行政院國家科學委員會專題研究計畫 期中進度報告

依P12的構造和功能而研發的避孕胜肽(2/3)
期中進度報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 96-3112-B-002-014-
執 行 期 間 ：96年05月01日至97年06月30日
執 行 單 位 ：國立臺灣大學生化科學研究所

計 畫 主 持 人：陳義雄

報 告 附 件 ：出席國際會議研究心得報告及發表論文

處 理 方 式 ：本計畫可公開查詢

中 華 民 國 97年06月04日

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

依 P12 的構造和功能而研發的避孕藥物

Contraceptives Based on the Structure and Function of P12

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC96－3112－B－002－014－
執行期間： 2007 年 5 月 1 日至 2008 年 6 月 30 日

計畫主持人： **陳義雄**
共同主持人：
計畫參與人員：

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

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：

中　華　民　國　　年　　月　　日

一、中文摘要

利用光化親合技術 (photo affinity technique)，已能將 P12 結合到小鼠精子頂體外的細胞膜，結合 P12 的精子蛋白已經由二維電泳法分離。摘下電泳膠片上的蛋白點並經由蛋白質體分析法而知 P12 在精子上的結合部位為 α- enolase 進一步由免疫染色法而確定 α-enolase 存於頂體外膜上。進一步也證實 α- enolase 的抗體可以抑制 P12 結合到精子的頂體外膜。

我們探討依 P12 的構造而設計的模擬胜肽的進度，有些延緩，因為我們無法如期從國內得到所需的合成胜肽。不得已情況下，只得要求國外廠商製備，這是相當費時，最近才從德國廠商得到幾個合成胜肽 [H-GPRIYDPV-NH2, H-GPLIYDPV-NH2, H-IYDP-NH2, Cyclo (TPA-PGPGPGAPLIYDPVCGGG), Cyclo (TPA-PGPGPGAPRIYDPVCGGG) (TPA: thiopropionic acid)]。這些胜肽對於 trypsin 活性和 P12 結合到精子的抑制能力正量測中。希望短期內能得到結果，以作為藥物設計的基礎。

我們將小鼠子宮液的蛋白經由 G-100 Sephadex column 的層析分成三個部份 (Fr I-III)。經由 Zymographic assay 而知子宮液的蛋白腖主要分佈於 Fr III。Fr III 所含 trypsin 活性可被 P12 抑制，顯示 Fr III 含有類似 trypsin 的蛋白腖。由於該蛋白腖含量十分少，現階段無法進一步經蛋白質體分析決定其結構，但不影響我們評估 Fr III 是否可以移走結合於精子的 P12。這一方面的探討正進行中。這在哺乳類的生殖有其重要意義，因為 P12 和精子的結合發生於受精後，精子遇到卵子前，在女性生殖道 P12 必須從精子移走，卵子才能受精。

中文關鍵詞：避孕胜肽、哺乳類精子、蛋白酶抑制蛋白、藥物設計

二、英文摘要

We succeeded in specifically crosslinking P12 to its binding site on the plasma membrane overlaying the acrosome of mouse spermatozoa by the photo affinity technique, using P12 conjugated with Sulfo-HSAB (N-Hydroxysulfosuccinimidyle-4-azidobenzoate), a photo affinity labeling reagent. The spermatozoal proteins complexes with P12 were isolated by 2-dimensional gel electrophoresis. Proteomic analysis of the protein spot excised from the 2D-gel demonstrated alpha enolase to be the P12-binding site on the sperm. The presence of alpha enolase on the acrosomal surface was confirmed by immunocytochemical staining using the antibody against enolase. Preincubation of spermatozoa with the alpha enolase antibody was able to inhibit the P12-sperm binding. We are ongoing to further study how the P12-enolase binding affects the sperm activity.

We delayed our progress for a certain extent on the study of peptide mimics based on the P12 structure, because we were frustrated to obtain the synthetic oligonucleotide we needed from the domestic sources. To make up the shortage, we ordered the customer-made product from Germany. It took time to receive the products. Recently, we got the materials including H-GPRIYDPV-NH2, H-GPLIYDPV-NH2, H-IYDP-NH2, Cyclo (TPA-PGPGPGAPLIYDPVCGGG), and Cyclo (TPA-PGPGPGAPRIYDPVCGGG) (TPA: thiopropionic acid), and we started to assay the effects of these peptides on the trypsin activity and the P12-sperm binding. We hope to summarize the results soon, in order to establish the basis for drug design.

