# 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?(1/3)
## 期中進度報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-3112-B-002-024-
執 行 期 間 ：95年05月01日至96年04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

處 理 方 式 ：本計畫可公開查詢

中 華 民 國 96年02月27日

# Development of contraceptives based on the structure and function of P12

# 依 P12 的構造和功能而研發的避孕胜肽


## 計畫主持人：陳義雄



## 計畫編號：95-3112-B-002-024



**Summary:** The contraceptive potential of proteins secreted from male accessory sexual glands has received scant consideration. Using mice as experimental animals, we aim to develop the contraceptives based on the structure and function of P12, a caltrin-like protease inhibitor consisting of 57 amino acid residues in seminal vesicle secretion (SVS), which shows strong affinity to the plasma membrane overlaying the anterior acrosome of mouse spermatozoa to inhibit the cellular $Ca^{2+}$-uptake. (1) We have demonstrated that :(a) there is a single-type P12-binding site (1.49 X $10^6$ sites/cell) with a $K_d$ value of 70nM on the mouse sperm (1); (b) $D^{22}$ and $Y^{21}$ on the P12 molecule are mainly responsible for the sperm-binding site which is distincted from the reactive site $R^{19}$ for trypsin inhibition (2); (c) an oligopeptide G-$^{18}$PRIYDPV$^{24}$ on the P12 molecule is able to inhibit the P12-sperm binding (2). This work demonstrated a slight impact of P12 on sperm capacitation, and demonstrated the removal of plasma membrane overlaying the acrosome region by immunoaggregation of P12 on mouse sperm. Further, we found that the N-terminal region, $^{43}$RKR$^{45}$, and the C-terminal region, but not $R^{19}$, $Y^{21}$, and $D^{22}$, are involved in the three epitopes that reside on one side and are three-dimensionally distant from $R^{19}$, $Y^{21}$, and $D^{22}$ on the P12 molecule. Based on the topology of multifunctional sties on the P12 molecule, we tested the contraceptive potential of the P12 antibody in female mice, and access the inhibitory effect of peptidomimics based on the oligopeptide on the P12-sperm binding.

**摘要：**哺乳類附屬性腺所分泌的蛋白質，能否發展成避孕藥物，至今幾乎沒有研究報告。以小鼠為實驗動物，我們以P12 的構造和功能為基礎，擬評估發展P12 為避孕藥物的潛能。P12 存於貯精囊液。是一個含 57 個氨基酸的蛋白酶抑制蛋白，會結合於頂體漿膜而抑制精子的吸鈣力，進一步證實：(a)精子上含單一型的 P12 結合位置(1.49 x $10^6$ sites/cell)，其解離常數($K_d$)為 70nM；(b)P12 經由其分子表面的$D^{22}$或$Y^{21}$結合於精子，而P12 抑制蛋白酶的活性氨基酸為$R^{19}$並不參與P12 和精子結合；(c)P12 分子上胜肽片段G-$^{18}$PRIYDPV$^{24}$會抑制P12 和精子的結合。執行本專題後，證實P12 不會引發精子的獲能反映，但它於精子頂體的免疫凝聚

則會移走頂體漿膜。進一步鑑定P12 分子的N-端胜肽片段，$^{43}$RKR$^{45}$和C-端胜肽片段為抗原區。R$^{19}$、Y$^{21}$和D$^{22}$不具抗原性，他們於P12 分子上，分佈於三個抗原區的反方向側面。基於P12 分子上多功能區的立體分佈，進一步測試P12 抗體的避孕能力。也測試G-$^{18}$PRIYDPV$^{24}$的模擬胜肽物抑制P12 和精子結合的能力。

