme



MATERIALS AND METHODS

Materials

1. Glass rods are with sanded-tips



2. Dry milk (250g / 200g H_2O).
3. Raw milk is form local meadow.
4. Raw milk is heated on 100 ℃, 1.5 min.
5. Dry milk, fresh milk (containing ultra-heated milk) from market of Taiwan.
6. Blocking solution (1％ gelatin/PBS): to avoid nonspecific binding.
7. Wash buffer(0.1％ gelatin + 0.05％ Tween 20/PBS).
8. Monoclonal antibodies (with HRP) prepared against heated β–lactoglobulin were screened initially for monoclonal that only reacted with dry milk using an enzyme-linked immunosorbent assay (ELISA), and the monoclonal antibodies were produced by researchers from Dr. Mao lab in College of Biology and Technology, National Chaio Tung University in Taiwan (Mao et al., 1990; Mao et al., 1988). Monoclonal antibodies with the highest binding affinity to dry milk were chosen as a specific reagent.
9. Color development (ABTS + 0.01％ H_2O_2).
10. Resolving gel (15％ 5 ml):
    (1) ddH_2O 2.6 ml
    (2) 30％ acrylamide mixed 1.0 ml
    (3) 1.5 M Tris-HCl, pH 8.8, 1.3 ml
    (4) 10％ SDS 0.05 ml

(5) 10％ ammonium persulfate

(6) TEMED 0.004 ml

11. Stacking gel(5% 1 ml) ：

(1) ddH_2O 0.68 ml

(2) 30％ acrylamide mixed 0.17 ml

(3) 1.0M Tris-HCl, pH 6.8, 0.13 ml

(4) 10％ ammonium persulfate 0.01 ml

(5) TEMED 0.001 ml

12. 5XSDS loading buffer：

(1) ddH_2O 0.9 ml

(2) 1 M Tris-HCl, pH6.8, 0.6 ml

(3) 50％ Glycerol 5 ml

(4) 10％ (w/v) SDS 2 ml

(5) β-mercaptoethanol 0.5 ml

(6) 1％ (w/v) bromophenol blue 1 ml

13. Marker (prestain)：

(1) β-Galactosidase 118 kDa

(2) Bovine serum albumin 86 kDa

(3) Ovalbumin 47 kDa

(4) Cartonic anhydrase 34 kDa

(5) β-Lactoglobulin 18.4 kDa

(6) Lysozyme 19 kDa

14. Coomassie Blue staining solution (50％ methanol，10％acetic acid，0.125％ [w/v] Coomassie Brilliant Blue R250) 。

Methods

1. Lowry Method (protein determination): 5 μl samples, 25 μl solution A, 200 μl solution B were ordered adding into ELISA plate each well. Wait 15 min for color development.
2. Monoclonal antibody as a specific reagent: Monoclonal antibodies prepared against heated β-lactoglobulin were screened initially for monoclonal that only reacted with dry milk using an enzyme-linked immunosorbent assay (ELISA). After which time, the one with the highest binding affinity to dry milk was chosen as a specific reagent.

3. Direct colorimetric immunoassay for denatured β-lactoglobulin in milk:

   A novel glass rod-based immunoassay was developed. In brief, the sanded tip of glass rod was dipped into test sample for 15 seconds, washed in buffered saline, blocked with 1% gelatin for 2.5 min, and followed by another wash. The tip was then incubated with monoclonal antibody conjugated with horse-radish peroxidase for 5 min and washed. Finally, the tip was dipped into ABTS for color development. Samples containing denatured β-lactoglobulin developed a color in green.

4. Gel electrophoresis and Western Blot: Containing 15% (wt/vol) polyacrylamide (unless specified) was used to find out the differences of raw milk, dry milk (containing ultra-heated milk), and fresh milk using a modified procedure (Yang and Mao, 1999) similar to that described (Oldfield et al.,1998). All the samples (milks) before loading was diluted 1:25 and mixed with sample buffers. Following the SDS-PAGE or native-PAGE, the gel was soaked briefly and instantly in solution containing methanol and transfer buffer (1:4) for 30 min. The gel was then immediately electrotransferred to a nitrocellulose membrane (Hybond-ECL extra; Amersham, Buckingham, UK) at 90 mA for 50 min in a semi-dry transfer cell (Bio-Rad). After that, membrane was immersed in 1% gelatin for 1 h with gentle shaking. The membrane was then treated with mAb or polyclonal antibodies and developed with 3-3'-diaminobenzidine (3, 3', 4, 4'-tetra-amino-biphenyl) according to the method previously described (Yang and Mao, 1999).

