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參展科別　生物化學

作品名稱　探討胃幽門螺旋桿菌毒性因子 GroES 之重要胺基酸組成及其致發炎機制

得獎獎項　大會獎：二等獎

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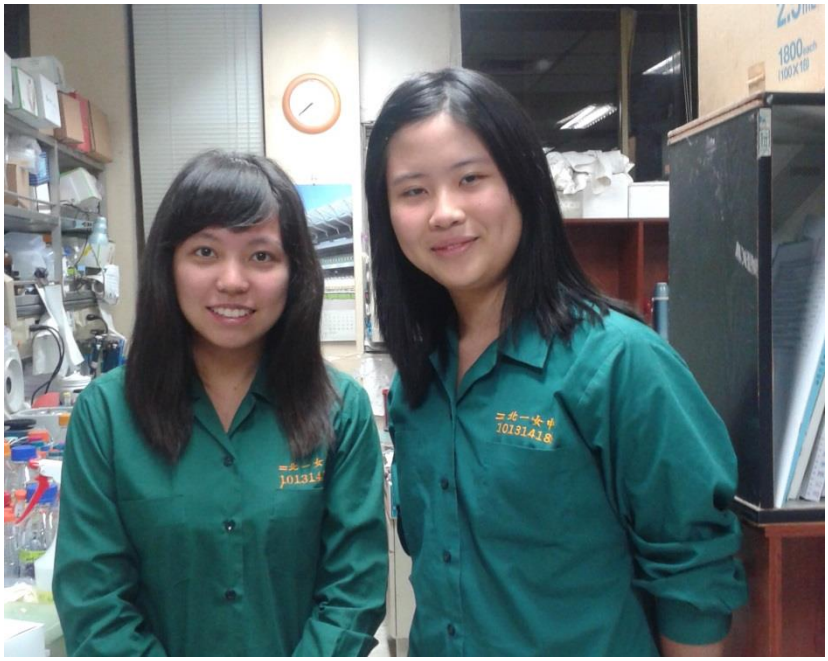
就讀學校　臺北市立第一女子高級中學

指導教師　許一懿、周綠蘋

作者姓名　陳俞禎、陳品心

關鍵字　GroES、胃炎、cysteine

作者簡介



我們是陳俞禎和陳品心，目前就讀臺北市立第一女子高級中學。學校設立的數理資優班讓我們有機會進入台大醫學院的實驗室參與生物化學方面的研究，於去年開始進行本專題研究。我們本身熱愛生命科學，閒暇時間經常前往聆聽一些生物研究的盛會，也參與中央研究院主辦的高中生命科學人才培育計畫。在實驗過程及學習生命科學的過程中，我們期勉自己能不斷探問並且永保熱情。非常感謝父母、師長、同學的鼓勵及教導，讓我們無論在實驗操作或者思考邏輯皆精進許多。未來期望能更進一步探討本研究的各個面向，使其於生活層面的應用更臻完善。

中文摘要

胃癌為全球癌症致死率第二高的癌症。胃幽門螺旋桿菌（*Helicobacter Pylori*）為其致癌最重要的因子之一，世界衛生組織更將其歸類於第一群確定性的致癌因子。幽門螺旋桿菌分泌毒性因子 GroES 蛋白，感染胃上皮細胞後能引起發炎反應；且發炎反應中，以介白素-8(Interleukin 8, IL-8)的釋放量最為顯著。GroES 蛋白（全長 1-118）在羧基端有 28 個延伸的胺基酸片段，刪去則 GroES 蛋白失去誘導細胞釋放 IL-8 之能力。因此我們希望進一步找出此延伸片段上最關鍵的致發炎片段及機制。

我們每次刪去 6 個胺基酸，探討 GroES 蛋白上與致成胃部發炎最為相關的胺基酸片段；接著以加入還原劑、加入螯合劑以及點突變的方式深入分析此致成胃發炎毒性因子的結構，探討可能的致發炎機轉。由實驗結果來看，GroES 蛋白羧基端半胱胺酸（cysteine）之間雙硫鍵形成的環狀結構能夠誘導胃上皮細胞釋放 IL-8，可能與致胃發炎有關；亦可能是組胺酸（histidine）與鎳離子之間的配位鍵引起細胞的發炎反應。Point mutation 結果則顯示 cysteine 之間雙硫鍵形成的環狀結構。

未來我們將更進一步探討此環狀結構存在的條件，也探討 histidine 與鎳離子之間的配位鍵對於致發炎的影響。我們希望能將研究成果發展成生物標記分子、疫苗以及單株抗體，進而建立一個應用平台，以儘早發現並治療胃部發炎等胃部疾病。

Abstract

Gastric cancer causes the second most death each year. *Helicobacter pylori* (*H. pylori*) secretes GroES, one of the critical virulence factors, which infects gastric epithelial cells and causes inflammation as well as gastric cancer. *H. pylori* GroES has the unique carboxyl extension of 28 amino acid fragment (91-118) in domain B, which is not found in GroES produced by other microbes. It was reported that the deletion of the extended C-terminal tail of *H. pylori* GroES reduced interleukin 8 (IL-8) release. Therefore, we try to investigate the critical amino acid residues of GroES involved in inflammatory response induction.

We deleted six amino acid residues once at a time from the C terminal end of GroES, and performed ELISA to investigate the critical sequences of the last 28 amino acids of GroES. The results indicated that the various truncated GroES mutants failed to induce IL-8 secretion in comparison with full-length GroES. Therefore, we further disrupted disulfide bonds (Cys94-Cys111 and Cys95-Cys112) by using Dithiothreitol (DTT) or site directed mutagenesis including C111A, C112A and C111A/C112A mutants to investigate the essential of the loop structure formed by the disulfide bonds in inflammatory response induction. The results indicated that single mutants of GroES, C111A and C112A, still have the ability to cause inflammation, whereas double mutant C111A/C112A does not. Taking together, the present study indicates that GroES unique structure could be developed for vaccines to prevent gastric cancer caused by *H. pylori* infection.

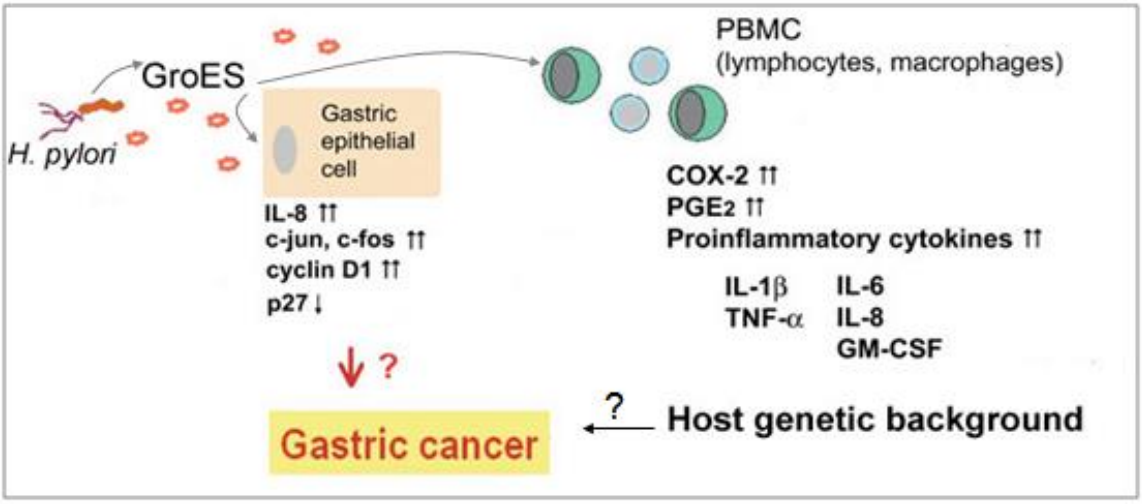
壹、前言

幽門螺旋桿菌為人類的病原菌，是一種寄生於人類消化道的革蘭氏陰性菌，能造成胃發炎、胃潰瘍、十二指腸潰瘍及胃癌等消化道疾病；而人體若遭受幽門螺旋桿菌反覆感染，會使得腸道細胞型態改變，產生免疫反應，造成消化不良消化性潰瘍等胃部疾病，更甚者會演變為胃癌。據 Nature Review Microbiology 於 2007 年的一份調查顯示：幽門螺旋桿菌感染世界上半數以上人口的胃部，特別是在開發中國家，五歲孩童已有 50 %以上的感染率，到了成人更是高達 90 %。而本實驗室先前利用二維免疫轉漬技術，針對胃癌及十二指腸潰瘍患者血清進行抗原性蛋白分析，發現幽門螺旋桿菌之 GroES 蛋白辨認次數在兩種病患族群間差異最大，亦發現 GroES 蛋白會刺激周邊血液單核球的 IL-1β、IL-6、IL-8 等的基因表現量上升，也會造成細胞增生相關因子表現量上升。又分析幽門螺旋桿菌 GroES 蛋白之序列，比較其他細菌的 GroES 蛋白序列後發現：幽門螺旋桿菌在羧基端(Carboxyl terminal, C-)的區域有 28 個胺基酸片段的延伸，在其他細菌並未發現有此胺基酸片段的延伸，刪去此片段，則 GroES 蛋白不能夠與細胞表面受體結合。

