行政院國家科學委員會專題研究計畫　成果報告

IgA1 蛋蛋酶蛋蛋蛋蛋蛋蛋蛋蛋蛋蛋蛋蛋蛋蛋蛋蛋蛋研究

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行政院國家科學委員會補助專題研究計畫■成 果 報 告
期中進度報告

TgA1蛋白酶的體外組裝及其在誘導人細胞分泌素產生的研究

計畫類別：■ 個別型計畫　　□ 整合型計畫
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成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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研究計畫中英文摘要

（一）計畫中文摘要。

關鍵詞: IgA1 蛋白酶, 分子體外組裝, 細胞分泌素, 定點突變, 分子識別

大量的研究證明：包括流行性感冒嗤血性桿菌在內的致病性細菌所產生的 IgA1 蛋白酶對於細菌的感染性及菌落形成之重要性與其酶的含量成正比。此酶一方面可以直接導致人體第一線防衛系統的衰減，另一方面又可誘導包括 IL-1、TNF-α、IL-6 和 IL-8 等在內的細胞分泌素的形成，對於細菌的感染、在感染區迅速形成菌落及最終導致內部發炎等起著極為重要的作用。在以往的研究中，我們已從 non-typable 流行性感冒嗤血性桿菌中克隆了 *iga* 基因，並分別表達及純化了 IgA1 蛋白酶的α-及β-鏈，還在體外實驗中證明瞭重組的α-鏈須在β-鏈存在時才能具有更好的蛋白酶之活性。本研在此基礎上進一步研究了 IgA1 蛋白酶中二條鏈的組裝方式的分子機制。為了研究分子識別, 我們完成了分子內二條鏈(α-鏈及β-鏈)中八個抗體識別殘基中的六個的突變體, 為第 93 年度的研究(即完成剩餘的二個突變體的分子識別及對細胞分泌素的誘導方式等)打下了基礎。突變體基因制備完成後已克隆到表達載體上並獲得了突變基因的表現。純化的突變體及正常酶所組裝雜合分子對人體 IgA1 水解的結果表明, 抗體分子識別位點的殘基的確對分子組裝及其對人體免疫球蛋白水解活性均有明顯的影響。

(二)計畫英文摘要。

Keywords: IgA1 protease, *in vitro* molecular assembly, cytokines, site-directed mutagenesis and molecular recognition.

IgA1 protease is an important protease produced by pathogenic bacteria including *Haemophilus influenzae*, the pathogenic potency of which is related to the relative amounts of the protease. This protease has also been found to induce cytokine production, such as IL-1, TNF-α, IL-6 and IL-8, suggesting that IgA1 protease is involved in both neutralising host defence molecule, IgA1, and the production of cytokines which contributes to infection of bacteria and inflammation at the sites of infection. In our previous study, we have achieved cloning, expression and purification of the recombinant α-chain (rα-chain), the surface domain of β-chain (srβ-chain) and the whole domain of β-chain (rβ-chain). Interaction between the rα-chain and the srβ-chain showed that the assembly of the two chains enhanced the proteolytic activity of the rα-chain. Also, we have gained antibodies to both α- and β-chain. In order to study how and why the β-chain of the protease enhances proteolytic activities of the α-chain and the role of IgA1 protease in infection and inflammation, with the aid of an NSC grant (NSC92-2311-B-110-006), we have created 6 of the 8 estimated residues from the antibody binding sites on both domains. Mutant DNA has been expressed with six-histidine tag in *E. coli*, a non-IgA1 protease production host cell, and the mutant enzyme has been purified to homology with affinity chromatography. In vitro assembly and proteolytic assays showed that the mutants are indeed affected the molecular assembly of the protease and thus the proteolytic activity ranging from 10-1,000-fold.

In the second year (supported by a grant numbered NSC93-2311-B-110-005) of research, we will go on straightforward to study cytokine production by IgA1 protease will be achieved by the following designs: 1) the rα-chain alone, 2) complex of rα-chain and srβ-chain, 3) complex of rα-chain and rβ-chain, 3) rβ-chain alone, 4) srβ-chain alone, as well as those already created mutants.