5. Enzyme linked Immunosorbent Assay (ELISA):

   Milk samples were added into 96 well ELISA plate (50 μL / well), and allow for coating 30 min. After washing three to four times, 350 μL of 1 % gelatin is added into ELISA plate each well, allowing for blocking 30 min. After another wash, following is antibody binding. The mAb solution (LG antibody: wash buffer= 1: 1000) is added into 96 well ELISA plate (50 μL / well) and allow 30 min. Last one is color development. ABTS with H_2O_2 (10000:1) was added into ELISA plate, and incubated for 30 min. Finally, the ELISA plate is read by ELISA reader.

6. Antioxidant role in milk with and without heat process: To test the antioxidant activity as a physiologic role in milks, plasma low density lipoprotein (LDL) isolated by ultracentrifugation was used. LDL was first incubated with the milk samples for 5 min followed by an addition of $CuSO_4$ as a free radical initiator. After incubation at room temperature for 2 h, the reaction mixture was stopped by 20% of trichloroacetic acid. Malondialdehyde, a free-radical generated product, was then measured by the addition of a chromogenic substance thiobarbituric acid. The solution was finally read at 540 nm by a spectrophotometer.

Results and Discussion

1. Development of a glass rod-based rapid method for denatured β-lactoglobulin:

   The general procedure was described in "Materials and Methods" and Fig. 1. The optimal durations for coating, blocking, and antibody binding were found to be 15 sec, 2.5 min, and 5 min, respectively (Fig. 2). A total of 8 min is sufficient for the whole procedure, washing time included. Using this method, it was found that the levels of denatured β-lactoglobulin in various processed milk samples were higher than that in the raw milk (Fig. 3).

2. Detection of high molecular weight denatured β-lactoglobulin by PAGE and Western Blot:

   High molecular weight β-lactoglobulins were not found raw milk (Fig. 4, lane 1) but in all processed milk (Fig. 4, lanes 2-8). The data indicated that β-lactoglobulin was denatured after processing.

3. Temperature- and time-dependent denaturing of β-lactoglobulin in raw milk:

   β-Lactoglobulin was found to be relatively stable at 60 ^{o}C. At 70 ^{o}C, the denaturing process is strictly time-dependent for up to 4 min. At higher temperature, the denaturing process accelerated. For instance, the denaturing of β-lactoglobulin became evident after heating at 80 ^{o}C for as short as 1 min (Fig. 5a, b)

4. Correlation of antioxidant activities and levels of denatured β-lactoglobulin: Samples contained various amounts of heated raw milk were prepared. The antioxidant activities and the amounts of denatured β-lactoglobulin were determined by antioxidant activity assay (Fig. 6a) and ELISA (Fig. 6b), respectively. It was also demonstrated that these two parameters were inversely correlated (Fig. 6c). It indicated that the processed milk which really contains the denatured β-lactoglobulin will has less antioxidant activity which is very good for our gastro intensity system.

Conclusions

1. I have successfully developed a novel eight-minute method to detect denatured β-lactoglobulin, which may be used as an indicator if milk was over heated.
2. Denatured β-lactoglobulin could easily be detected if raw milk was heated over 80 ^{o}C longer than one minute.
3. The antioxidant activity in milk would decrease when processed milk was overheated, and that the activity was inversely correlated to the levels of denatured β-lactoglobulin in the milk samples

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Acknowledgement

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Legends

Fig. 1.

Experimental steps for the glass rod-based immunoassay: All as described in "Materials and Methods."

Fig. 2.

Determination of optimal durations for coating, blocking and antibody binding. The coating time. Sanded tips of glass rods were dipped into raw milk or dry milk for 0.25, 0.5, 1.5, 2.5, 5, 10, 20, 30 min, respectively, following a 30-min gelatin-blocking. Subsequently, these glass rods were then incubated with horseradish peroxidase (HRP) conjugated monoclonal antibody against denatured LG for 30 min. Following three-time washes, ABTS was used to develop the colorimetric presentation at 415 nm. The blocking time. All glass rods were coated with milk samples for 0.25 min followed a time-course blocking to find out the optimized blocking time. The following procedures were

same as described above. The antibody binding time. All glass rods were first coated for 0.25 min and a subsequent 2.5-min block. However, the HRP-conjugated-Ab were allowed to bind for variable time. The other procedures were as described above.