我們的實驗即是藉由 ELISA 分析 GroES 序列上後 28 個胺基酸，找出和胃發炎以至於胃癌相關之具潛力的胺基酸片段；探討其可能的致發炎結構。將研究結果發展為單株抗體及疫苗，從根本解決胃部疾病引發的問題。

貳、實驗背景

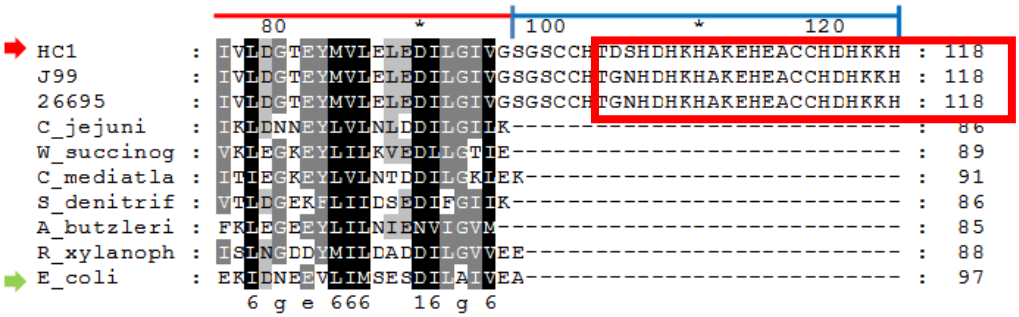
一、毒性因子 GroES 會致成胃發炎



圖一、GroES 誘導胃發炎示意圖

幽門螺旋桿菌分泌的毒性因子 GroES 能誘導人類的周邊血液單核球釋放出多種發炎性細胞激素，引起發炎反應，也能誘導胃上皮細胞釋放細胞激素以及促使一些基因表現量上升，引起胃發炎、胃潰瘍，甚至有可能致成胃癌。

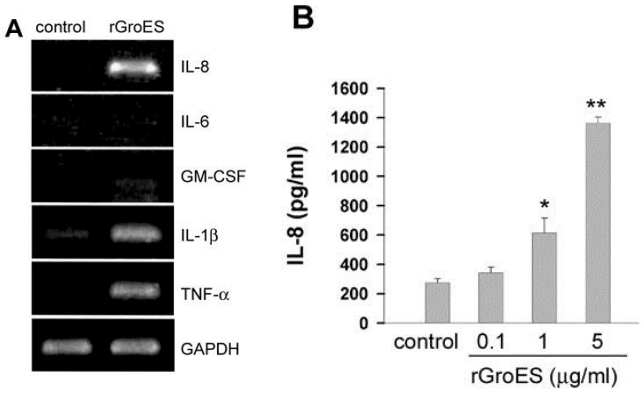
二、GroES 蛋白



圖二、幽門螺旋桿菌的 GroES 蛋白序列

GroES 蛋白又稱熱休克蛋白-10(Hsp10)。許多細菌上都有 GroES 蛋白， 但唯有幽門螺旋桿菌分泌的 GroES 蛋白具有延伸的 28 個胺基酸（圖二），序列全長共 118 個胺基酸，為幽門螺旋桿菌的致病因子之一。

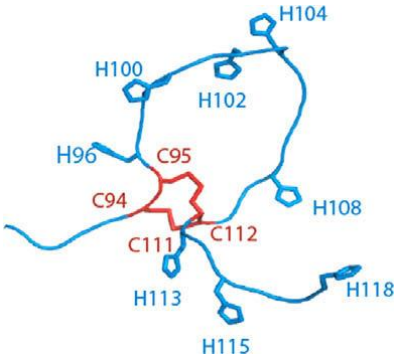
三、GroES 誘導 KATO-III 細胞釋放不同細胞素



圖三、 GroES 蛋白誘導 KATO-III 細胞釋放細胞素
(A) RT-PCR 結果圖 (B) ELISA 結果圖

經由圖三 A 圖的 RT-PCR(反轉錄酶 PCR)結果則可以看到，GroES 誘導釋放的細胞素中，以 IL-8 的表現量最為顯著，因此我們的實驗便以細胞釋放 IL-8 的濃度來當做細胞發炎的指標。

四、GroES 蛋白結構圖



圖四、GroES 蛋白結構圖

GroES 蛋白羧基端分布有 Cysteine（圖中以 C 表示）及 Histidine（圖中以 H 表示），而 Cysteine 和 Cysteine 之間雙硫鍵會構成環狀結構，histidine 則會和金屬離子產生配位鍵。

參、研究方法或過程

以每次刪去 6 個胺基酸的方式，即分別使用 112、106、100、94 個胺基酸，並分別命名為 GroES 1-112、GroES 1-106、GroES 1-100、GroES 1-94，來測定最主要的致發炎因子。

一、 設計 primer

上 NCBI 網站查詢 *H .pylori* GroES 的基因序列→找出 Forward 和 1-112, 1-106, 1-100, 1-94 的 Reverse 序列後分別加上限制酶 BamHI 和 XhoI 切位。

GroES Forward : 5′ - GGATCCATGAAGTTTCAGCCATTAGGAGA - 3′

GroES 1-112 StopReverse : 5′ - CTCGAGTTAACAGCAAGCTTCATGCTC - 3′

GroES 1-106 StopReverse : 5′ - CTCGAGTTATTTAGCATGTTTATGGTC - 3′

GroES 1-100 StopReverse : 5′ - CTCGAGTTAATGATTACCTGTATGACA - 3′

GroES 1-94 StopReverse : 5′ - CTCGAGTTAACAAGAGCCTGAGCCCAC - 3′

(綠色字體為限制酶切位，紅色字體為 stop codon)

二、含目標基因質體轉殖至勝任細胞

1. 抽取幽門螺旋桿菌之 genomic DNA (Extraction of genomic DNA)

   刮取一個培養基的菌量，離心 5min 後去除上清液，接著使用 PUREGENE DNA purification kit 抽取幽門螺旋桿菌之 genomic DNA。

2. 聚合酶連鎖反應 (Polymerase Chain Reaction, PCR)

   利用聚合酶連鎖反應放大標的基因，使用 KAPA HiFi^{TM} PCR kit，並以上述抽取之幽門螺旋桿菌 genomic DNA 做為模板(template)，其反應時

使用配方如下：

成份	體積(μL)
KAPA HiFi DNA polymerase	0.5
DNA Template	1
*Primer forward (10 μM)	0.30
*Primer reverse (10 μM)	0.30
dNTP (10 mM)	0.75
5X KAPA HiFi fidelity buffer	5
Sterile water	17.15
Total volume	25

完成後，利用 1%洋菜膠體電泳分析 PCR 產物。

將要回收的 DNA 片段從洋菜膠體上切下，使用 Gel/PCR DNA Fragments Extraction Kit 進行 PCR 產物回收儲存於-20 ℃。

3. GroES-112、GroES-106、GroES-100、GroES-94 與 pET28a 質體之接合反應

(1) 質體 DNA 的製備與微量抽取

實驗使用的 pET28a 質體原轉型於 *E. coli* JM109 中保存，所以先將帶有 pET28a 的 JM109 菌養開在培養基上，之後挑出單一菌落培養，菌液再使用 High Speed Plasmid mini Kit 抽取 pET28a 質體，儲存於-20 ℃。

(2) 限制酶水解

將 pET28a 質體與 PCR 產物回收的 DNA 進行限制酶水解，反應配方如下:

成份	比例
質體或 DNA 片段	1000~2000ng
10X NEB buffer	5μL
10X BSA	5μL
BamHI	1μL
XhoI	1μL
Sterile water	補體積
Total volume	50μL

在 37℃下反應 2 小時，之後加入 calf intestinal alkaline phosphatase (CIP) 1mL 以防止自我接合，利用 DNA 電泳分析其 DNA 片段，最後使用 Gel/PCR DNA Fragments Extraction Kit 將 DNA 片段回收，儲存於-20 ℃。

(3) 與 pET28a 質體之接合反應

被限制酶水解後的四個 DNA 片段與質體 pET28a 進行酵素結合反應，反應條件如下:

成份	比例
質體 pET28a	50 ng
DNA 片段	50 ng
T4 DNA ligase (1 U/μL)	1μL
10X ligation buffer	1μL
Sterile water	補體積
Total volume	10μL

將混合好的樣品於 16 ℃下培養 20 小時，接著轉型至勝任細胞 Rosetta

4. 轉型作用

將勝任細胞 Rosetta 和接合反應後的產物混勻後置於冰上，之後於 42℃下進行熱休克效應(heat shock) 45 sec，再置於冰上，接著加入 LB 液並於 37

℃下培養使細菌恢復並增殖，培養過後的菌液離心後，去除上清液至剩下 50 μL，再將菌液均勻塗在含有 Kanamycin 之 LB 培養基上培養。

5. 以 colony PCR 之方式篩選轉型基因庫

用 tip 尖端沾取 LB 培養基上單一菌落後加入離心管滅菌水中，再以 pET28a 上之 T7 與 T7 terminator 的序列做為 primer 以及前述之四個基因上之專一性 primer 來進行 PCR 反應。選擇有增殖出正確 DNA 分子量之菌株，將這些菌株培養後進行微量質體 DNA 之抽取，質體保存於-20 ℃，