報告內容

Introduction

On about four hundred square meters' surface of mucus layer in human body, the secreted immunoglobulin A (IgA) is the predominant molecule to protect the body from foreign substances and opportunistic microorganisms against infection. IgA carries out agglutinating activity to form bacteria-immunoglobulin complexes that become trapped in the mucus layer, preventing the pathogen from making physical contact with the epithelial surface in a process called immune exclusion (1). Immune exclusion also limits the amount of time that the pathogen has to excrete virulence factors, which would impair host defences and damage the cells lining the mucous membranes. There are two subclasses of IgA, IgA1 and IgA2. There is a sequence consisting of thirteen amino acids within the hinge region of IgA1, which is absent in IgA2 (2), which makes IgA1 susceptible to proteolysis by a family of enzymes called IgA1 proteases (3).

The IgA1 proteases are constitutively expressed by pathogenic bacteria (4), which colonise and infect a diverse range of mucosal surfaces in the human body (table 1). The bacterial IgA1 proteases specifically target human IgA1, as they do not cleave any other classes of immunoglobulin (3, 5). IgA1 proteases cleave human IgA1 to produce antigen-binding fragments and Fc fragments (6). The IgA1 proteases cleave post-proline peptide bonds within the duplicated octapeptide sequence composed of proline, serine, and threonine residues in the hinge (2). Until recently, human IgA1 was believed to be the only substrate of the IgA1 proteases (3, 7, 8), however several substrates have been identified including the IgA1 protease translation product (autoproteolytic cleavage) (9), the LAMP1 protein (a membrane glycoprotein of lysosomes) (10, 11), outer membrane proteins of *Neisseria gonorrohoeae* (12) and IgA1 from chimpanzees, gorillas and orang-utans (13). These alternative substrates also contain proline rich sequences similar to the sequence in the IgA1 hinge region. The cleavage of proline-serine bonds by IgA1 protease has been called Type 1 cleavage and the cleavage of proline threonine bonds Type 2 cleavage. To date, the Neisseria and Haemophilus species have been found to produce both types of IgA1 proteases. Some of the non-typeable *Haemophilus influenzae* IgA1 proteases can cleave IgA1 to produce two Fabα fragments of unequal size, which is thus called Type 3 cleavage. The Type 3 cleavage pattern could be the result of two active sites within the protease or the bacteria producing two types of IgA1 protease enzymes (14, 15).

The IgA1 proteases of the different bacterial species can be split into three catagories, according to their enzymatic properties and their gene sequences. The IgA1 proteases of *Haemophilus influenzae*, *Neisseria gonorrohoeae* and *Neisseria meningitidis* are serine proteases

(16), the streptococcal IgA1 proteases are metalloproteases (3) and the Prevotella species produce cysteine proteases (17). The IgA1 protease activity requires metal ions and optimal activity is around pH 7, although it does have activity between pH 5 and pH8 (4).

The *iga* genes exist as a single copy in the bacterial genome (18, 19) and the *iga* genes from several pathogenic bacteria have been sequenced. These include *H. influenzae* serotype d (20), *H. influenzae* serotype b (19), *S. pneumoniae* (21), *N. gonorrohoeae* (9), *N.meningitidis* (22) and *S. sanguis* (23). The *iga* genes coding for the serine proteases have been revealed that similar gene structures and a high degree of sequence homology between these *iga* genes are present in pathogenic bacteria (24, 18). However, the sequences contain many regions of non-homologous sequences interspersed with regions of great homology, which gives the gene a 'mosaic –like organisation' and is believed to be the result of deletions, insertions and exchange of genetic material within and between species (25 - 28). The streptococcal *iga* genes also show homology within the species (21, 28, 29), however they have no significant homology to the serine protease genes. The gene sequence of the cysteine protease of *B. melaninogenicus* (30) has yet to be cloned and sequenced.

The bacterial IgA1 proteases cleave human IgA1 within the hinge region of the heavy chain of the IgA1 molecule to produce antigen binding (Fabα) and intact Fcα fragments, thereby destroying the normal protective functions of IgA1 in host primary defence mechanisms. The Fabα fragments are still able to recognise and bind to bacterial surface antigens, however the secondary function of the IgA1 molecule is lost with the Fc portion (31). The bacterial pathogen coated with Fabα fragments block potential antigen binding sites, which protects the bacteria from any intact and functional immunoglobulins such as IgG and IgA2 (32, 33).