Fig. 3.

Relative amounts of denatured β-lactoglobulin in milk samples determined by the rapid method. Lane 1: raw milk; Lanes 2-8: processed milk (lanes 2-6: five different brands of fresh milk, lanes 7-8: two different brands of dry milk). All ateps are as described in " Materials and Methods."

Fig. 4.

Analysis of milk proteins by gel electrophoresis. Lane 1: raw milk; Lanes 2-8: processed milk (lanes 2-6: five different brands of fresh milk, lanes 7-8: two different brands of dry milk). 12.5% dry milk dissolved in PBS. All milk samples were first diluted for 25 times and then 5 μL of diluted samples were used to analyze in coomassie blue. In Western Blot, mouse monoclonal Ab and were used as primary and secondary antibodies, respectively. Finally, diaminobenizidine (DAB) solution containg H_2O_2 was used to developed the color.

Fig. 5.

(a) Time and temperature effect for denaturation of β-lactoglobulin in raw milk.

(b) Correlation of levels of denatured β-lactoglobulin and heating t ime at 70 ^{o}C.

100 μL of raw milk in each 0.5 ml eppendorf was heated at 50, 60, 70, 80, 90 ℃, and incubated for 0.25, 0.5, 1, 2, 4, 8, and 16 min, respectively. The heated samples were immediately chilled on ice until analysis. In subsequent ELISA, 50 μL of heated samples were used as antigen and the following steps were same as described in " Materials and Methods."

Fig. 6. Antioxidant activities and denatured β-lactoglobulin contents in milk samples contained various amounts of heat raw milk in unheated raw milk. (a) Variable percentage of heated raw milk was prepared by mixing with original raw milk. The prepared samples were then used to perform LDL peroxdation assay. The horizontal axis was presented as percentage of heated raw milk. Antioxidant activity was calculated by the equation: Relative of antioxidant activity = {1- [(OD sample – OD 100% original raw milk) / (OD without milk – OD 100% of original raw milk)]} x 100%. (b) denatured β-lactoglobulin content in samples. Variable percentage of heated raw milk was taken as coating antigen to perform ELISA. Specific against denatured LG monoclonal Ab was used as primary Ab and goat ant mouse IgG was used as secondary. Others steps were some as described in methods**.** (c) Linear regression analysis.