We resolved the protein components of mouse uterine fluid into three fractions, Fr I-III by gel chromatography on a G-100 Sephadex column. Zymmergraphic assay for the protease activity manifested predominant distribution of trypsin-like proteases in Fr III. Hydrolysis of the trypsin substrate N-benzoyl-phe-Val-Arg *p*-nitroanilide by Fr III was inhibited to a certain extent by P12, indicating that mouse uterine fluid contains trypsin-like protease which can be specially inhibited by P12. However, very trace amount of the protease in the uterine fluid hindered our attempt to determine its structure by proteomic analysis. Instead, we are planning to assess whether P12 on its sperm-binding site can be removed by Fr III. This plays an important role in the mammalian reproduction, because the P12-sperm binding take place in the semen, and removal of P12 from sperm in female reproductive tract should predate the sperm-egg encounter to proceed fertilization.

英文關鍵詞：Contraceptive；Mammalian sperm；Protease inhibitor；Drug design

三、報告內容

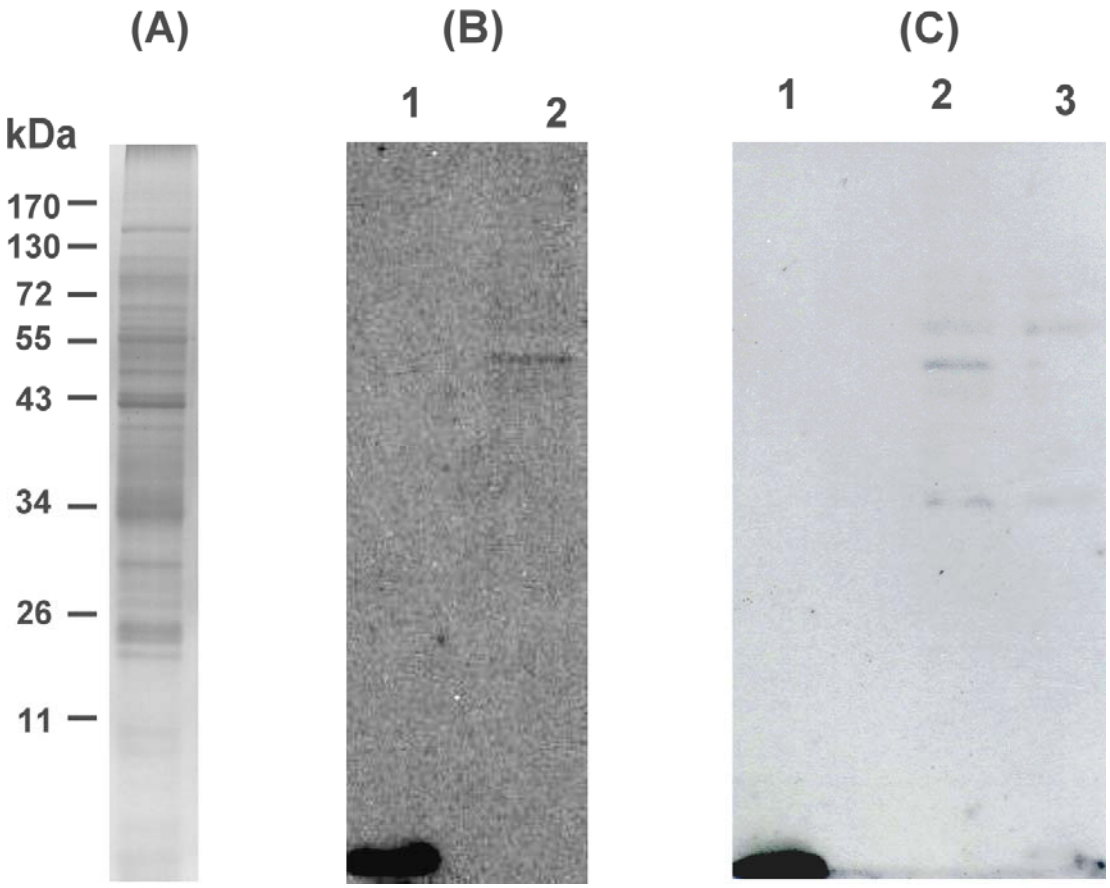

Fig. 1. Covalent association of P12 to its receptor on sperm by photo affinity labeling. (A) Sperm membrane protein characterization by SDS-PAGE. The sperm membrane protein was subjected to electrophoresis on a 12% slab gel. The protein patterns were visualized after silver staining the gel. (B) & (C) Identification of P12 receptor on mature intact sperm. (B) 50 μg of P12 was reacted with a crosslinking