The biological significance of IgA1 proteases is not clear, as there is only indirect evidence to support the role of IgA1 protease as a virulence factor in bacterial infection and colonisation. However many investigators have discovered that only the pathogenic members of a bacterial species produce IgA1 proteases (34) including *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis*, which are the three main causes of bacterial meningitis (7, 35) and the particularly virulent clone of *Haemophilus influenzae* biogroup aegyptius, which causes Brazilian Purpuric Fever (36).

On another hand, the IgA1 protease-producing pathogenic bacteria are all mucosal pathogens and the predominant immunoglobulin in the mucosal secretions is IgA. The bacterial pathogens may have evolved with this enzyme to carry out this specific function of destroying the main mucosal antibody.

Thirdly, some of the IgA1 proteases have been found to be highly immunogenic and antibodies against IgA1 proteases have been detected *in vivo* in disease, convalescent secretions and in harmless colonisation (37-41). The IgA1 proteases produced by *Haemophilus influenzae* clones have displayed antigenic polymorphism and more than thirty antigenic types have been identified by neutralising antibodies (15). Frequently changing antigenic types is thought to be a mechanism of evasion from the host's immune system (24, 42). Re-occurring bacterial infections could be attributed to the action of IgA1 proteases on pre-existing IgA1 antibodies from a previous exposure to the bacteria (34). A bacterial infection may be the result of a disruption in

the balance between the neutralising antibodies at the mucosal surfaces and the activity of the IgA1 proteases as described in a 'hypothetical model for the pathogenesis of invasive infections' by Reinholdt and Kilian (43, 44).

Fourthly, the virulence of pathogenic bacteria could be related to the quantity of IgA1 protease activity, which has been found to be higher in meningitis patient samples of cerebrospinal fluid and blood in comparison to samples taken from asymptomatic carriers (45).

Finally, Studies of the role of IgA1 protease on cytokine production have revealed an alternative function of IgA1 proteases, and have found the recombinant IgA1 protease of *Neisseria gonorrohoeae* stimulates the release of TNFα, IL1β, IL6 and IL8 from peripheral blood mononuclear cells (46). However, Beck *et al*. (47) have found contrasting results, which conclude that IgA1 protease inhibits TNFα. These results show that the IgA1 protease may have other properties, which are yet to be discovered.

IgA1 proteases are attacked by lactoferrin, a protein found in milk, secretions and neutrophils, by extracting the preprotein from the cell membrane, hence making the protein more accessible to antibodies. Lactoferrin also inhibits the Hap adhesin protein (48) and as these two proteins are autotransporters and they are thought to be important in bacterial adhesion to mucosal surfaces, the lactoferrin may be contributing to host defence mechanisms by inhibiting successful colonisation of the bacteria. How the lactoferrin protein is able to extract the preprotein from the bacterial membrane is yet to be determined (49).

The C terminus of the IgA1 protease pre-protein contains a sequence, which forms a membrane binding pore structure referred to as the 'helper domain' or beta domain (50-53). The beta domain is composed of amphipathic anti-parallel beta pleated sheets, which allows the translocation of the pre-protein through the bacterial outer membrane (54, 55). The function of the beta domain is thought to be to transport the protease domain into the extracellular environment where the IgA1 protease attains its active conformation. The N terminus of the beta domain is composed of hydrophilic amino acids and can be highly variable in size (53), whereas the C terminus portion contains a highly conserved, 31KDa protein sequence, which inserts into the membrane (52). The beta domain may have other functions in addition to the function as a transmembrane transporter protein.

Aims of the project

The role of IgA1 protease has been thought to relate to specific cleavage and inactivation of IgA1, facilitating bacterial colonisation and infection. However recent data has suggested this enzyme may be also a potent pro-inflammatory reagent inducing cellular production of cytokines such as IL-1, TNF-α, IL-6 and IL-8. We have recently cloned, expressed and partially purified the α domain (containing the active site), β surface domain and complete β domain of IgA1 type 1 from *Haemophilus influenzae*. In addition we have generated specific antibodies to the α and β chains of the enzyme.

The aims of the study are to investigate in detail the assembly of the two chains in the reaction with human IgA1 and the role of the α and β chains in the production of inflammatory cytokines from bronchial epithelial cell lines and monocyte/macrophages. In addition the effect

of both domains assembled together *in vitro* will be assessed spectroscopically. Moreover, the ability of the antibodies to abrogate any effects will be studied. Finally, the individual residues pertaining to the binding and proteolytic activities will be determined by site-directed mutagenesis. This will provide a wide understanding of this enzyme in the pathogenesis of COPD and chemical role of individual residues of the enzyme.