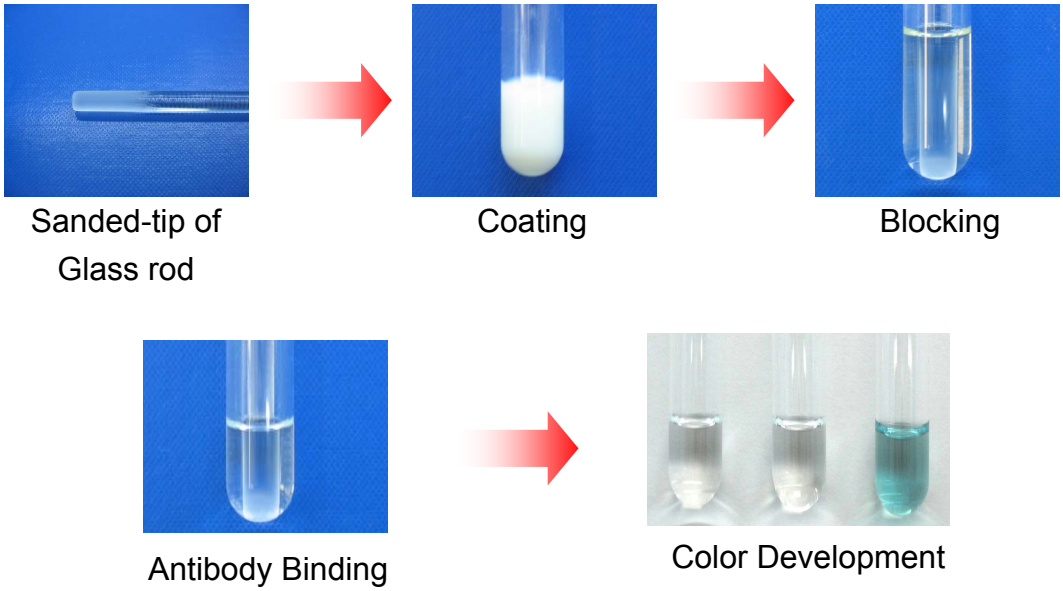

Sanded-tip of
Glass rod
Coating
Blocking
Antibody Binding
Color Development

Fig. 1

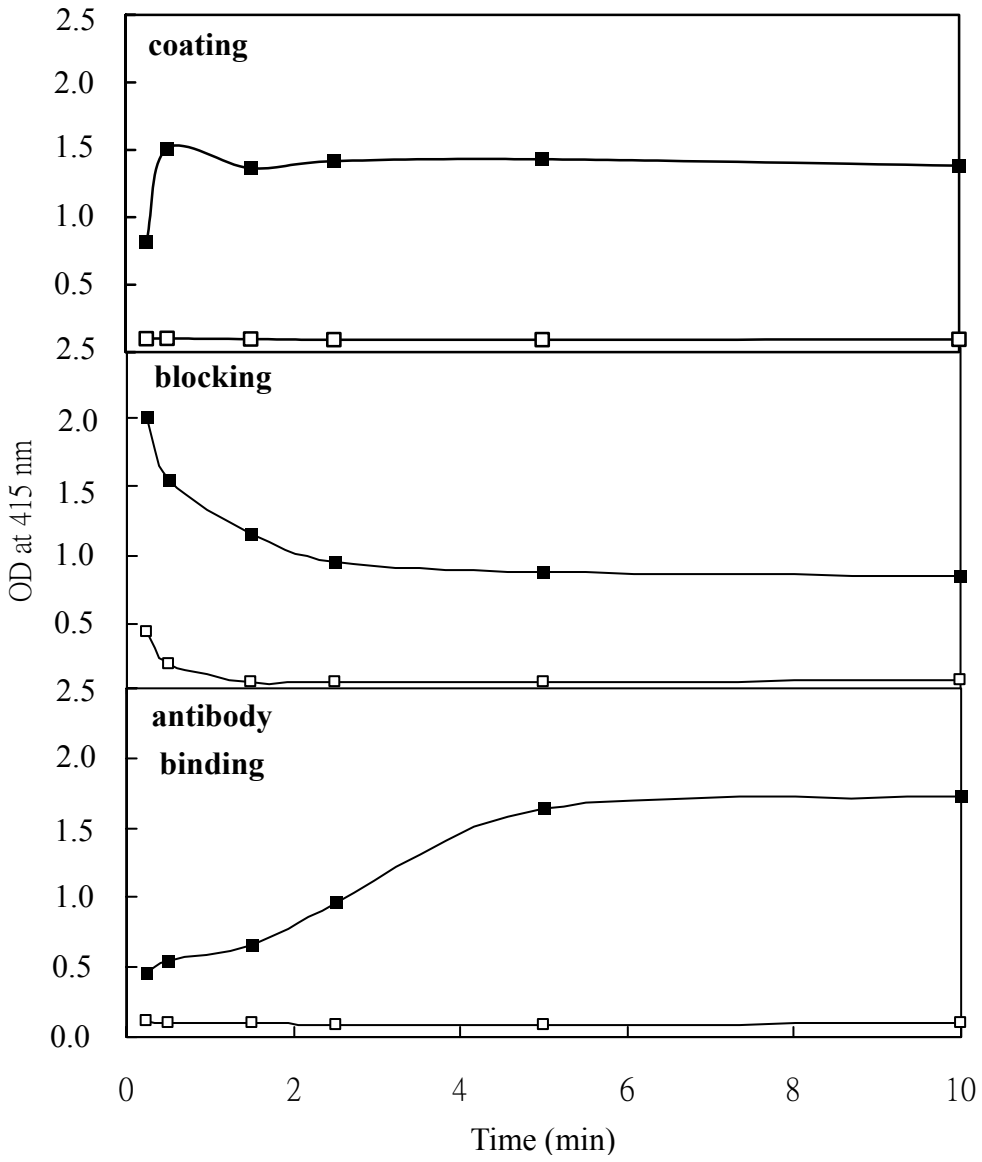

coating
blocking
antibody
binding
OD at 415 nm
Time (min)
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10