**Methods:** (1) We prepared ten P12 variants by recombinant techniques, including six single-site mutated mutants (R19L, Y21V, D22G, R43G, K44S, and R45T), two multisite mutated mutants (R43G/K44S/R45T and L50H/R52G/K53A), and two deletion mutants (Nd10 and Cd8) in which 10 and 8 residues were deleted from the N- and C-terminals, respectively. Their immunoreactivities to the P12 antibody were compared. Oligopeptides linked with thiopropionic acid (Tpa) of Tpa -PP(h)PP(h)PP(h)APLIYDPVC and Tpa-PGPGPGAPLIYDPVCGGG were synthesized and further oxidized to disulfide bond for the ring closure via the assistance of Prof. S. T. Chen, Institute of Biological Chemistry, Academia Sinica. The P12 antibody was purified from the antiserum collected from female mice being intrasplenically immunized with P12 purified from SVS by affinity chromatography. Fertility in terms of date of parturition after coitus (dpc) and the little size at delivery was examined for female mice being intrasplenically immunized with P12 or being received an i.v. injection of either the P12 antibody. The inhibitory effect of the oligopeptide on the P12-sperm binding was assayed to assess whether the structural feature of Tyr and Asp in the cyclic peptides is suited to the sperm-binding site of P12.

**Results:**

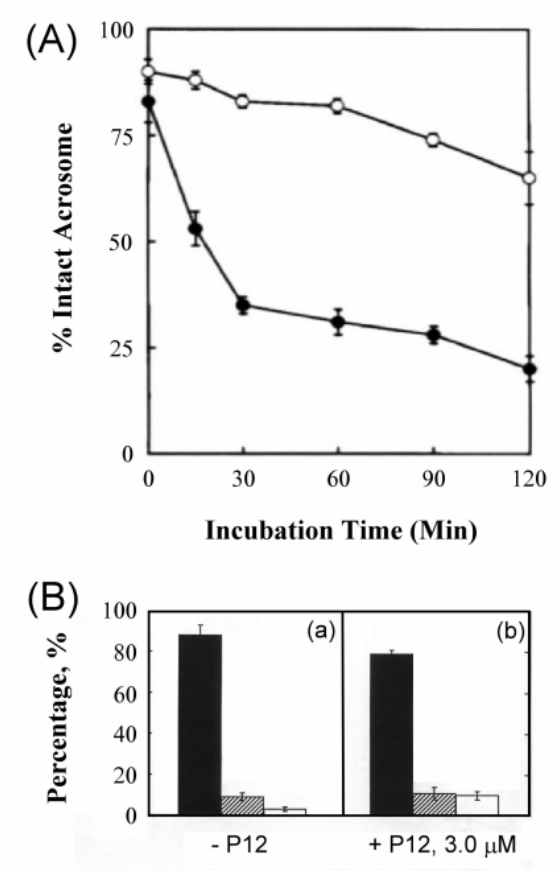


**Figure 1. Examination of the status of mouse spermatozoa**

Freshly prepared mouse sperm from the caudal epididymis in modified Tyrode's solution (2X $10^6$ cells/ml) devoid of $CaCl_2$ were incubated at specific condition in a 5% $CO_2$ incubator at 37°C. (A) Immunoagglutination of P12 on sperm caused the acrosome removal. The cell incubation in the presence of 3.0 μM P12 (○) or preincubated with P12 for 10 min and further exposed to rabbit P12 antibody (1.0μg/ml, ●) was proceeded at 0-120 mins. The acrosomal status of sperm was scored by the Coomassie-blue staining technique as described in "Materials and Methods". The date represent the average of three determinations in which 200 cells were counted per determination, and error bars represent the S.D. (B) A slight effect of P12 on the spermatozal status. The cells were incubated in the absence of P12 (a) or in the presence of P12 (b) for 30 mins. The CTC fluorescence method described in the text was exploited to score the population of uncapacitated cells (solid bars), capacitated cells (hatched bars) and acrosome-reacted cells (open bars). Data represent the means of five individual trials counting 200 cells per trial and error bars represent the SD.