reagent, N-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HSAB) at a molar ratio of 1:15 in PBS, pH 7.4 under dark at room temperature for 1 h. Labeled P12 was allowed to react with mature intact sperm at 4μg/108 sperm cells in the dark for 15 min. at 37oC. Photoactivation was performed for 10 min. by exposing the sample to a very short-wavelength. Lane 1, HSAB-labeled P12(control), lane 2, sperm membrane protein extracted after reacting HSAB-labeled P12 with intact sperm. The gel was immunodetected by Western blot analysis using the rabbit antiserum P12 as the primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody. (C) The specificity of crosslinking was confirmed by photo reaction in the presence of 100 fold molar excess of purified P12. Lane 1, HSAB labeled P12(control), lane 2, normal crosslinking reaction, and lane 3, crosslinking with excess cold P12.

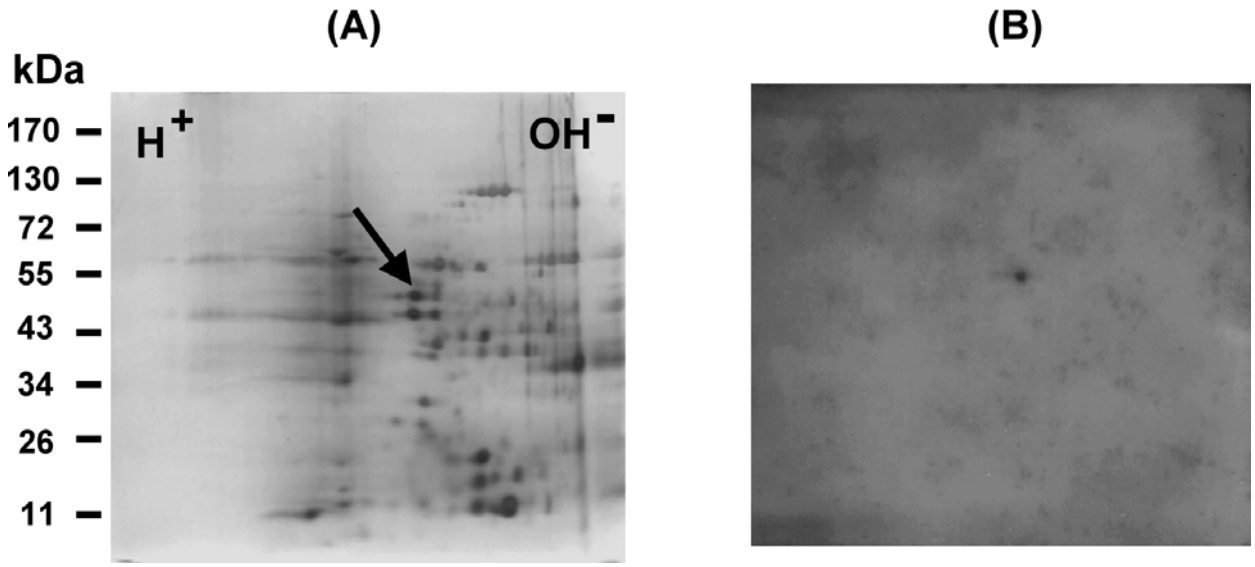

Fig. 2. Separation of P12 receptor complex by 2-dimensional gel electrophoresis. (A) The P12 receptor complex was separated by 2-dimensional gel electrophoresis and silver stained. The silver staining was coupled with western blot by using P12 antibody. (B) The first dimension was focused using ampholytes (pI range 3-10). The second dimension is a 10% SDS-poly acrylamide gel. The acid(H+) and alkali(OH-) ends of the gel are marked. The P12- receptor complex (spot marked with the arrow in Fig. A) was in-gel digested and subjected to MS/MS analysis. The complex was identified as 47 kDa mouse alpha non-neuronal enolase.