Fig. 2

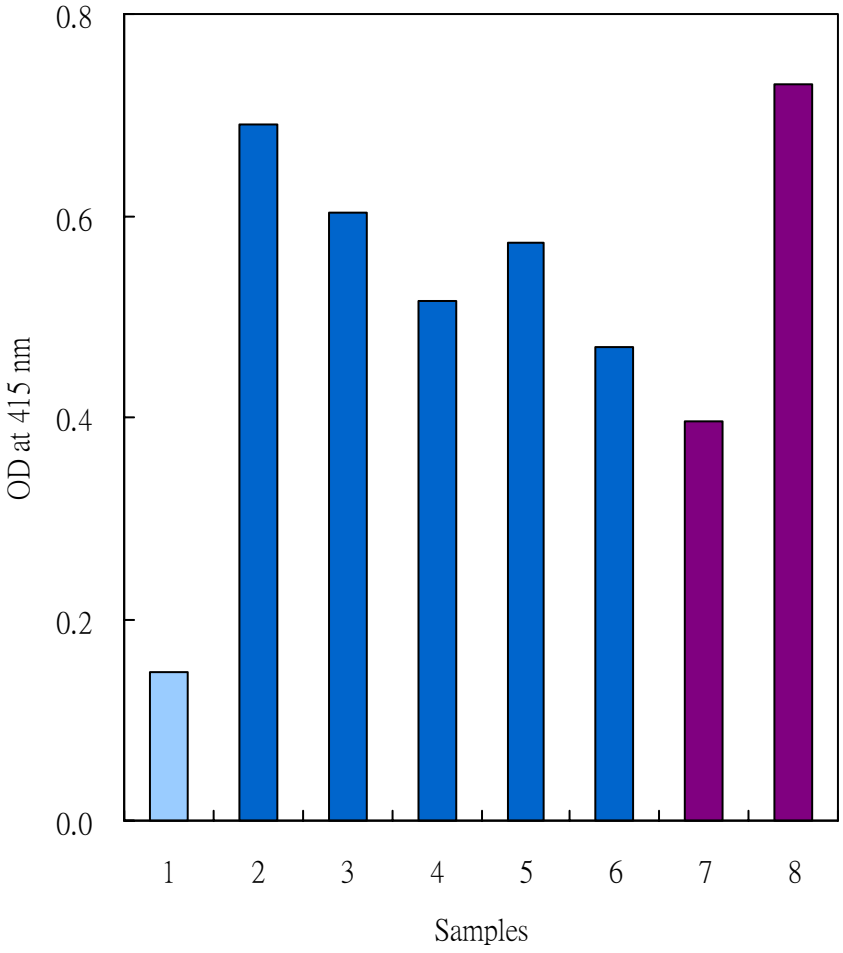


Fig. 3