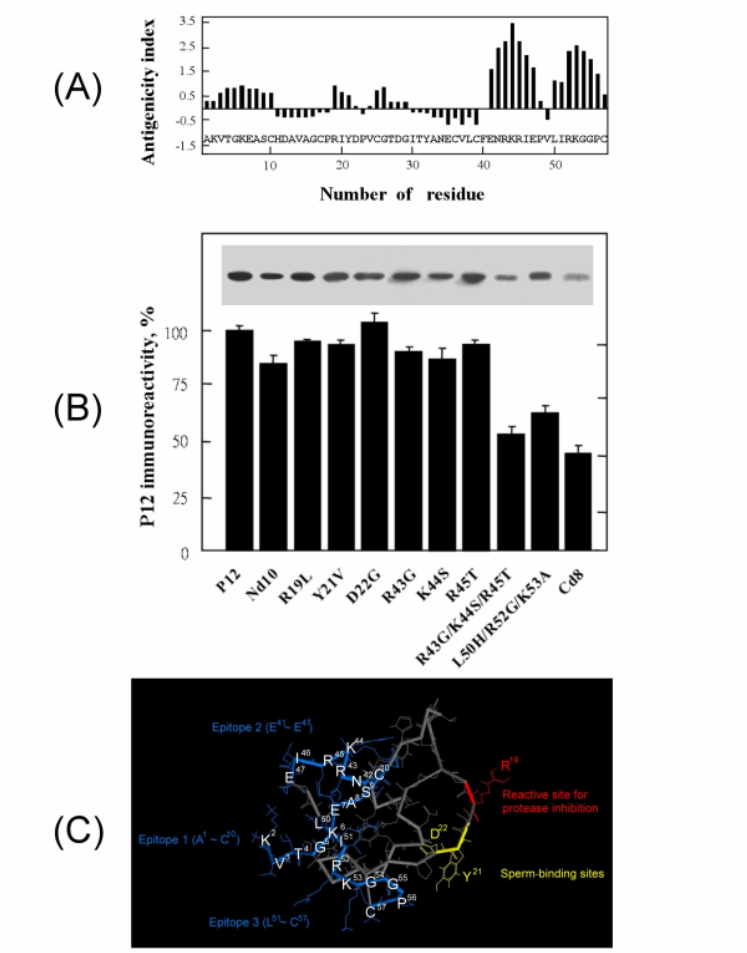


**Figure 2. Three epitopes on the P12 molecule**

Prediction of potential epitopes along the amino acid sequence. (B) Immunoreactivity of ten P12 variants. Equal amount of each purified protein sample was subjected to SDS/PAGE on a 15% polyacrylamide gel slab (6.5×10.5×0.075 cm). The gel was stained with commassie brilliant blue (not shown) and immunodetected by Western blot using the P12 antibody from the rabbit antiserum as the primary antibody and horseradish-conjugated anti-rabbit IgG as the secondary antibody (Top panel). The intensity of each immunoblot band was normalized to its corresponding protein band intensity, and the immunoreactivity of each P12 variant was relative to that of the parent protein which was referred to as 100%. Error bars represent the S.D. of the means for three determinations. (C) Molecular structure of P12. The tertiary structure was generated by homology modeling, using the automated Swiss-model service. The tubing diagram shows the polypeptide backbone consisting one α-helix which stretches from $E^{35}$ to $R^{43}$, and a small antiparallel β-sheet which comprises the three twisted strands of $^{23}$PVCG$^{26}$, $^{27}$TDGI$^{30}$, and $^{53}$KGGP$^{56}$. The side chains of amino acid residues are displayed by stick structure. The three-epitopes (blue) are distant from both the sperm-binding site (yellow) and the reactive site for protease inhibition (red).