6. DNA 定序

萃取之質體 DNA 送交台大醫學院第一共同儀器中心進行 DNA 定序，確認其 DNA 序列無誤。

三、得到目標蛋白並培養細胞

1. 以2 mM IPTG，37°誘導目標蛋白，每隔1小時測一次吸光值，待到OD600吸光值介於0.5～0.7之間為止。藉由蛋白質電泳確認目標蛋白確實被誘導。

2. 以西方墨點法檢測重組蛋白質為 GroES 蛋白

裁剪 PVDF membrane，先以 100%甲醇浸潤，再浸泡在轉印(transfer)緩衝液中(配方如下)。將四張浸泡過轉印緩衝液的濾紙、PVDF membrane，膠片由下而上依序平鋪在電泳轉印槽石墨板上，最後再鋪上三張浸泡過轉印緩衝液的濾紙，然後蓋上負極的上層石墨版。使用的電流為每平方公分約 1 mA，電壓設 10 V，通電 4 小時。轉印好的 PVDF membrane 取下先以 Fast green 蛋白質染劑染色約 2 分鐘，再以二次蒸餾水漂洗褪色，待背景回復白色後，再風乾裁切進行抗體偵測。

轉印緩衝液(transfer buffer)	
CAPS	10 mM
Methanol	10% (v/v)
pH11.0	
Fast green	0.1 g
Acetic acid	10 mL
Ethanol (95%)	26.3 mL
ddH2O	64 mL
Total volume	100 mL

將裁好的 membrane 先以 100%甲醇潤洗後泡在含有 5% (w/v)脫脂牛奶的 TN 緩衝液中，接著於室溫搖晃 1 hr 進行 blocking，倒掉反應液後再以 TNT 緩衝液漂洗 4 次，每次 10 秒後，即可進行初級抗體(primary antibody)結合反應。初級抗體以含有 1% (w/v)脫脂牛奶的 TNT 稀釋，而初級抗體即 anti-GroES，稀釋比例為 1:10000，並於 4℃下反應 16 小時，之後倒去初級抗體溶液並以 TNT 緩衝液漂洗 6 次，每次 8 分鐘。接著加入次級抗體溶液 HRP-conjugated rabbit Ab，一樣以含有 1% (w/v)脫脂牛奶的 TNT 稀釋 1: 2000，室溫搖晃 1 小時，倒去次級抗體溶液並以 TNT 緩衝液漂洗 6 次，每次 8 分鐘，接著即可以 ECL 呈色方法看結果。

TN	
Tris-HCl	20 mM
NaCl	0.15 M
pH7.5	

TNT	
Tris-HCl	20 mM
NaCl	0.15 M
Tween 20	0.05% (v/v)
pH7.5	

ECL 呈色，吸掉 PVDF membrane 上多餘的緩衝液後，均勻加入適量的反應物(ImmobilonTM Western Chemiluminescent HRP substrate，A 與 B 兩種溶液等體積混合)，反應後將多餘的受質溶液吸掉，利用 LAS-4000 影像分析儀分析 membrane 上的冷光反應。

3. 重組分泌性蛋白質之純化 (Purification of recombinant proteins)

收集之上清液和製備完成之 Ni^{2+} Chelating Sepharose beads 混合後，在 4℃下進行震盪培養 16 小時，使帶有 His-tagged 之 GroES 結合在 beads 上。

binding buffer	
Tris-HCl	20 mM
NaCl	500 mM
Immidazole	5 mM
pH7.9	

實驗中用到純化蛋白的方法是以樹脂管柱(resin column)去結合帶有 His-tag 的重組蛋白質，因此必須配製 Ni2+管柱。取 Chelating Sepharose beads (50% slurry in ethanol) 3 mL，配製成 1.5 mL 樹脂管柱，待液體流至欲乾，依序加入 4.5 mL ddH2O、7.5 mL charge buffer (50 mM NiSO4)使管柱配製成鎳離子管柱(nickel column)，接著加入 7.5 mL binding buffer 以平衡管柱，最後必須再加入 binding buffer 7.5 mL 使管柱內環境與溶解蛋白質的溶液相同。

當沉澱的重組蛋白質利用 binding buffer 回溶後，先以 15000 rpm，15 分鐘離心以沉澱無法再被溶解的部份與殘骸，接著取上清液並且和已配製完成的 Ni2+ beads 輕輕混勻後置於 4℃以上的低溫處輕輕搖晃 overnight，讓帶有 His-tag 的重組蛋白與 Ni2+ beads 充分結合。

隔天先將重組蛋白與 Ni2+ beads 溶液以 1500 rpm，3 分鐘離心後，去除

上清液並加入 40 mL binding buffer 輕輕搖混五分鐘，之後再以相同條件離心，重覆三次以洗去沒有和 Ni2+ beads 結合的雜質；再將溶液注入管柱中，先以 20 mL binding buffer 沖洗管柱，接著加入 20 mL 的 wash buffer (含有 50, 100, 150, 200, 250, 500 mM immidazole 之 8M binding buffer7.5 mL)之 elution buffer 將 His-tagged 蛋白質競爭下來，每 1.5 mL 收集一管，並以 15% SDS-PAGE 檢視其純度和進行蛋白質定量。

4. 重組分泌性蛋白質之定量

蛋白質以 660 nm Protein Assay Reagent 定量，蛋白和 reagent 以 10:150 比例室溫混合 5 分鐘，即可以分光光度計測定 660 nm 波長可見光吸收值。將測得數值與已知蛋白質濃度的標準品數值做比較，就可藉由標準曲線圖推得重組蛋白質的濃度。

5. 十二烷基磺酸鈉-聚丙烯醯胺膠電泳法

(1) 膠體玻璃之架設

取兩塊在稀酸中浸泡過之平面玻璃(mini gel: 10 x 10.5 cm)，其中一塊上方呈凹型，用清潔劑將玻璃清洗乾淨後以二次水潤濕，放入烘箱烘乾。在兩塊玻璃中間底部夾有乳白色橡膠做為 spacer，用鐵夾將玻璃兩側夾緊，再將此裝置直立放置，準備注入膠體。

(2) 配置聚丙烯醯胺膠體 (Preparation of polyacrylamide gel)

將預先配置好之 A solution、B solution 以及 C solution 混合進行膠體製作，A solution、B solution 以及 C solution 詳細配方如下：

A 液	
Acrylamide	30 %
N-N’-methylene-bis-acrylamide	0.8 %
B 液	
Tris-HCl, pH 8.8	1.5 M
SDS	0.4 %
C 液	
Tris-HCl, pH 6.8	0.5 M
SDS	0.4 %

依據下表配置下層 separating gel 以及上層 stacking gel:

溶液	separating gel 15 %	stacking gel
A solution	4.0 mL	0.3 mL
B solution	2.0 mL	—
C solution	—	0.5 mL
ddH_2O	2.0 mL	1.2 mL
TEMED	5.0 μL	3.0 μL
10 % APS	55.0 μL	9.0 μL

首先配置 separating gel：依照上表依序在小錐形瓶中加入 ddH_2O、B solution、A solution、TEMED 以及 10% APS (ammonium persulfate)，混合均勻後迅速將凝膠溶液注入玻璃裝置中，並於凝膠上方加入甲醇溶液使膠體平整，並維持玻璃裝置在室溫下，待介面形成後，倒出甲醇溶液，準備注入 stacking gel。接著配置 stacking gel：依照上表依序在小錐形瓶中加入 ddH_2O、C solution、A solution、TEMED 以及 10% APS，混合均勻後將凝膠溶液倒入 separating gel 上層，並插入一梳狀模板(comb)使膠體形成凹槽，待膠體凝固後可將梳狀模板取下，供蛋白質樣品注入。

(3) 分泌性蛋白質樣品的處理 (Sample preparation)

將樣品加入適量之 2X sample buffer，夾上防爆夾，於 95℃水浴中加熱 10 分鐘。配方如下：

2X sample buffer	
0.5 MTris-HCl pH6.8	1 mL
10% SDS	4 mL
50% glycerol	2.4 mL
β-Mercaptoethanol	0.2 mL
ddH_2O	2.4 mL
Bromophenol blue(BPB)	0.01%
Total volume	10 mL

(4) 電泳之操作 (Protocol of electrophoresis)

將膠片周圍之橡膠條拿掉，玻璃片以鐵夾固定在電泳裝置上，使有凹槽的一面朝向電泳槽，取 1 X 電泳緩衝液(10 X 電泳緩衝液配方如下)注滿上下電泳槽，將準備好之蛋白質樣品加入樣品槽中，電泳槽上層接上負極，下層接上正極，設定 17 mA、170 V 情況下進行電泳，

10 X 電泳緩衝液	
Tris	25 mM
Glycine	250 mM
SDS	0.1 %
pH 8.3	

本實驗所採用之標準蛋白質分子量(Low-range Protein Molecular Weight Markers)如下表所示：

蛋白質	分子量(kDa)
Phosphorylase B	97.4 kDa
Bovine Serum Albumin	66.2 kDa
Ovalbumin	45.6 kDa
Carbonic Anhydrase	31.0 kDa
Soybean Trypsin Inhibitor	21.5 kDa
Lysozyme	14.4 kDa