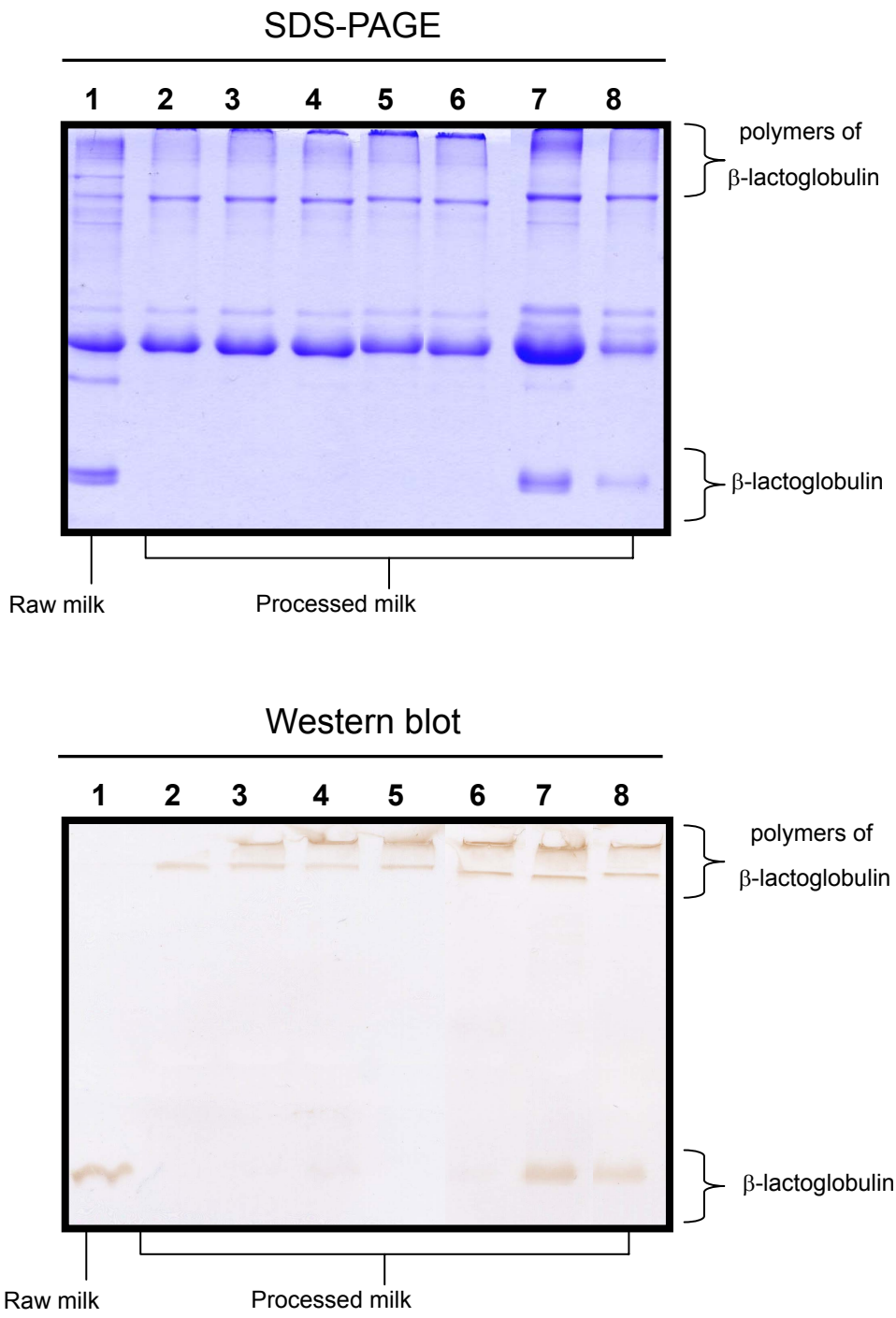
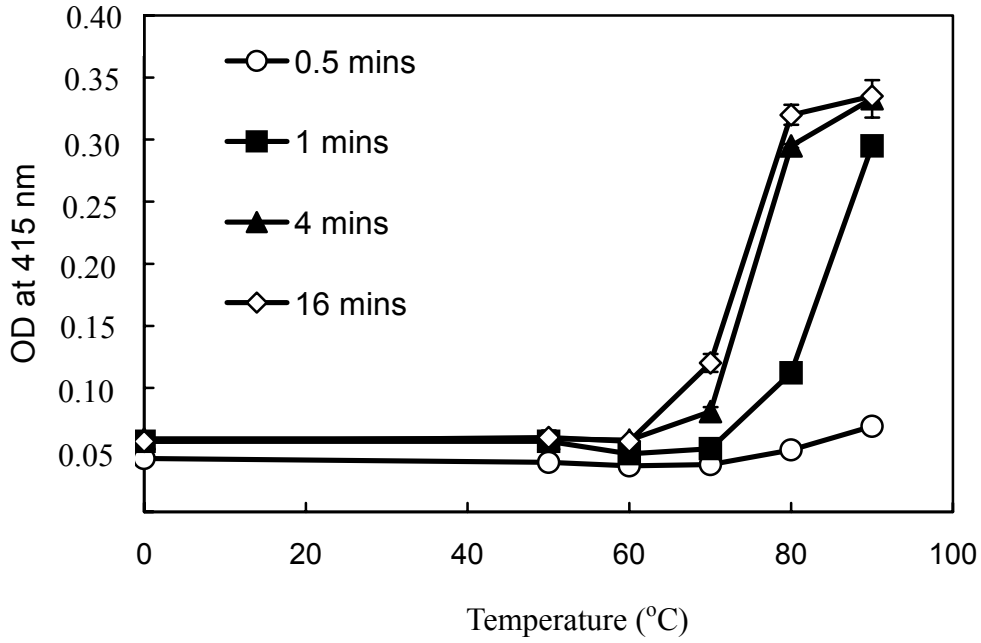