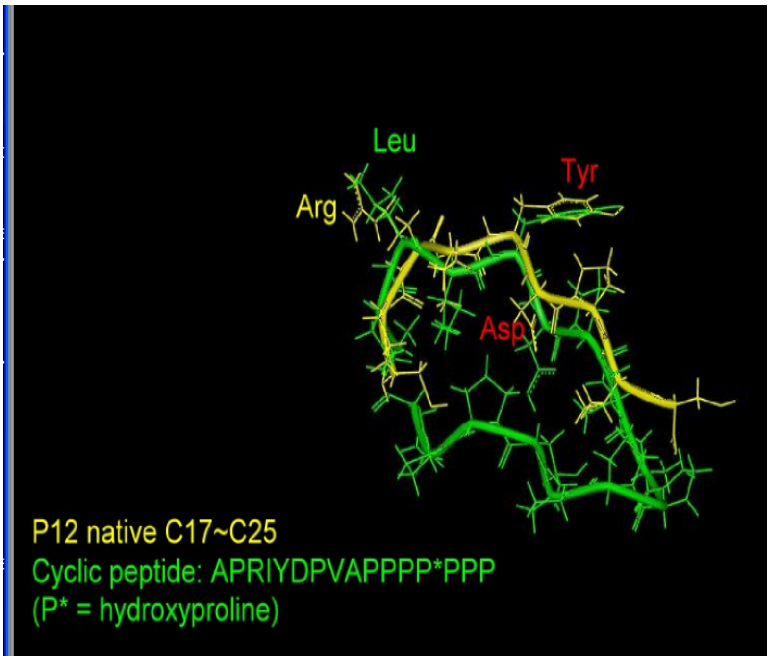


**Figure 3. The structure of a cyclic-peptide formed by thio group oxidation of $^{17}$CPRIYDPV-(proline/hydroxylproline)n-thiopropionic acid (n=6)**

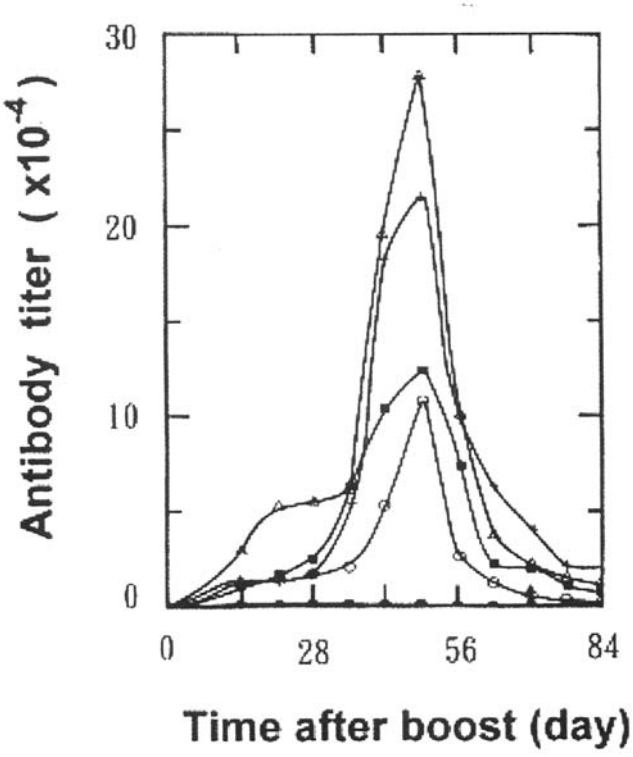


**Figure 4. The change of antiserum titer during the intrasplenical immunization of mice.**

The antiserum titer of each animal was assayed by ELISA as described in "Material and Methods". The change of serum end-point titer in each animal is represented by one symbol. The serum end point titer of the control animals was assayed to be less than 100 (●)