(5) 電泳膠片之染色與脫色

完成電泳後，將膠體小心取下並浸泡於染色液(Coomassie blue staining solution)中約 30 分鐘，再將染色液倒出，加入脫色液(Destaining solution)浸泡膠體直到膠體背景呈現透明無色，最後將膠體置於 ddH_2O 中保存。染色液與脫色液配方如下:

染色液	
Methanol	12 %(v/v)
Acetic acid	10 %(v/v)
Coomassie brilliant Blue	4 tablet

脫色液	
Methanol	25 %(v/v)
Acetic acid	7 %(v/v)

四、胃上皮系 MKN45、KATO-Ⅲ細胞之培養

胃上皮系 MKN45、 KATO-Ⅲ細胞為貼附性細胞，將細胞以 RPMI1640（含 10 % FBS）在 37℃、5 % CO_2 的條件置於 75T 的細胞培養瓶進行培養，待細胞生長至培養瓶底部面積七分滿時，將培養液倒除，以無菌 PBS 清洗細胞兩次，加入 1mL 0.5%胰蛋白酶緩衝液(EDTA-Trypsin)在 37℃、

5 % CO_2 培養箱處理 5 分鐘，將細胞懸浮並以無菌吸量管將細胞團塊沖散，並加入 3mL 培養液中止胰蛋白酶對細胞的作用，將此細胞液取至 50mL 離心管，以 500xg 室溫下離心 10 分鐘後去除培養液，以無菌 PBS 將細胞再次懸浮清洗，並再次以 500xg 室溫離心 10 分鐘，加入培養液並調整細胞液濃度分配至培養瓶進行繼代培養，或是調整細胞液濃度至 $2x10^5$/mL，以每孔 200μL 分配至 96 孔盤的培養孔中，放入 37℃、5 % CO_2 培養箱中隔夜培養。

五、以重組蛋白處理細胞

將上述分配並貼附至 96 孔培養盤之 MKN45、KATO-Ⅲ三種細胞，去除培養液，以無菌 PBS 清洗一次，以 RPMI1640(（不含 FBS）)配置不同濃度之重組蛋白液後，處理 96 孔培養盤中的細胞 24 小時後，收取培養液保存於-20℃，準備酵素連結免疫吸附分析。

六、 ELISA

1. Coating：在 96 孔反應盤中，加入 100μL（1μg/mL in PBS）的 IL-8 多株抗體，置於 4℃中隔夜培育。

2. 隔日取出後，倒去多餘抗體溶液，以 wash buffer 清洗兩次

3. Blocking：加入 200μL 含有 4 %牛血清白蛋白（Bovine Serum Albumin, BSA）的 PBS 進行 Blocking，置於室溫培育至少一小時。

4. 去除覆披溶液後，以清洗溶液 wash buffer 清洗三次，並拍乾小孔中殘餘液體

5. 配製 standard curves：加入 100μL 經純化之 IL-8 重組蛋白（濃度分別為 0 pg/mL、15.625 pg/mL、31.25 pg/mL、62.5 pg/mL、125 pg/mL、250 pg/mL、500 pg/mL in 4% PBS/BSA），用以製備標準曲線

6. 待測之細胞培養液，以 blocking 溶液進行 100 倍稀釋後，取 100μL 加入反應孔中，並置於室溫中培育 2 小時。取出後小心去除多餘檢體溶液

7. 以 wash buffer 清洗三次，並拍乾小孔中殘餘液體後

8. 在每個反應孔中加入 100μL（0.05μg/mL in 4% PBS/BSA）的生物素標記之 IL-8 偵測抗體（biotin-labeled IL-8 detecting antibody,），置於室溫下培育 1 小時

9. 完成後倒掉多餘抗體溶液，以清洗溶液清洗三次，並拍乾反應孔中的殘餘液體。

10. 接著加入 100 μL HRP-conjugated Streptoavidin，置於室溫下培育 30 分鐘

11. 完成後倒掉多餘溶液，以 wash buffer 清洗三次，最後一次清洗時，要盡量拍乾微孔中的殘餘液體

12. 最後加入 100 μL 的 3,3’,5,5’- 四甲基聯苯胺（3,3’5,5’, Tetramethylbenzidine, TMB）作為受質進行呈色，置於室溫避光培育約 30 分鐘。

13. 加入 0.18M 100μL 硫酸（H_2SO_4）終止反應，並以 450 nm 的波長偵測吸光值，利用標準曲線使用內插法求各處理組別 IL-8 之濃度。

七、加入 DTT

1. 在 GroES 蛋白中加入 5 mM DTT，作為實驗組；與未加入 DTT 的對照組操作 Non-reducing gel 蛋白質電泳，分析結果，確認 DTT 確實發揮破壞雙硫鍵的工作。

2. 用加入 DTT 的 GroES 蛋白處理胃上皮系 KATO-III 細胞，與未加入 DTT 的對照組操作 ELISA（步驟如六）。

八、加入 EDTA

用加入EDTA的GroES蛋白處理胃上皮系KATO-III細胞，與未加入EDTA的對照組操作 ELISA（步驟如六）

九、Point mutation

1. 設計 C112A 及 C111A primer 序列

C111A/C112A primer design :
F: A A H D H K K H stop
gct gct cat gat cac aaa aaa cac taa
R: same as C111A-R

2. 在 primer 中加入 T4 PNK 使其 5'端磷酸化，37°C 30min，使日後得以接合

3. Clean up PNK

4. 以目標質體為 plasmid 進行 PCR

5. Gel extraction

6. Ligation

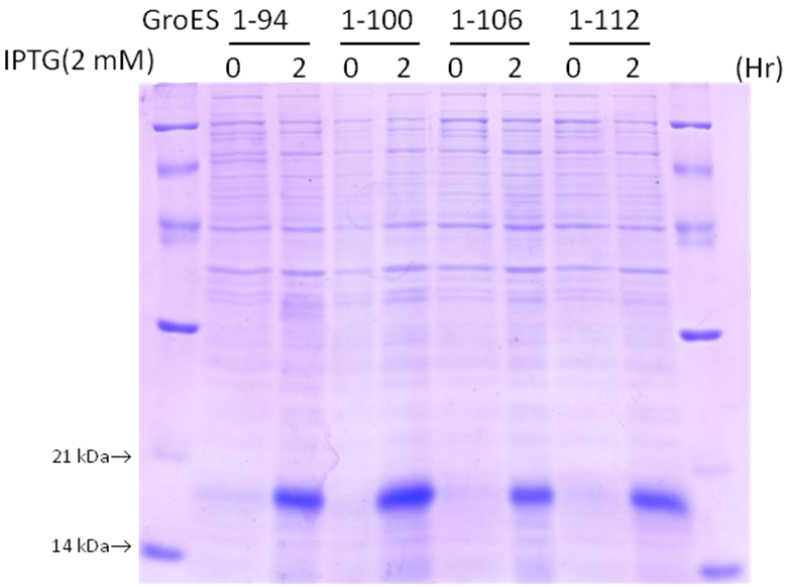
DNA 10～20 ng	10～20 ng
10X buffer	
T4 ligase	
Total volume	10 μL