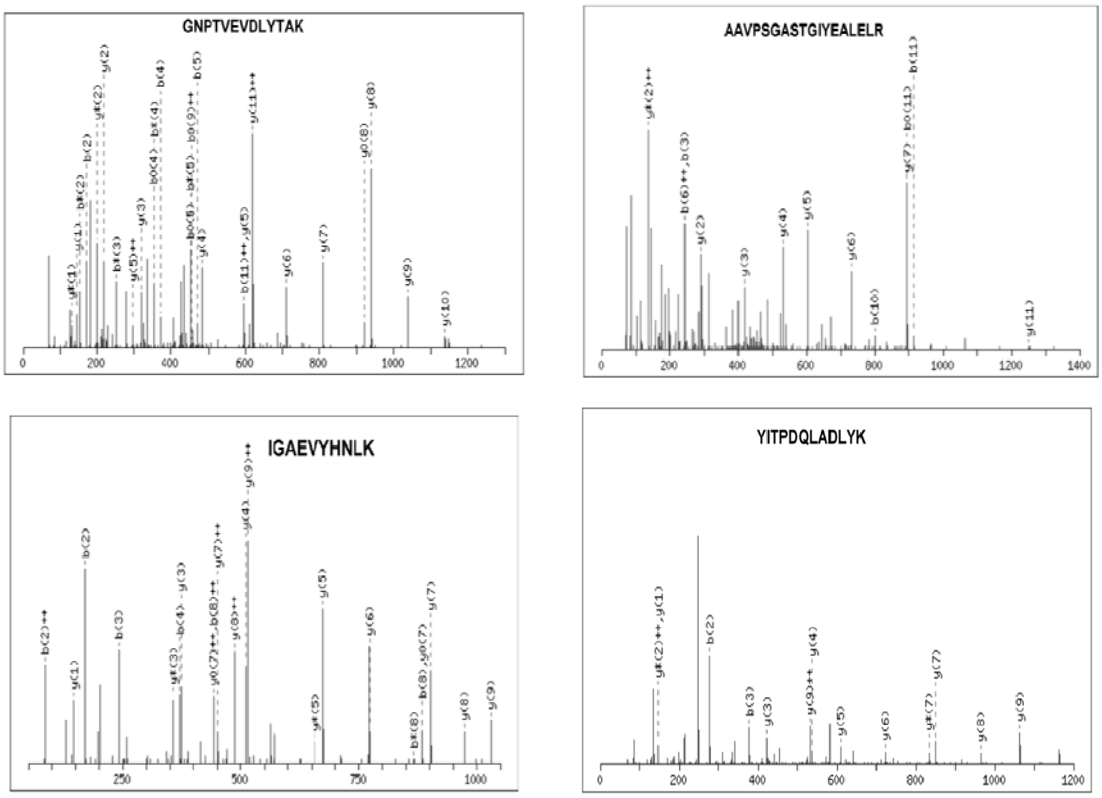

Fig. 3 Mass spectral analysis of P12-receptor complex. MS/MS spectra of a peptide from trypsin-digested alpha enolase. Predicted sequences and ions of type b and y are shown for four fragments.

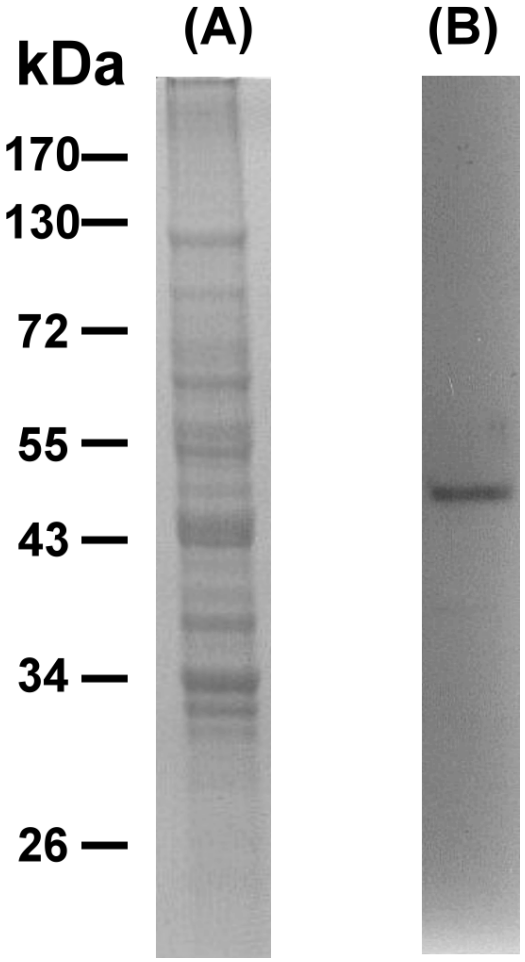

Fig. 4. Immunodetection of mice alpha enolase from acrosome with human alpha enolase antibody. (A) Reducing 12 % SDS-PAGE profile of sperm acrosome protein. (B) Immuno detection of mice alpha enolase by western blot with polyclonal antirabbit human alpha enolase antibody (dilution 1: 1000) as primary antibody and goat-anti- rabbit HRP conjugate as secondary antibody.