Materials and methods

1. *Expression and purification*: The gene *iga* for α- and β-chains from non-typable *H. influenzae* were fused in pET233-2, respectively, in which *Nco*I and *Bam*H1 restriction sites had been made by PCR. Cells were grown in LB medium to transformation density before addition of IPTG to final concentration of 0.1mM to induce gene expression. The α-chain recombinant protease domain was found in periplasmic space and the β-chain stayed in cytosol with loosely attached to membrane.
2. *Affinity purification of recombinant IgA1 protease*: The cellular extracts (α-domain in osmotic buffer and β-domain in PBS containing 0.2% Tween-20) were loaded onto affinity column, respectively, to purify the recombinant protease, using the added poly-histidine tag. SDS-PAGE was used to identify the purity of the recombinant protease. Generally 90-95% purity could be achieved.
3. *Assembly and proteolytic activity assays*: WT and mutant protease assembled on the surface of isolated prokaryotic membrane fragments according to 1:1 ratio of α- and β-chains as determined by spectroscopy. Control assembly was also carried out in the absence of membrane fragments. Human IgA1 was then added and incubated at 37℃ with various of time scale (25 min to 14 hr) to trace the kinetic changes in proteolytic digest of human IgA1. The digested fragments were separated on SDS-PAGE to examine the activity of assembled protease.
4. *Site-directed mutagenesis*: The residues in the antibody recognition site in α-domain (D272, E276 and D281) have been replaced with valine residue. The antibody-recognition residues in the putative surface region of β-domain (A71, K72, K77) which recognized by antibody have also been changed to the same valine residue respectively. Expression and purification of the mutants were carried out as described above.

Results and discussion

The successful expression enables us to carry on the experiments as designed and purification shows that the recombinant α- and β-chain can be purified to homology in affinity column (Fig. 1 and Fig. 2). Results showed that the expression and purification have met all the requirement for our proposed research scheme of *in vitro* assembly with mutants (first year) and induction of human cytokines (second year).

1. Types of non-typable IgA1 protease

We have so far examined 75 isolates of non-typable *Haemophilus influenzae*, 17 of which

contain IgA1 protease. Clinical identification followed by genomic identification confirmed these isolates as non-typable *Haemophilus influenzae* (Fig. 1).

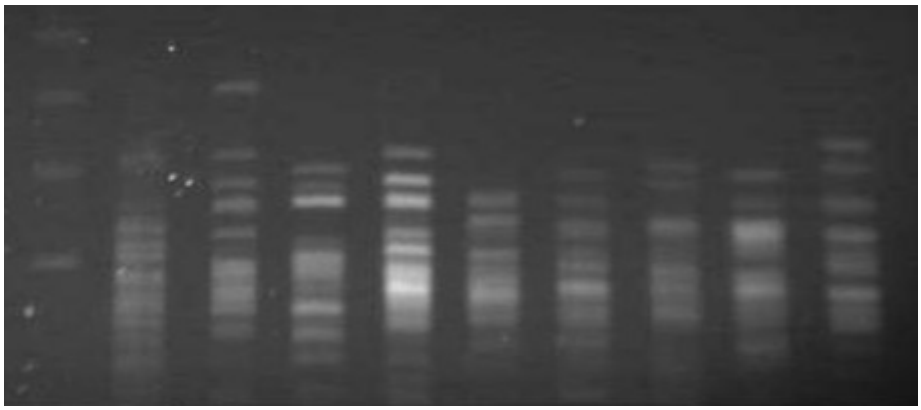

Figure 1. Genomic identification of non-typable *Haemophilus influenzae.* Lane is DNA marker and the rest are non-typable *Haemophilus influenzae* isolates. After clinical identification, genomic DNA were isolated as PCR templates.

Characterization of IgA1 protease showed that there are two types of IgA1 protease in ono-typable *Haemophilus influenzae* isolates (Fig. 2). Lanes 1 and 3 isolates are type 3 IgA1 protease, and lane 5 isolate is type 2 IgA1 protease. Among the 17 isolates containing IgA1 protease, 16 of them belong to type 3 protease and only one of them belongs to type 2. This suggests that majority of non-typable *Haemophilus influenzae* in Taiwan are type 3.