7. Transformation（其後步驟與二 4.之後的步驟同）

肆、研究結果與討論

一、得到目標蛋白

1. 以 IPTG 誘導表現 GroES 蛋白之結果確認能夠誘導表現出目標蛋白

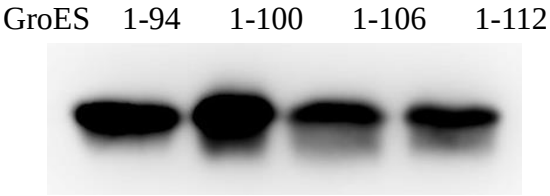


圖五、以 IPTG 誘導表現目標蛋白的蛋白質電泳結果

2. 以西方墨點法及破菌後離心確認目標蛋白為 GroES 蛋白且狀態正確

(1) 西方墨點法檢測結果

此 4 段 GroES 蛋白皆被 GroES 抗體辨識出，確認得到產物為 GroES 蛋白。

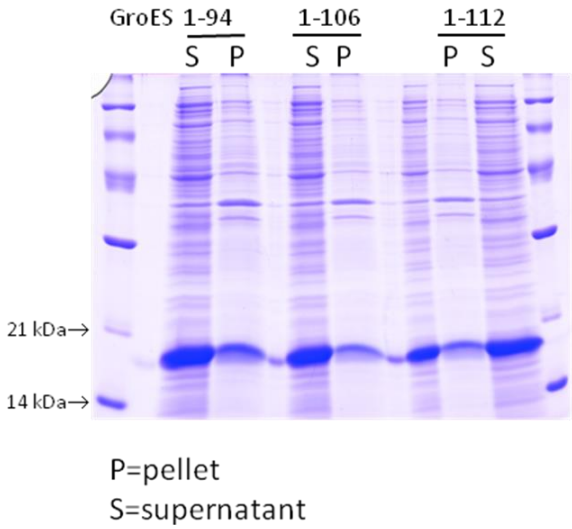


圖六、以西方墨點法辨識目標蛋白的結果

(2) 透過離心分析上清液及沉澱物的蛋白質電泳結果

所得到的 GroES 多位在 supernatant，確認得到的蛋白質處於正確

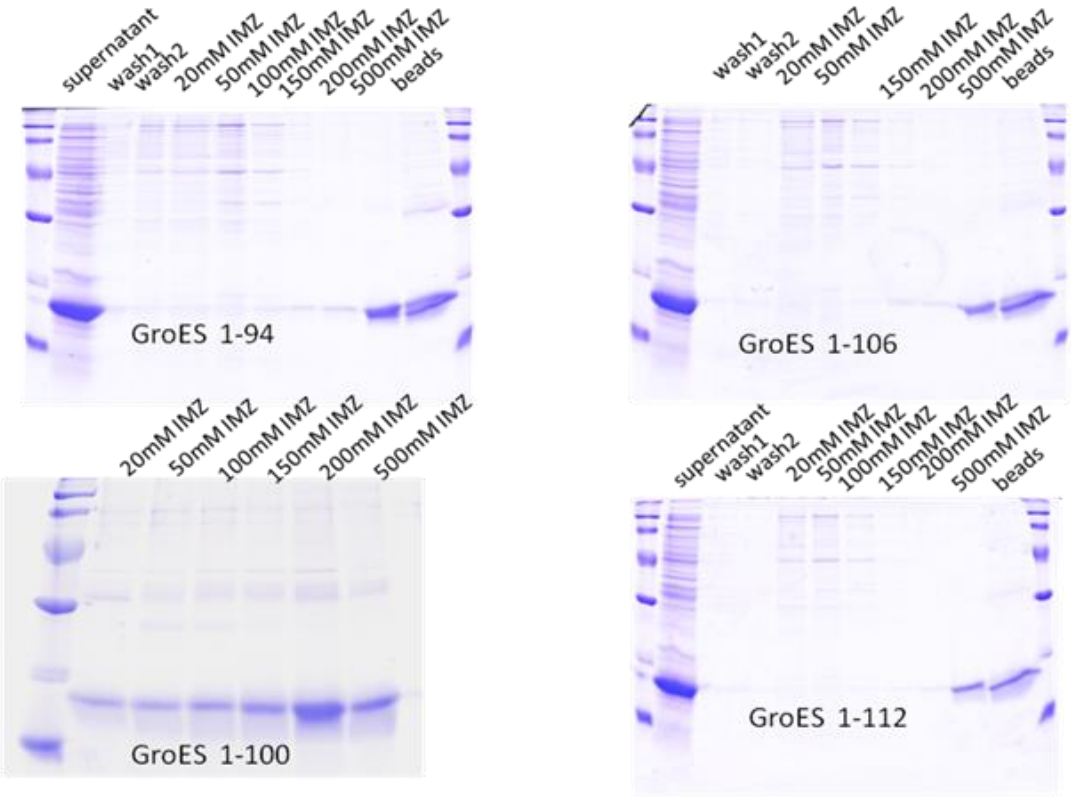
的狀態。



圖七、 等體積上清液及沉澱的蛋白質電泳結果

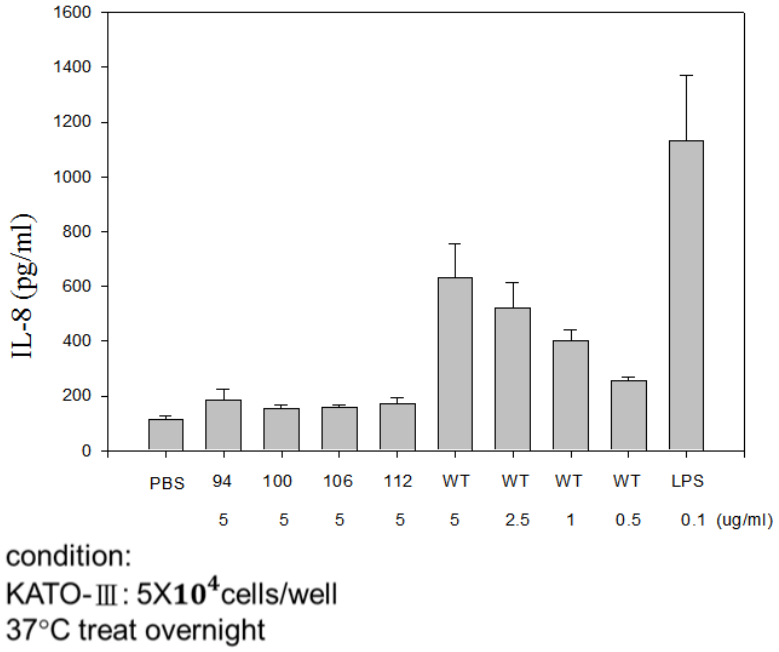
3. 4 段 GroES 蛋白 IMZ 競爭結果

得到適合此蛋白純化的條件：500 mM IMZ



圖八、以 IMZ 競爭目標蛋白的蛋白質電泳結果

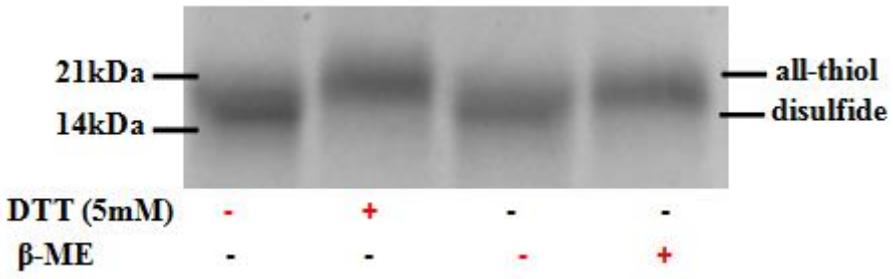
二、 初步 ELISA 結果



圖九、GroES 在 5 種長度下的 treat 細胞的 ELISA 結果

三、加入 DTT 後的電泳及 ELISA 結果

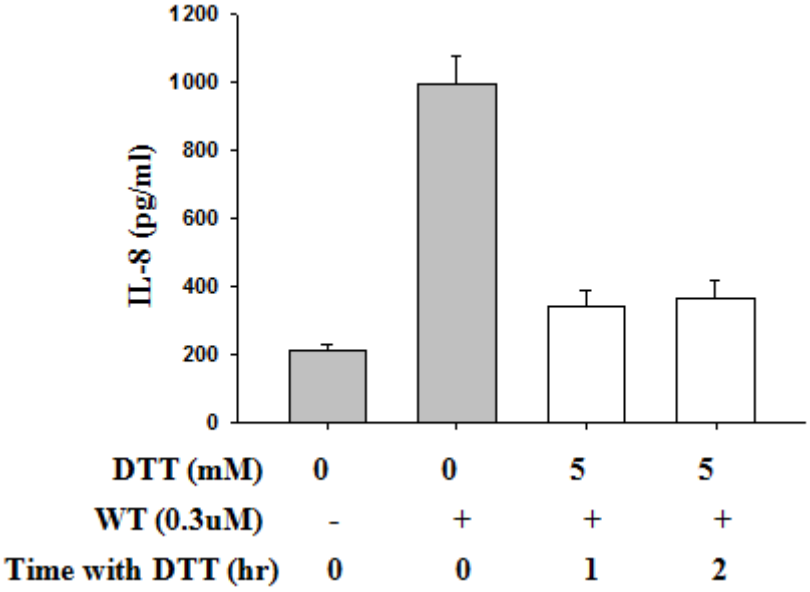
1. 電泳結果



圖十、加入 DTT 的電泳結果

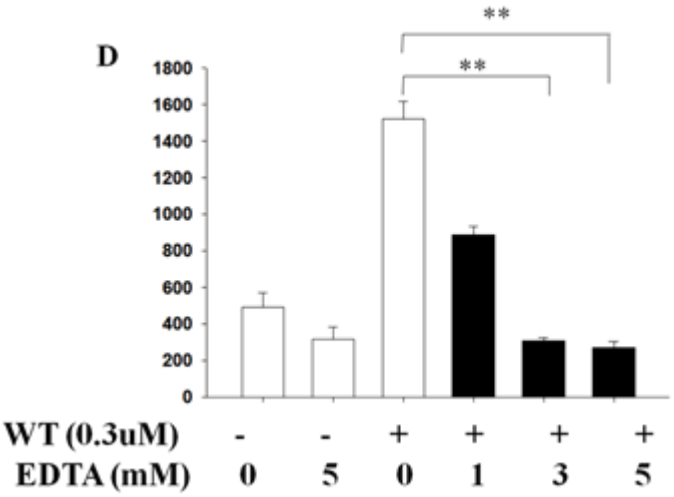
蛋白質 non-reducing 電泳結果，用來分析 DTT 對 GroES WT 結構的影響。由此結果確定 DTT 確實破壞雙流鍵，使 GroES 蛋白呈現鏈狀。