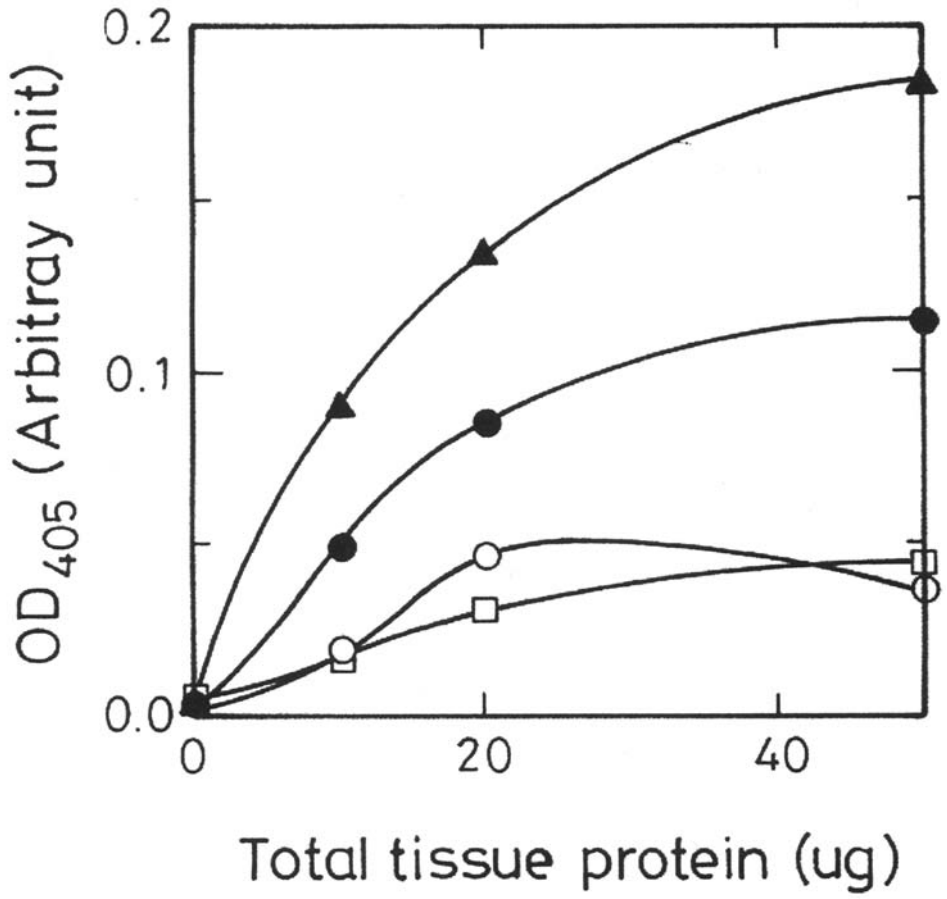


**Figure 5. Distribution of P12 antibody in the reproductive tracts of immunized female mice.**

Five females mice were intrasplenically immunized with P12 as described in "Materials and Methods". Six weeks after the last booster of antigen, the antibody in the protein extract of tissue homogenates prepared from vagina (□), uterus (▲), oviduct (●) and ovary (○) was determined by ELISA as described in Figure 4. The $OD_{405}$ on ELISA plates was recorded.

**Table 1. Fertility of female mice following intrasplenical immunization with P12[a]**

| Animal | Antibody Titer ($1x10^{-4}$) | Parturition Date[b] | Litters Size[c] |
|---|---|---|---|
| I | 1.5 | 21 | 14 |
| II | 14.5 | 37 | 10 |
| III | 21.0 | 54 | 4 |
| IV | 21.0 | 56 | 10 |
| V | 28.5 | 46 | 14 |
| control | <0.01 | 21 | 12.3±1.4[d] |

a. Forty female mice (8 we-old) were intrasplenical immunization with P12. Two weeks later, the animals were cohabited with a fertile male mouse. All of them were pregnant and labored as the control females. Ten days after parturition, the animals were received another booster of antigen. After 42 days, five animals were randomly chosen and the relative antiserum titer of each animal was determined before it was cohabited with one fertile male mouse on the same day. Control female (body weight: 27.4±1.1g) received on similar schedule with the same injection without P12.

b. Days after cohabitation with a fertile male mouse.

c. Number of offspring at delivery.

d. Average litter size of 3 control females.