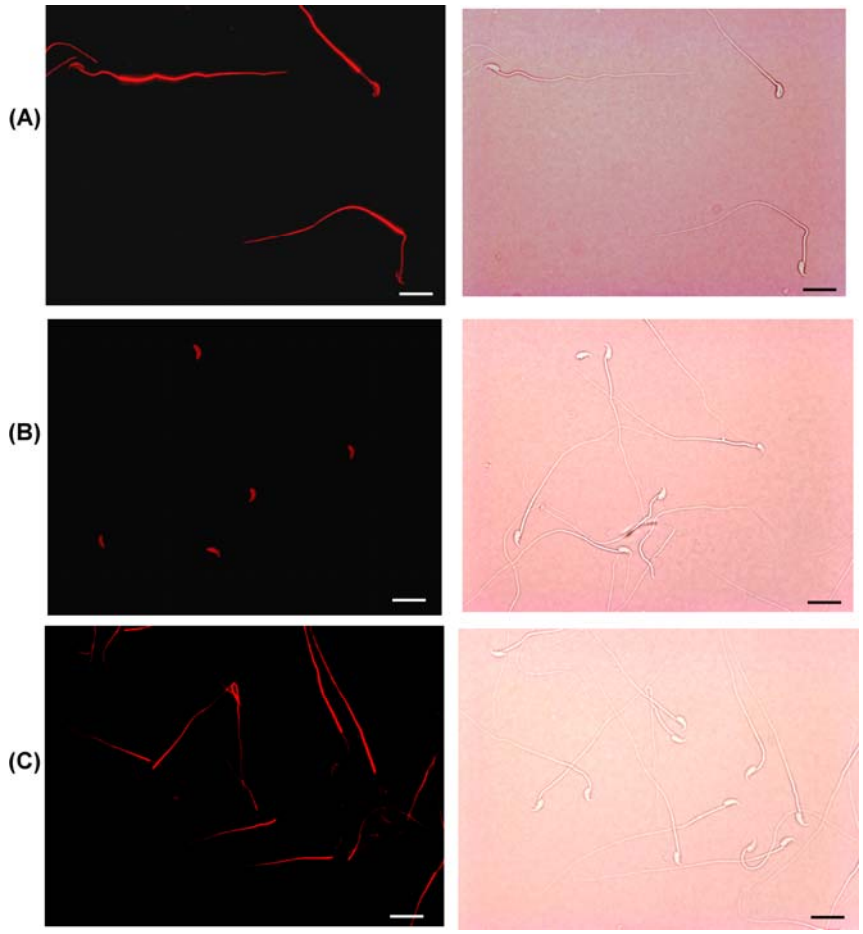

Fig. 5 Immunolocalization of binding zone of P12 on intact sperm. Indirect immunoflourescence was performed on mature intact spermatozoa collected from the cauda epididymis and washed twice in PBS. The cell suspensions were pipetted onto microscope slides, air dried and fixed with ice cold 100 % methanol for 5 minutes. Slides were incubated with human alpha non-neuronal enolase antibody(diluted 1: 50 in 1% BSA in PBS) for 30 minutes, washed in PBS 3 X 5 minutes, incubated with TRITC-conjugated anti-rabbit immunoglobulins (diluted 1:100 in 1% BSA in PBS) for 30 minutes and washed in PBS as before. Bar = 10 μm. (A) Sperm was incubated with alpha enolase antibody. Fluorescence appeared on the acrsomal region. (B) Sperm was incubated with P12 followed by incubation with P12 antibody. (C) Sperm was pre-incubated with alpha enolase antibody followed by incubation with P12 and P12 antibody. No fluorescence appeared on the acrosome region. Pre-incubation of the sperm with alpha enolase antibody inhibited further binding of P12 to acrosome thus confirming alpha enolase as the receptor for P12.