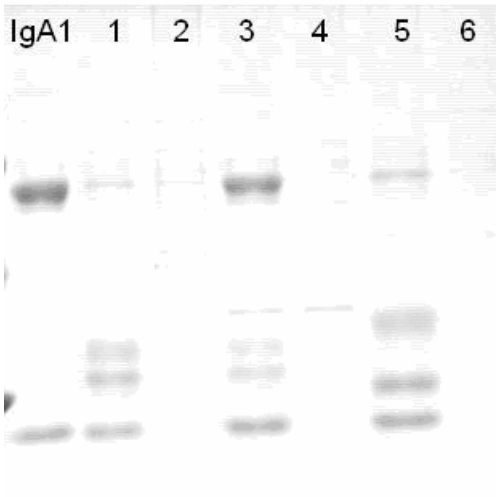

Figure 2. Hydrolysis of human IgA1 by non-tyopable *Haemophilus influenzae.* Lanes 1, 3 and 5 are human IgA1 incubated with IgA1 protease from three different *Haemophilus influenzae* (non-typable) isolates.

2. Large-scale purification of IgA1 protease

In the last report, we have achieved expression and purification of recombinant IgA1 protease from non-ypable *Haemophilus influenzae*, but the scale was limited, preventing large-scale titration of protease. Thus we developed a large scale expression in 3L LB medium and allowed the protease to be transferred to supernatants. Purification showed that the recombinant IgA1

protease had been purified (almost) to homology (Fig. 3). Figure 3 shows that fractions 16 to 20 all contain a single band which is located at 106 kDa. Activity titration showed that all the fractions contain IgA1 protease activity, suggesting that the single band is the α-chain of IgA1 protease.

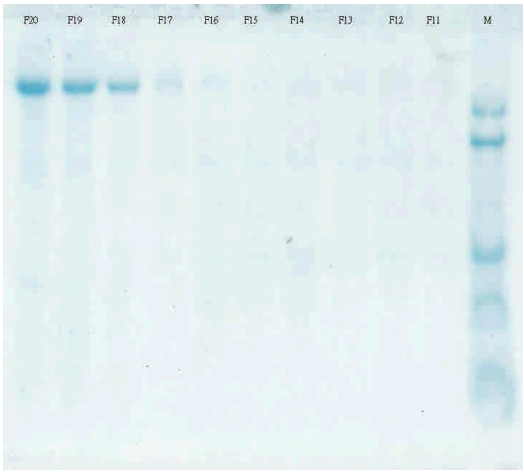

Figure 3. Purification of recombinant type 2 IgA1 protease. The labeled number on the top of each lane is the fraction number. All fractions containing the single band have strong IgA1 protease activity.

3. Engineered β-chain, expression and purification

This is an additional work not designed in our proposal, but as the research undergoes well, we added this part in order to identify the exact sequence on which different types of IgA1 protease cleave. Thus we have engineered the α-protein, surface domain and β-chain to express them independently (Fig. 4).

These domains have been cloned in expression vector, and some of them have been purified to homology (Fig. 5).

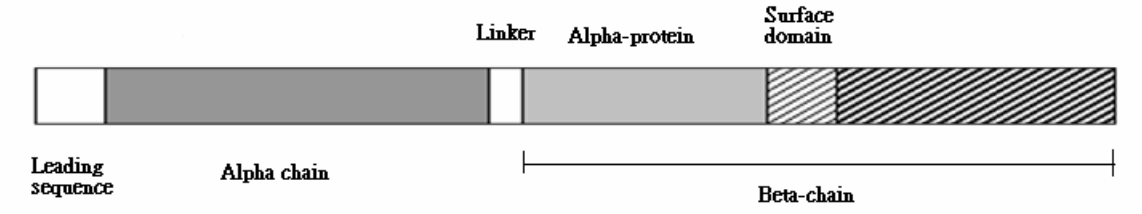

Figure 4. Structure of non-typable *Haemophilus influenzae* IgA1 protease gene (*iga*). Domains from left to right are leading sequence, α-chain, linker, α-protein, surface domain and β-core.

Figure 5. Purification of recombinant protein containing α-protein and surface domain. The framed lanes are different fractions as numbered on the top of each lane.

4. Cytokine production under the induction of IgA1 protease

This is the only part of our research project which is not completed due to the blood source problem. Although we have now used immortal thorax carcinoma instead neutrophils, the whole process is therefore delayed.