2. ELISA 結果



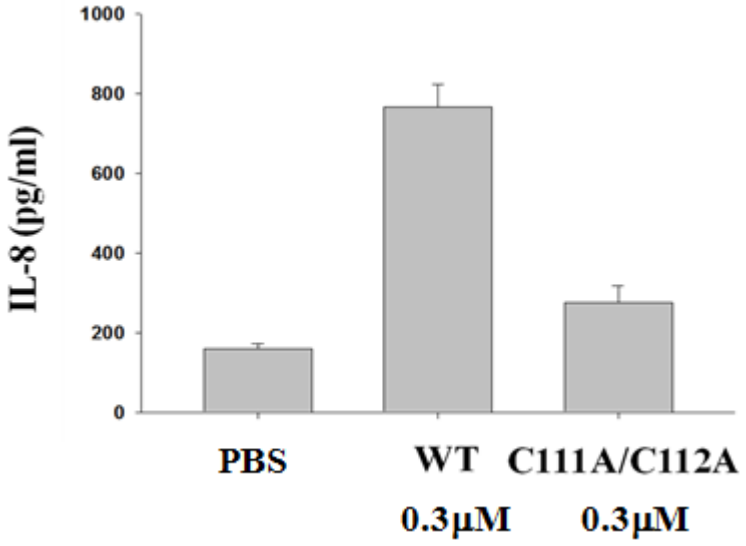
圖十一、加入 DTT 的 ELISA 結果圖

四、加入 EDTA 的 ELISA 結果



圖十二、加入 EDTA 的 ELISA 結果圖

五、Point mutation 結果



圖十三、Point mutation 的 ELISA 結果圖

圖（十一）（十二）（十三）ELISA 結果圖，用來測定 GroES 在同時突變兩對雙硫鍵（double-mutation）。X 軸上欄不同條件的 GroES，Y 軸代表將樣品吸光值代入標準曲線所求出的濃度。p value <0.01

伍、結論與應用

一、由 GroES 1-112, 106, 100, 94 誘導釋放 IL-8 的 ELISA 結果可知：

GroES 唯有全長時能夠誘導細胞釋放 IL-8；截斷的 4 個片段則不具此作用。GroES 的最後 28 個胺基酸會形成環狀結構，這是由 Cysteine 兩對雙硫鍵(C94/C111 和 C95/C112)鍵結而成。將 GroES 以每次刪去 6 個胺基酸的方式分段可能會影響到環狀結構，並影響誘導細胞釋放 IL-8。推論可能是 GroES 蛋白羧基端之環狀結構造成如此結果；GroES 蛋白羧基端亦分布有 Histidine，而 Histine 與蛋白溶液中的鎳離子之間會形成配位鍵（參考文獻四），故我們也推論可能是此配位鍵影響誘導細胞釋放 IL-8

二、探討 GroES 可能的致發炎結構

1. 由全長 GroES 蛋白加入還原劑 DTT 的電泳和 ELISA 結果可知：環狀結構會影響 IL-8 分泌 DTT 破壞 GroES 的雙硫鍵，使蛋白無法形成環狀結構。ELISA 結果顯示加入 DTT 後，GroES 誘導細釋放 IL-8 的濃度顯著下降，因此環狀結構可能影響 IL-8 分泌。

2. 由全長 GroES 加入螯合劑 EDTA 的 ELISA 結果可知：和金屬離子的鍵結能力會影響 IL-8 分泌螯合劑螯合金屬離子，使 Histidine 和鎳離子之間無法形成配位鍵，ELISA 結果顯示加入 EDTA 後，GroES 誘導細釋放 IL-8 的濃度顯著下降，因此和鎳離子之間的配位鍵亦可能影響細胞釋放 IL-8。

3. 由 Point mutation 的結果可知： GroES 羧基端上 Cysteine 之間兩對雙硫鍵構成的環狀結構影響 IL-8 分泌同時突變 C111 及 C112 為 A111 和 A112，則 GroES 誘導細胞釋放 IL-8 的濃度顯著下降，推論 Cysteine 之間兩對雙硫鍵(C94/C111 和 C95/C112)構成的環狀結構可能影響 IL-8 分泌。

陸、討論和未來展望

一、 GroES 致胃發炎的機制推測有二：

機制 1：GroES 羧基端上 Cysteine 之間兩對雙硫鍵(C94/C111 和 C95/C112)構成的環狀結構使其與胃上皮系細胞表面受體結合。

機制 2：GroES 蛋白上的 Histidine 和蛋白溶液中的鎳離子形成配位鍵。未來工作為確認此一配位鍵在致胃發炎過程中扮演的角色。

二、我們目前是同時突變 C111 和 C112，故僅能確認 GroES 在兩對雙硫鍵皆存在時，與致胃發炎相關，而無法得知單一雙硫鍵存在時，GroES 是否與致胃發炎相關。未來工作即分別突變 C111 和 C112，更深入地探討 GroES 的環狀結構對致胃發炎的影響。

三、找出 GroES 致胃發炎的確切機制後，我們希望能將其發展成生物標記分子、疫苗以及單株抗體；建立一個應用平台，以儘早發現並治療胃部發炎等胃部疾病。

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1. Introduction

1.1 H. pylori induces cytokines which are linked to gastric cancer.

Helicobacter pylori is a Gram-negative, microaerophilic bacterium that selectively colonizes in the human stomach. *H. pylori* infection has been proven as a major cause of chronic gastritis and peptic ulcer disease, and is highly related to gastric cancer. *H. pylori* was also classified as a class I carcinogen by the World Health Organization(WHO) in 1994. Despite a recent decline in infection rates, the prevalence rate of *H. pylori* remains at nearly 50% of the world's population and the associated gastric cancer is the second leading cause of cancer-related death worldwide. The most remarkable feature of persistent *H. pylori* infection is that it causes inflammatory responses, which is an important risk factor for malignancy.

The cytokines induced by *H. pylori* infection include TNF-α, IL-1β, IL-6, and IL-8 [2]. It is well recognized that release of these proinflammatory cytokines is closely linked to the pathogenesis of *H. pylori*-associated gastric cancer. Induction of cytokines secretion by *H. pylori* depends on both host genetic background and microbial virulence. IL-8 was the most significantly up-regulated gene and appears to play a major role in the epithelial cell response to *H. pylori* infection and in the pathological processes leading to gastric disease. Several authors have demonstrated increase in IL-8 in response to *H. pylori* in both in vivo and in vitro studies. Gastric mucosal IL-8 levels have shown a positive correlation with the degree of stomach corpus inflammation, and IL-8 is also highly increased in gastric cancer. IL-8 is secreted from gastric epithelial cells by *H. pylori* virulence factors such as cag pathogenicity island (cagPAI) and OipA. However, it has been shown that cagPAI and OipA are not the sole factors responsible for induction of proinflammatory cytokines. We have previously reported that *H. pylori*

GroES (GroES) can induce the production of proinflammatory cytokines including IL-8, IL-6, IL-1β and TNF-α in human PBMC and trigger IL-8 production in gastric epithelial cells.

1.2 H. pylori GroES has the unique carboxyl extension, which possibly leads it to cause the induction of proinflammatory cytokines.

GroES, also referred to as the heat-shock protein A (HspA), is an unusual homolog of the essential bacterial GroES chaperonin family. In general, GroES serves as a co-chaperonin of the heptameric GroEL-GroES barrel complex, which mediates the refolding of a variety of nonnative proteins. In addition to the highly conserved GroES chaperonin domain (domain A), GroES contains a C-terminal extension (domain B), which is absent in most of the other GroES members. domain B consists of 28 amino acids including and 4 Cys residues and confers a unique conformational structure which form two disulfide bonds and generate a closed-loop structure. Except its co-chaperone activity, GroES has been reported to play additional important roles, being involved in nickel homeostasis and urease activation. Besides intra-cellular locations, GroES is able to gain access to the extracellular compartment and shown strong antigenic properties. GroES represent strong antigen that is specifically recognized by the sera from *H. pylori*–infected patients, and it then considered as a potential candidate for vaccine development and diagnosis of *H. pylori* infection.

The upregulation of proinflammatory cytokines by GroES has been shown contributed to the gastric inflammation. However, the molecular mechanism of induction proinflammatory cytokines production has not been elucidated. Here we investigated the possible involvement of GroES induce IL-8 release in gastric cancer cells. When every 6 amino acid were truncated from the end of GroES domain B, we

found that only full length GroES can induce IL-8 release, but not the truncated forms. Moreover, when the disulfide bonds of domain B were disrupted by reducing agent DTT or site-directed mutagenesis, we found that the conformation structure is collapsed and failed to reduce IL-8 production. These data indicate that unique domain-B of GroES may act as a potent virulence factor in gastric cancer cells and a therapeutic target for the inflammatory diseases.

2. Materials and Methods

Mutants construction

Truncated mutants (GroES 1-94, GroES 1-100, GroES 1-106, GroES 1-112) of GroES were prepared by polymerase chain reaction (PCR). Preparation of point mutants (C111A, C112A, C111A/C112A) was carried out using the Phusion site-directed mutagenesis system (Thermo Scientific). Primer sequences were listed in Table 1 and 2. The GroES genes were digested by restriction enzymes BamHI and XhoI and then ligated with pET28a plasmid for the truncated mutants or pQE30 plasmid for the mutants. The PCR amplified mutant-containing vectors were transformed into *E. coli* Rosetta. Colony PCR was conducted, using T7 terminator on pET28a as a primer, to select colonies which proliferated the correct molecule weight of the DNAs. To confirm the presence of the desired mutation, the DNA sequence of the mutants were determined.