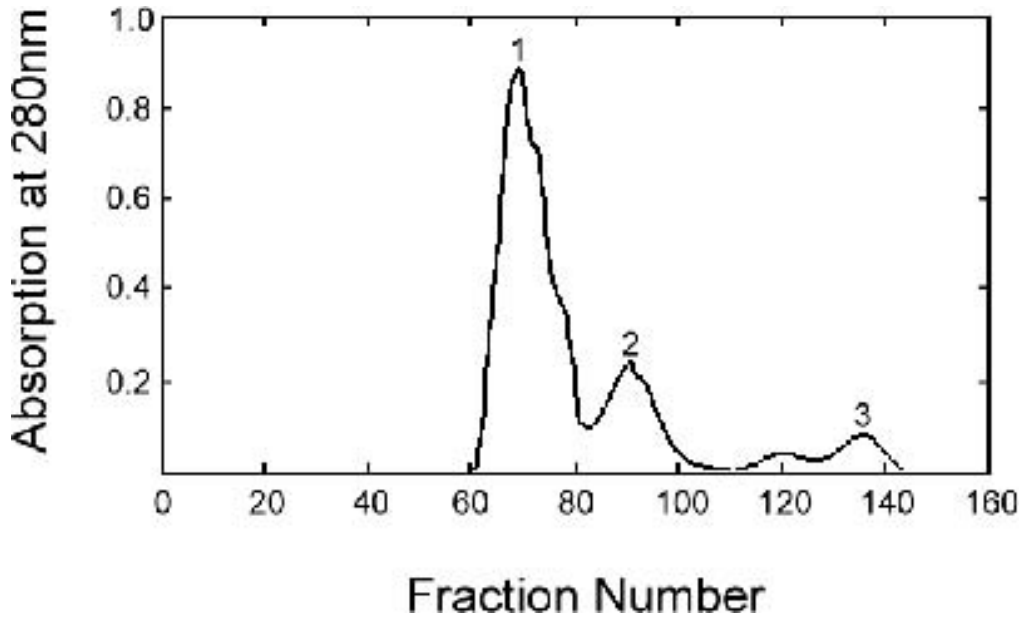

Fig 6. Fractionation of proteins in uterine luminal fluid by gel chromatography.

Four milliliters of uterine luminal fluid freshly prepared from DES-stimulated baby mice were applied to a Sephadex G-100 (2.5 x 90 cm) pre-equilibrated with 0.1M ammonium acetate. A 2ml sample was collected for each fraction and monitored the absorption at 280 nm. Each peak fraction were pooled for further study.

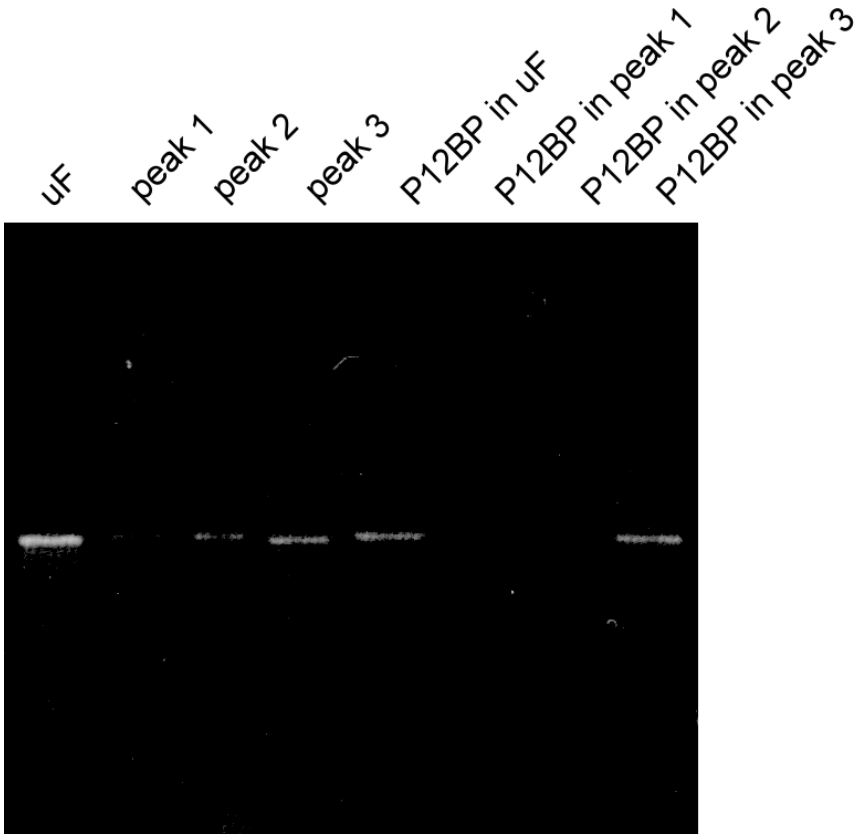

Fig 7. Zymographic Illustration of protease activity in uterine fluid. Around 200 ug protein from uterine fluid (uF), peak 1, peak 2, peak 3 in 75 ul PBS were respectively incubated with 75 ul Affigel bead being covalently conjugated with P12. Each bead was exhaustively washed with PBS, and followed the bead collection by centrifugation. Suitable amount of protein from UF, peaks 1-3 of Fig6, and the Affigel beads prepared as mentioned was subjected to SDS/PAGE on 12% polyacryamide gel slab (8x12 cm, 0.75 mm thicker) containing 200 uM Boc-Gln-Ala-Arg-MCA. The gel slab was washed twice with 2.5% Triton X-100 in cold water for 10 min, and the incubated in 50mM Tris – HCl – 10mM CaCl2-0.005% Triton X-100 (pH 7.5) at 37℃ for 30 min. The gel slab was UV- irradiated to reveal the protease protein band