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Supplementary: results from last grant (NSC92-2311-B-110-006)

The following results have been recorded in the last report numbered 93B36151 (NSC92-2311-B-110-006).

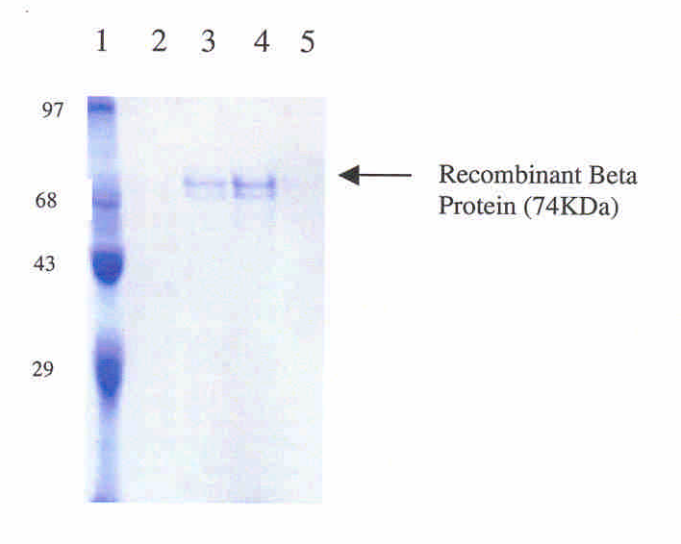

Figure 1. Purification of recombinant β-chain of IgA1 protease in affinity chromatography. The putative β-chain is 74 kDa and the single band in the SDS-PAGE is located the putative range of 74 kDa, suggesting that the β-chain has been purified to homology.

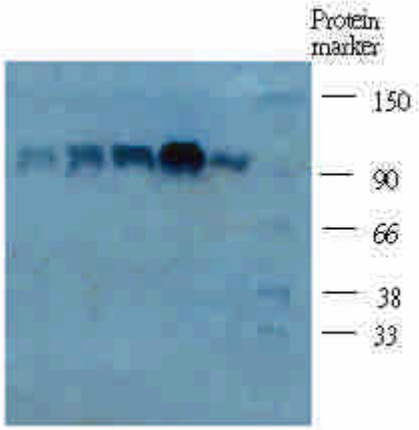

Figure 2. Purification of recombinant α-chain of IgA1 protease in affinity chromatography. The putative molecular weight of α-chain is 110 kDa and the single band after affinity purification is located in the putative range on SDS-PAGE, suggesting that the recombinant protease α-chain has been purified to homology. The five lanes are from five different fraction collected from affinity chromatography.

The purified recombinant contains highly proteolytic activity in digestion of human IgA1 immunoglobulin (Fig 3). The results clearly suggest that the *H. influenzae* IgA1 protease recognizes one specific sequence in human IgA1 immunoglobulin, so that the heavy chain is

cleaved into two different fragments. This is different from Type I which cleaves human IgA1 into two equal sized fragments, so that only a single band can be observed on SDS-PAGE, nor from Type II which cleaves human IgA1 resulting one 45 kDa and the other 5 kDa (normally it was run off the 8% SDS-PAGE as front line) (Fig 3, lane 3).