Expression and purification of GroES

Expression of each protein was induced in *E. coli* strain Rosetta for 4 hr at 37℃ with 1 mM isopropyl β-D-thiogalactoside (IPTG), using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm whether the target proteins have been induced. The soluble recombinant proteins were purified on a Ni^{2+}-chelating Sepharose column. All proteins were ＞95% pure as assayed by Coomassie blue staining on SDS-PAGE.

Culture the human gastric epithelial cells: KATO-Ⅲ

Human gastric epithelial cells KATO-Ⅲ were obtained from the American Type Culture Collection (HTB-103) and cultured in RPMI1640, supplemented with 10 % FBS and 100 units/ml of penicillin-streptomycin and grown at 37 ℃ with 5 % CO_2. Wait until cells cover 70% of culture plate, then pull over the medium. The cells were washed by Phosphate buffered saline (PBS, Gibco). 1c.c. EDTA-Trypsin was added, under 37°C with 5% CO_2 of for 8 minutes. The cell groups were broken up by sterile pipette, then medium was added to suspend trypsin action, then the cells were taken out to the 50 mL centrifuge tubes and centrifuged at the following terms: 500xg for 10 minutes. After that, the culture medium was removed, resuspended and washed by sterile PBS, centrifuged at the same terms again. Finally, 1/5 of the cells were taken out for sub-culturing and the remained 4/5 cells were kept for assignment to the 49-well plate at 37°C with 5% CO_2. The cells were kept overnight.

ELISA

KATO-III cells were co-cultured with 0.3 μM WT and mutants for 16 hr. Cell-culture supernatants were collected. KATO-III cells were dissolved with medium, and trypan blue was added. Observe and count the amount of the cells. Then interleukin-8 polyclonal antibody (IL-8 Ab) and stock buffer were added for coating. After blocking (Bovine serum albumin, BSA), wash thrice by wash buffer. The cells were taken out from the 96-well plate to eppendorf, centrifuged at the following terms: 1500 rpm for 5 minutes, then the supernatant was taken out to another eppendorf. Standard curves were prepared simultaneously in different concentration: 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL, 15.625 pg/mL, 7.8125 pg/mL, 3.90625 pg/mL. Both samples plus sample buffer and standard curves plus sample

buffer were placed at room temperature for 1 hour. After washing three times, biotin-IL-8Ab was added at room temperature for 1 hour. Again wash for three times, then HRP streptavidin (Merck) was added at room temperature for 30 minutes. Again wash for three times, then TMB (Sigma) was added in a very short of time. Wait until the color changed. At last, stop solution was added in a very short of time.

Truncation mutants expression and functional assay

Every 6 amino acid codons were truncated from the end of GroES gene, and each of them were digested and ligated to pET28a. Single mutant C111A and C112A and double mutant C111A/C112A were constructed as well. The plasmids were transformed into competent cell Rosetta, and then colony PCR was conducted to select the correct colonies for DNA sequencing. Target proteins were expressed by IPTG induction, then protein expression and purification were performed. At the same time, KATO-Ⅲ cells were cultured using RPMI1640. KATO-Ⅲ cells ($5X10^4$ cells/well) were treated with GroES overnight at 37°C. ELISA was performed under the concentration of 0.3 μM WT and truncated GroES to detect the release of IL-8 in inflammatory response induction.

DTT Treatment

Reducing agent DTT was added to disrupt the disulfide bonds between cysteines. SDS-PAGE was performed to check if DTT served its function. ELISA was performed afterward under the concentration of 5 mM DTT and 0.3 μM WT.

Statistical analysis

All experiments were carried out in triplicate and data are expressed as the mean ±S.D. The Student t test was employed to compare treated versus control samples. For all tests, $p < 0.05$ was considered statistically significant.

3. Results

3.1 The truncated proteins failed to induce IL-8 production.

In a previous study, it was reported that only GroES 1-118 can induce PBMC to release IL-8, but not GroES 1-90 (domain A) or GroES 91-118 (domain B) [1], which indicates that the extended 28 amino acids are indispensable in causing inflammation. Therefore, we try to investigate the critical amino acid residues in GroES domain B. We deleted six amino acids once at a time from the end of GroES, and ELISA was performed to detect the level of IL-8 release. The result showed that only WT GroES (GroES 1-118) can induce IL-8 release, but not the four truncated forms (Fig. 2).

3.2 Disulfide bonds are critical for IL-8 induction.

The result of the truncation inspired us that maybe it is the structure that influence the capability to induce IL-8 release [3]. It is known that there are two disulfide bonds formed by four cysteines in GroES domain B. Therefore, we used DTT to disrupt the disulfide bonds and ELISA was performed to detect the level of IL-8 release. The result showed that the DTT-treated GroES lost the capability to induce IL-8 release (Fig. 4), which displayed that the disulfide bonds formed by cysteine residues were critical in causing the inflammation.

3.3 Single point mutation shows the essential of C94/C111 or C95/C112 disulfide bonds for GroES activity.

Knowing that the two disulfide bonds in GroES domain B is critical, we further constructed single point mutant,C111A or C112A, to investigate the importance of either of the two disulfide bonds. Site-directed mutagenesis was used and the level of IL-8 release was detected by ELISA. The result showed that when losing one of the two

disulfide bonds, GroES still has the ability to induce IL-8 release and cause inflammation (Fig. 5), which means that either of the two disulfide bonds is important.

3.4 Double point mutation, C111A/C112A GroES displays the indispensability of the loop structure.

By site-directed mutagenesis C111A/C112A, both of the disulfide bonds were deduced and then the level of IL-8 release was detected by ELISA. The result showed that C111A/C112A GroES mutant lost the capability to induce IL-8 release (Fig. 6). It is known that the loop structure did not exist anymore when losing the both disulfide bonds. Therefore, we demonstrated that the loop structure in GroES domain B is essential in causing the inflammation.

4. Discussion

H. pylori infection has been reported to cause chronic inflammation with production of proinflammatory cytokines. Numerous studies showed that *H. pylori* infection enhances IL-8 production in gastric epithelial cells. In this study, we focused on GroES-induced IL-8 secretion in human gastric epithelial cells. We observed that only full-length GroES can induce IL-8 secretion. In contrast, C terminal truncated mutants, point mutants of GroES failed to induce IL-8 production.

Bacterial HSPs have been reported to activate proinflammatory cytokines production. GroEL and DnaK of *E. coli* induce IL-6 and TNF-α in monocytes and endothelial cell [6]. GroEL of *C. pneumonia* is known to activate inflammation and contributes to coronary disease (CAD) [7, 8]. *M. leprae* Hsp65 induces inflammation and is associated with airway hyperresponsiveness [9]. These studies indicate that bacterial Hsps are closely associated with immune responses to bacterial infection. However, in the family of GroES, only *H. pylori* GroES and GroES of *Mycobacterium* have been reported to modulate immune responses previously. In contrast, *E. coli* GroES was reported unable to induce proinflammatory cytokines production [6]. Thus, it is likely the unique domain B of *H. pylori* GroES may contribute to GroES-induced immune responses. A previous study examined whether domain A or domain B is the immunogenic domain of *H. pylori* GroES. They performed ELISA using *H. pylori* GroES, domain A, and domain B as antigens and identified the dominant serological response was against domain A. In this study, we found that neither truncated mutants nor point mutants of *H. pylori* GroES can induce IL-8 production in gastric epithelial cells. To reveal this conflict, we proposed that domain B of *H. pylori* GroES may assist immunogenic domain A to induce immune responses in gastric epithelial cells.

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Laboratory of Dr. Lu-Ping Chow in the Institute of Biochemistry & Molecular Biology, National Taiwan University College of Medicine, Taipei, Taiwan

PI: Lu-Ping Chow

Researcher: Yu-Lin Su

Laboratory of Dr. Jung-Yaw Lin in the Institute of Biochemistry & Molecular Biology, National Taiwan University College of Medicine, Taipei, Taiwan

PI: Jung-Yaw Lin

Laboratory of Dr. Kwang-Huei Lin in the Graduate Institute of Biomedical Sciences, Chang Gung University, Taoyuan, Taiwan

PI: Kwang-Huei Lin

Taipei First Girls High School, Taipei, Taiwan

Biology teacher: Yi-Yi Hsu

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6. References

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Figure Legends

Fig. 1 Confirmation of the expression of the truncated proteins examined by SDS-PAGE (A) and immunoblotting (B).

Fig. 2 Only WT GroES (GroES 1-118) can induce IL-8 release, but not the four truncated forms. KATO-III cells were incubated with 0.3 μM GroES WT and the truncated forms of GroES for 16 hours and IL-8 release was determined.

Fig. 3 Non-reducing SDS-PAGE was performed to confirm the disulfide bonds were deduced after GroES was treated with DTT.