SDS-PAGE
1 2 3 4 5 6 7 8
polymers of
β-lactoglobulin
β-lactoglobulin
Raw milk
Processed milk
Western blot
1 2 3 4 5 6 7 8
polymers of
β-lactoglobulin
β-lactoglobulin
Raw milk
Processed milk

Fig. 4


0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
OD at 415 nm
0.5 mins
1 mins
4 mins
16 mins
0
20
40
60
80
100
Temperature (°C)

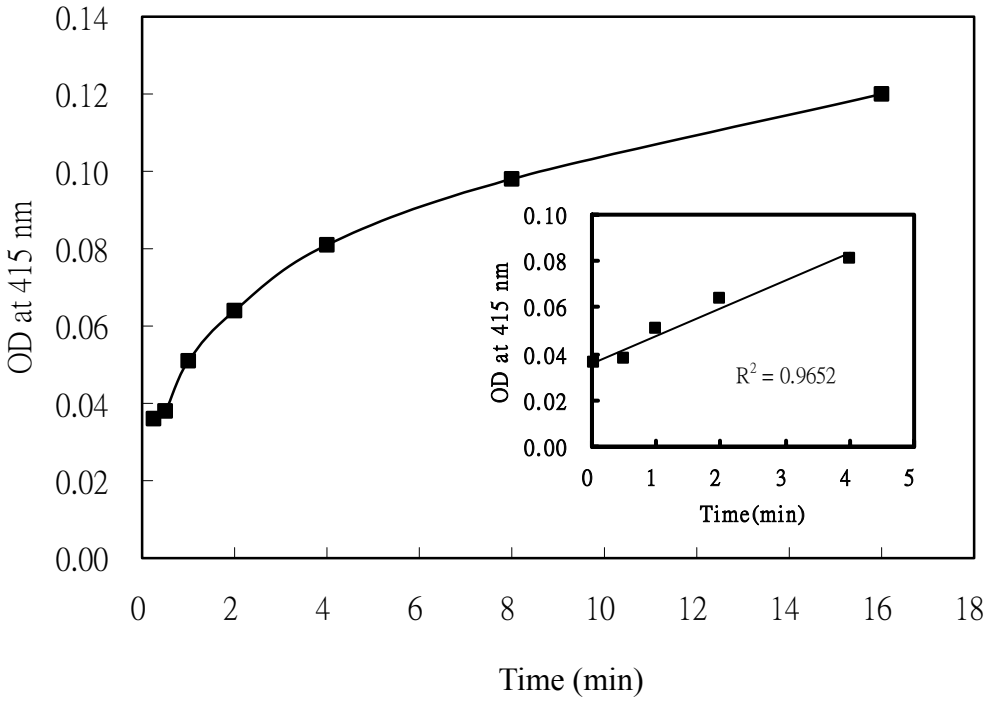
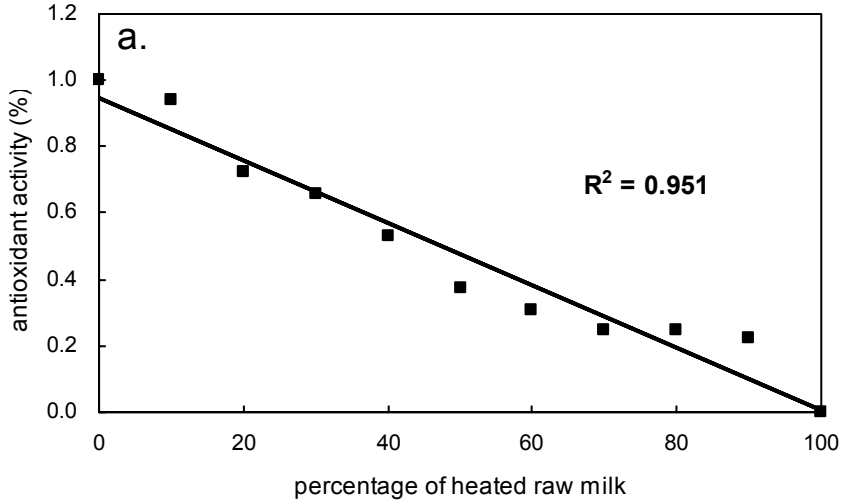
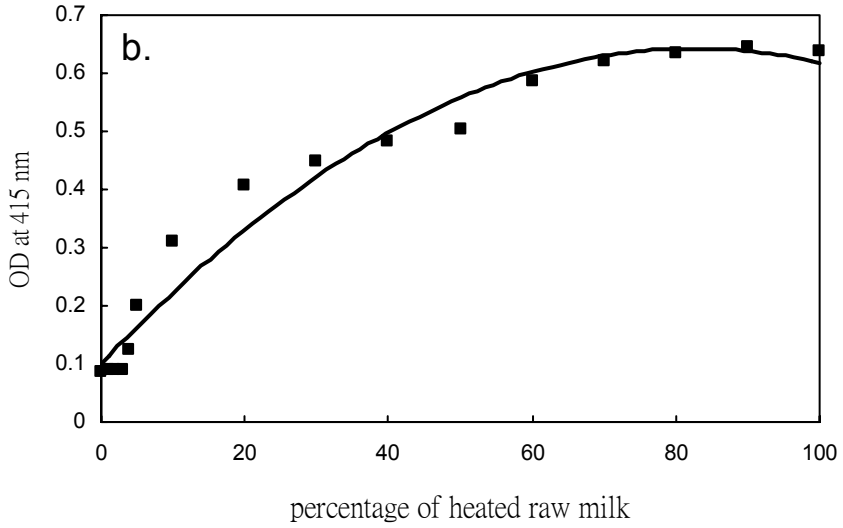