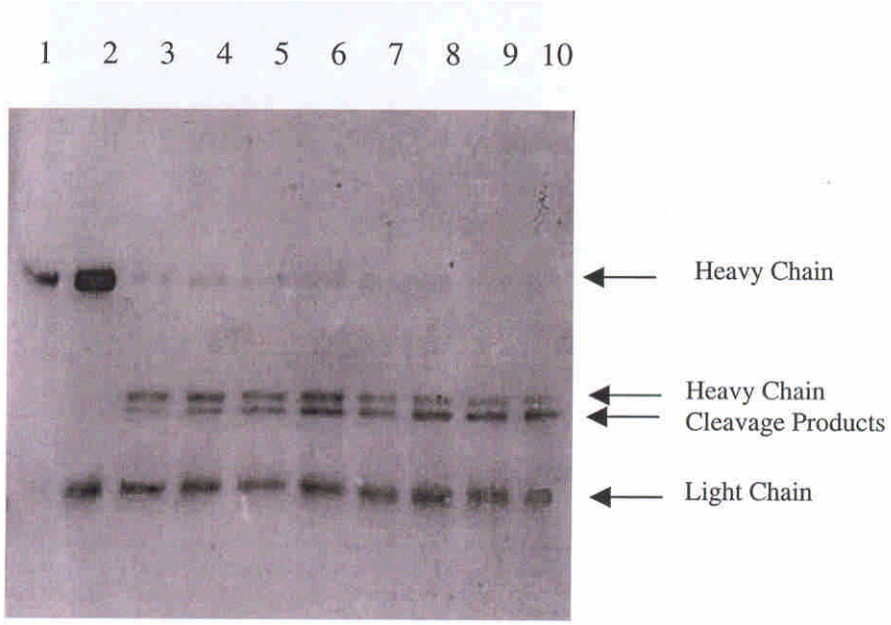

Figure 3. Western blot from SDS-PAGE of human IgA1 digested with *H. influnzae* IgA1 protease. Each lane contains 1μg human IgA1 and 5ng of recombinant IgA1 protease and incubates for 4 hr before inactivating the enzyme in SDS-PAGE loading buffer.

The *in vitro* assembly (Table 1) arises surprised results, not only the enzymatic proteolytic activity changes dramatically, but the cleavage sites seem not exactly the same as the WT.

Table 1: Assembly design and the relative assay loading in figure 4.

Mutants		β-chain mutants	
α-chain mutants		βA71V	βK72V
	αD272V	αD272V/βA71V (lane 3)	αD272V/βK72V (lane 7)
	αE276V	αE276V/βA71V (lane 4)	αE276V/βK72V (lane 6)
	αD281V	αD281V/βA71V (lane 5)	Not enough protein

Figure 4 clearly shows that the cleavage specificity locates at the antibody binding site, presumably related to active site residues, namely the α-E276 residues. This is a good progress in IgA1 protease studies since not a single report across the world about active site residues pertaining to the proteolytic activities of the enzyme.

Membrane attached assembly was also carried out. The results (data not shown due to the membrane proteins affect the identification of hydrolytic human IgA1) also show that membrane assembly enhances the proteolytic activities of the enzyme.

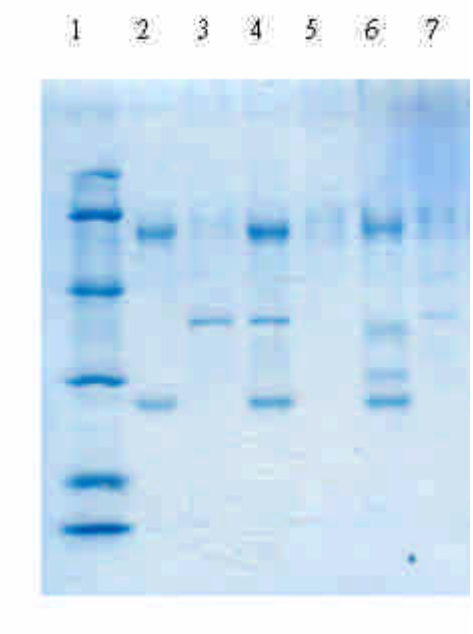

Figure 4. SDS-PAGE of human IgA1 fragments cleaved by recombinant mutant IgA1 protease. The gel contains incubation of human IgA1 with αD272V/βA71V (lane 3), αE276V/βA71V (lane 4), αD281V/βA71V (lane 5), αE276V/βK72V (lane 6), and αD272V/βK72V (lane 7) respectively. Lanes 1 and 2 are protein marker human IgA1 alone, respectively.

附件二

可供推廣之研發成果資料表

□ 可申請專利　□ 可技術移轉　　日期：93 年 10 月 26 日

國科會補助計畫	計畫名稱：TgA1 蛋白酶的體外組裝及其在誘導人細胞分泌素產生的研究 計畫主持人：何世屏 計畫編號：NSC93－2311－B-110－005　學門領域：BE
技術/創作名稱	N/a
發明人/創作人	N/a
技術說明	中文： N/a （100~500 字）
	英文： N/a
可利用之產業及可開發之產品	N/a
技術特點	N/a
推廣及運用的價值	N/a

※　1.每項研發成果請填寫一式二份，一份隨成果報告送繳本會，一份送　貴單位研發成果推廣單位（如技術移轉中心）。

※　**2.本項研發成果若尚未申請專利，請勿揭露可申請專利之主要內容。**

※　3.本表若不敷使用，請自行影印使用。