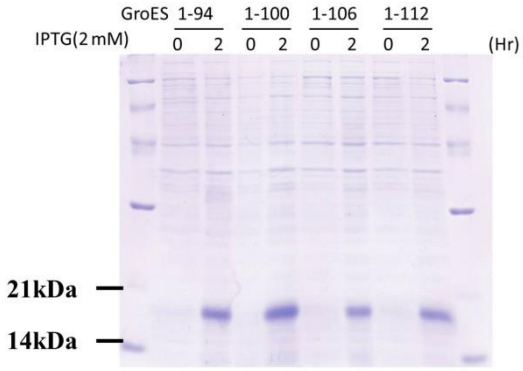
Fig. 4 The loop structure of domain B generated by disulfide bonds is required for GroES to induce IL-8 release. KATO-III cells were incubated with 0.3 μM GroES WT, which were treated with 0 mM, 1mM, 5mM DTT beforehand, for 16 hours and IL-8 release was determined.

Fig. 5 GroES still stimulates inflammatory response in KATO-III cells when losing one of the two disulfide bonds. KATO-III cells were incubated with 0.3 μM GroES WT, C111A or C112A for 16 hours and IL-8 release was determined.

Fig. 6 GroES cannot stimulate inflammatory response in KATO-III cells when losing both of the disulfide bonds. KATO-III cells were incubated with 0.3 μM GroES WT or C111A/C112A for 16 hours and IL-8 release was determined.

Figure 1

A



B

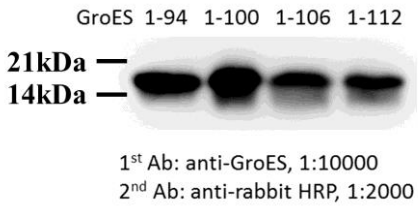


Figure 2

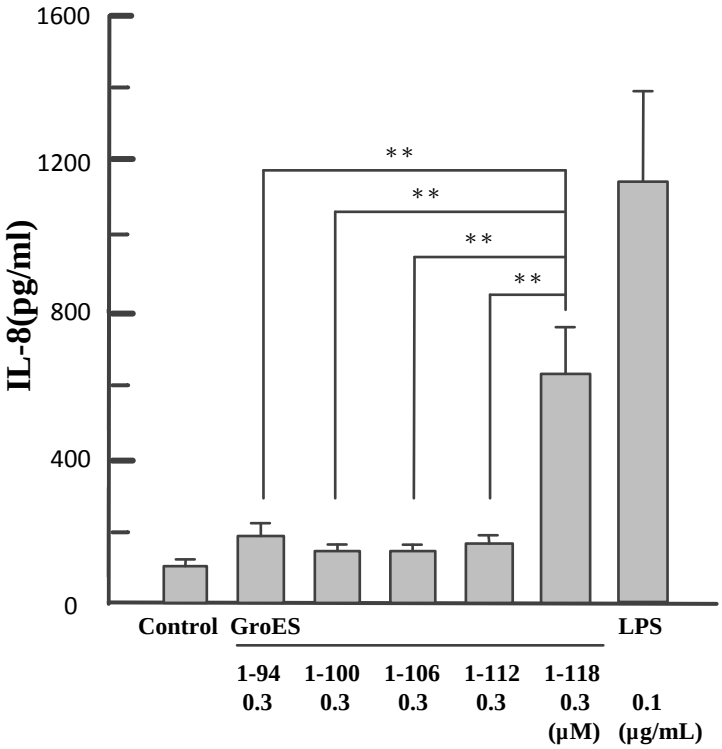


Figure 3

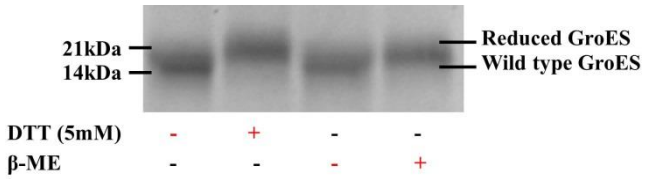


Figure 4

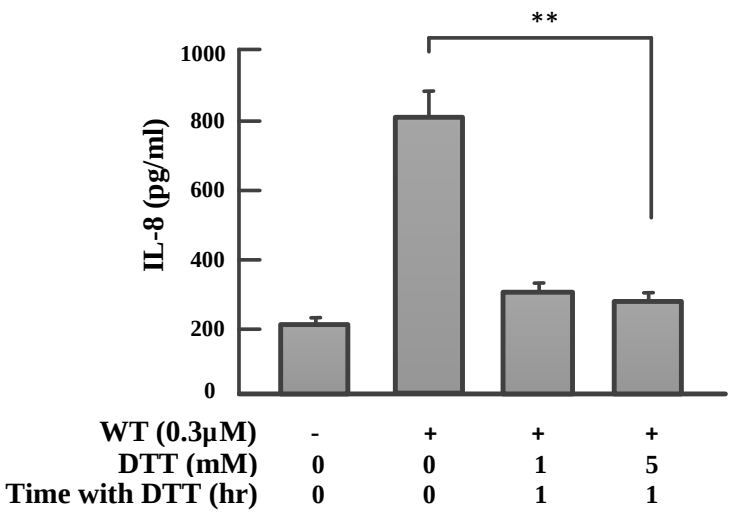


Figure 5

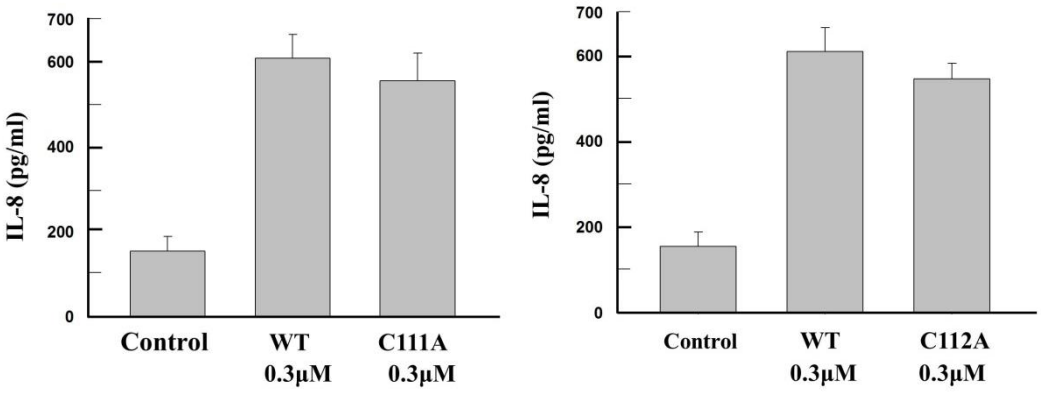


Figure 6

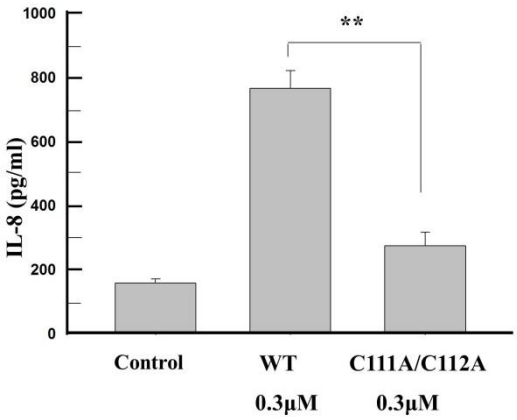


Table 1 The primers we designed for truncation

Truncated GroES		Primer sequence
Forward		5′ - GGATCCATGAAGTTTCAGCCATTAGGAGA - 3′
Reverse	GroES 1-112	5′ - CTCGAGTTAACAGCAAGCTTCATGCTC - 3′
	GroES 1-106	5′ - CTCGAGTTATTTAGCATGTTTATGGTC - 3′
	GroES 1-100	5′ - CTCGAGTTAATGATTACCTGTATGACA - 3′
	GroES 1-94	5′ - CTCGAGTTAACAAGAGCCTGAGCCCAC - 3′

Table 2 The primers we designed for site-directed mutagenesis

Mutant		Primer sequence
C111A	Forward	**A** C H D H K K H gct tgt cat gat cac aaa aaa cac
	Reverse	A E H E K A H K agc ttc atg ctc ttt agc atg ttt
C112A	Forward	**A** H D H K K H stop gct cat gat cac aaa aaa cac taa
	Reverse	C A E H E K A H gca agc ttc atg ctc ttt agc atg
C111A/C112A	Forward	**A** **A** H D H K K H stop gct gct cat gat cac aaa aaa cac taa
	Reverse	A E H E K A H K agc ttc atg ctc ttt agc atg ttt

評語

1. 本研究提出幽門螺旋桿菌 GroES 的羧基端 28 個延伸胺基酸片段在致發炎機制上的角色，本研究製備了數個不含 C 端的重組蛋白，皆不具有誘發胃細胞株產生 IL-8。

2. 本研究有必要胜肽生成 91-118 片段，讓其形成完整的雙硫鍵，再探討此片段有沒有能力誘發細胞株產生 IL-8。

3. 製備抗(91-118)片段的專一性抗體，看會不會影響 GroES 誘發產生 IL-8。