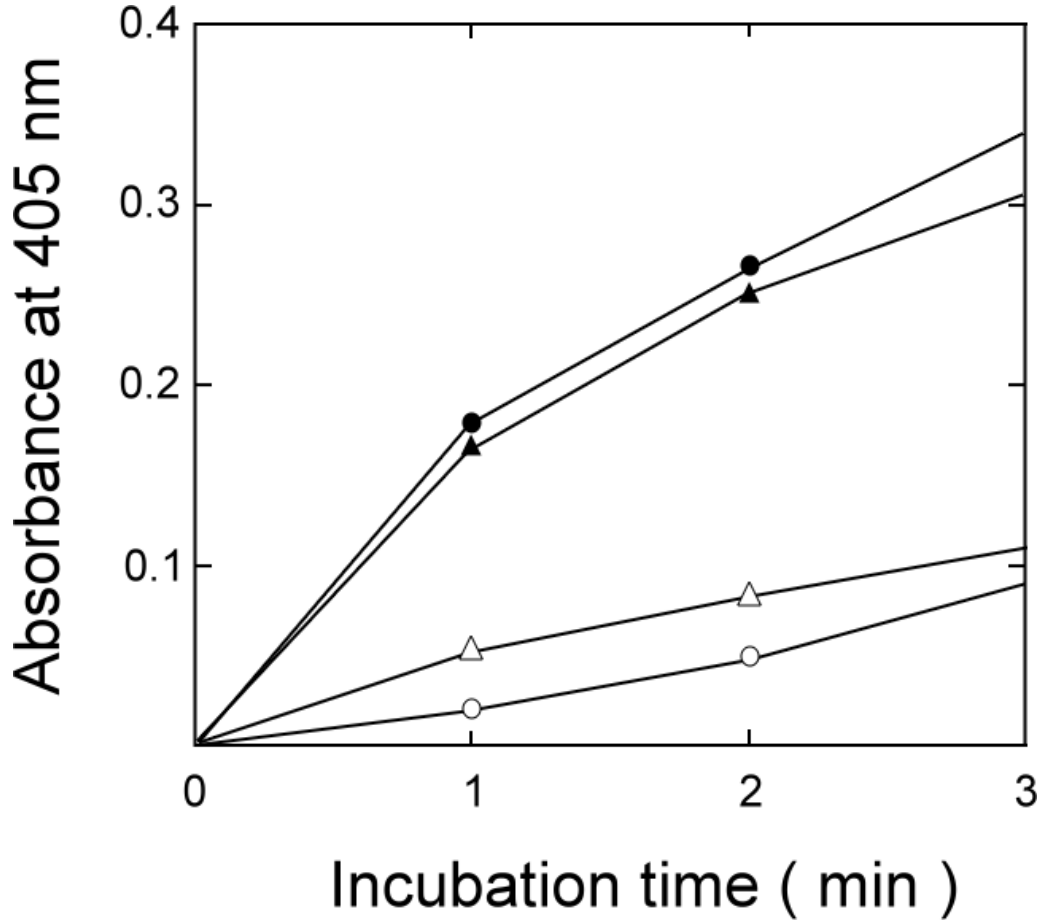

Fig 8. Demonstrate of the P12 – inhibited protease in uterine fluid. N-benzoyl-Pro-Phe-Arg-*p*-nitroanilide HCl was used as substrate. The time course of the substrate hydrolysis was monitored by the change in absorbance at 405 nm. In 1.0 ml reaction buffer of 0.1M Tris-HCl-20 mM CaCl2 at pH8.0, 100 ug of peak 3 of Fig 6 alone (●), or in the presence of 6.5 nM P12 (○) was incubated with 60 uM substrate. The substrate hydrolysis by 12.5 nM trypsin alone (▲), or in the presence of 6.5 nM P12 (△) was also measured for comparison.

四、結論

1. Our progress up to date achieves two aspects: (1) We demonstrated the α-enolase on the plasma membrane

2. overlaying the anterior acrosome of mouse sperm as the P12-binding receptor; (2) We proved the P12-inhibited

3. protease in uterine fluid. Finding the reproductive significance of these two discoveries is ongoing right now.