0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
OD at 415 nm
0
2
4
6
8
10
12
14
16
18
Time (min)
0.10
0.08
0.06
0.04
0.02
0.00
OD at 415 nm
R2 = 0.9652
0
1
2
3
4
5
Time(min)

Fig. 5


a.
antioxidant activity (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
R^2 = 0.951
percentage of heated raw milk


b.
OD at 415 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
percentage of heated raw milk

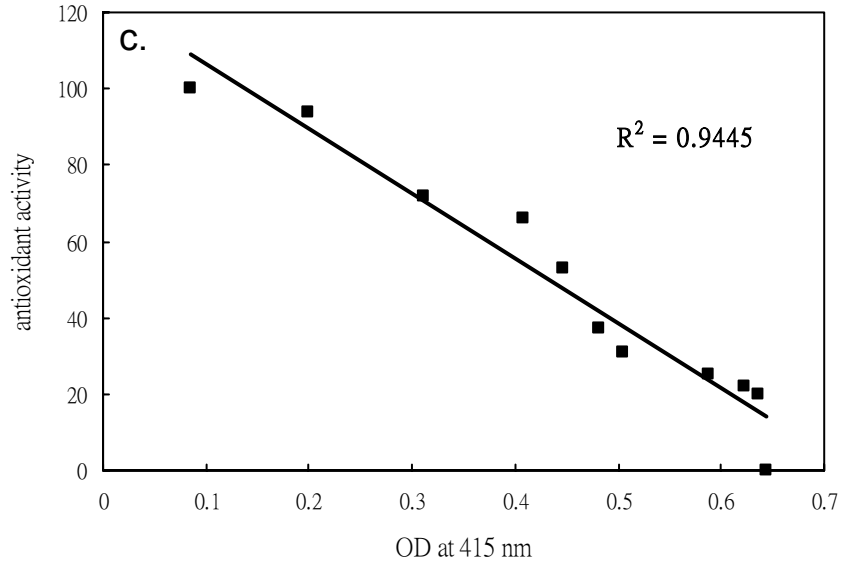

c.
antioxidant activity
120
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
R^2 = 0.9445
OD at 415 nm

Fig. 6

評語及建議事項

彭同學的實驗動機之原創性均具可圈可點之處，其實驗結果可能具實用價值，表達能力及實驗工作態度，均能令人留下深刻之印象，其科學作品成果，仍須進一步印證，綜合之，此科學作品具科學價值。