附件一

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

依 P12 的構造和功能而研發的避孕胜肽(2/3)

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號：NSC 96-3112-B-002-014-
執行期間：96 年　05　月　01　日至　97　年　06　月　30　日

計畫主持人：陳義雄
共同主持人：
計畫參與人員：

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

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
■出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：國立台灣大學生化科學研究所

中　華　民　國　97 年　06 月　03　日

參加第 41 屆生殖研究學會 (The Society for the Study of Reproduction, SSR) 年會心得報告

國際有關生殖學的研究，SSR 是現今最大的學會。該組織是一個美洲學者專家發起的民間組織，會員遍及世界各地。每年於美加地區選擇一城市舉辦年會。第 41 屆學會於今年 5 月 26 至 30 日於夏威夷大島的可納市(Kailua-Kona, Big Island, Hawaii)舉辦。與會約 1000 人左右，共有 766 個論文報告。不論是邀請演講人、口頭報告人皆需張貼壁報定時討論，因此壁報討論佔有大會約 50-60%的時間，依個人經驗，參與有興趣的壁報討論，有較長的時間探討主題，常能獲得有用的資訊。生殖學的重要性在任何時代皆被重視，至今已累積了可觀的論文報告，但大多止於表像觀察，較缺少分子層次的深入探討。因此分子生殖學，特別是哺乳類的研究已漸成顯學。這是一門新興學門，所涉及的期刊論文的科學影響系數目前尚不高，但將來的提升是可預料的。國內在這一個領域的學者、專家較分散，參與這一次大會約有 15 人左右(包括學生)。生殖學是一門相當廣泛的跨領域研究。這次大會的研討分類也較雜，勉強分成幾個領域：(1)神經內分泌學；(2)雄性生殖腺和配子的分化和生長機制；(3)複製技術；(4)受精、著床和懷孕；(5)子宮的生理和病理研究；(6)幹細胞的研究；(7)排卵研究；(8)荷爾蒙作用和生長因子；(9)精子功能、活化和獲能的研究；(10)睪丸的形成和分化；(11)受精研究；(12)卵細胞的減數分裂；(13)非變異性的基因調控 (epigenetics)；(14)生殖組織的基因表現；(15)生殖免疫的研究；(16)環境對生殖的影響；(17)生殖品種的保存和生殖毒物的研究；(18)卵巢各種細胞的研究。與會者可依其興趣選擇領域參與探討。本人參與了(2)、(4)、(9)、(13)、(14)等領域，得到一些資訊，有助於往後研究。我所提出的專題 “An interlocking meshwork of covalent cross-links between SVS I-III from mouse seminal vesicle are involved in copulatory plug formation”，闡釋 SVS I-III 三個蛋白分子之間的雙硫鍵和類胜肽鍵(isopeptide bond)的交錯鍵結於交配栓形成的生理意義，可說是這一方面的研究至今最深入的探討。這是一個先驅型的研究，現在較不吸引注意，但其重要的生理意義定會被重視。

An Interlocking Meshwork of Covalent Cross-Links between SVS I-III from the Mouse SeminalVesicle are involved in Copulatory Plug Formation

Yee-Hsiung Chen[2,4], Huan-Chin Tseng[2], Han-Jia Lin[3], and Chia-Yih Wang[2]

[2]Institute of Biochemical Sciences, College of Life Science, National Taiwan University, Taipei, Taiwan, [3]Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan and [4]Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan

No monomer forms of SVS I-III are found in mouse seminal vesicle secretion (SVS), despite detection of these within the SVS by reduced SDS-PAGE. Instead, they ware found to have been selectively cross-linked by inter-polypeptide disulfide bridges to give various high molecular weight complexes (HMWCs). Although SVSI-III can be immunochemically stained in cross sections of a mouse copulatory plug, heating the clotted lump in SDS-PAGE sample buffer in the presence of DTT does not release into solution any SVSII and only a trace of SVSI and SVSIII. This suggested that there are other types of covalent cross-links between the SVS I-III proteins present in the plug other than disulfide bonds. Type 4 Transglutaminase (TG_4) from the mouse coagulating gland may be involved and was purified to homogeneity. TG_4 was compared to Type 2 Transglutaminase (TG_2) from guinea pig liver in terms of enzymatic ability to cross-link proteins in the SVS. It was found that TG_4 showed much greater activity than TG_2 when catalyzing cross-links between HMWC proteins. This contrasts with the fact that TG_4 is less active than TG_2 when cross-linking any of the free SVSI-III proteins released from the HMWC after reduction by DTT.