**Table 2. Fertility of female mice fellows the *i.v.* injection of P12 antibody[a].**

| Animals | Dosage of Antibody Injected (μg of antibody/g of body wt.) | Parturition Date (dpc) | Litter size[b] |
|---|---|---|---|
| Group I | 0 | | |
| 1 | | 21 | 17 |
| 2 | | 21 | 17 |
| 3 | | 21 | 14 |
| 4 | | 20 | 10 |
| Group II | 1.0 | | |
| 1 | | 21 | 17 |
| 2 | | 24 | 6 |
| 3 | | 24 | 18 |
| 4 | | 21 | 12 |
| Group III | 4.0 | | |
| 1 | | 25 | 11 |
| 2 | | 25 | 14 |
| 3 | | 24 | 17 |
| 4 | | 25 | 18 |
| Group IV | 7.0 | | |
| 1 | | 35 | 15 |
| 2 | | 33 | 14 |
| 3 | | 55 | 15 |
| 4 | | 43 | 16 |

a. sixteen fertile female mice (11 week-old, body weight: 29.7±1.2g) were divided into four groups. The animals were received an *i.v.* injection of 100 μl sterile saline alone or in the presence of antibody. After 3 h, each animal was cohabited with one fertile male mouse.

b. Number of offspring at delivery

**Discussion and Conclusion:**

The results of this work indicate that: (a) P12-sperm binding does not affect the spermatozoal status; (b) *in vitro* sperm incubation in the presence of P12 and its antibody mimics the acrosome-reaction; (c) the P12 antibody is able to pass through the blood circulation into the sexual organs of female mice upon immunization with the antigen. P12 may not detach from the ejaculated spermatozoa immediately when they are forced through the cervix into the uterus because of the high affinity of P12 to the sperm. Because of the increase in both vascular permeability and luminal secretion of the reproductive tract during the proestrus stage, the P12 antibody is likely to be secreted into the uterine lumen.

This work together with our previous study sheds light on the location of multi-functional sites on P12. According to the molecular model (Fig. 2C), the three epitopes are on the side opposite to the location for sperm-binding site. The topology of these functional sites enables us to elucidate the structural basis of P12-sperm binding that is suited to immunoaggregation of P12 on the acrosome of sperm. The binding of D22/Y21 on the P12 molecule to the sperm head from one direction would turn R19 towards the other direction and expose the three epitopes. Such a structural feature allows not only the immunoaggregation of P12 on the sperm head, which causes the removal of membrane overlaying acrosome (Fig. 1), but also the binding of a trypsin-like protease to the reactive R19 from the other direction. Among the sexual organs of male and female, P12 is predominantly secreted from the luminal epithelium of male accessory sexual glands, and the P12-sperm binding takes place after ejaculation. Since the ejaculated spermatozoa reside for a significant period in the female reproductive tract of naturally inseminated mice, to carry on capacitation, induction of a sufficient amount of P12 antibody in the female reproductive tract is likely to immunoaggregate P12 on the ejaculated sperm before sperm-egg encounter. Thereby, the sperm would become infertile. Our result of fertility trial seems to support the possibility.

Immunocontraception has received increased interest in the past few decades. As our knowledge of molecular reproduction grows, more members of the functional molecules, which participate in the fertilization, development or transportation of gametes, have been assessed as potential candidates for the development of antifertility vaccines. In this regard, most of the studies thus far have focused on the immunoresponse of reproductive protein hormones and gamete-specific antigens and. However, serious side effects, such as hormone imbalance, orchiditis or ovaritis, may develop. Using P12, which is secreted from the male accessory sexual glands, as the contraceptive vaccine may avoid the problems mentioned above. Such a conceptual strategy awaits future study

**Reference:**


1. L.Y. Chen, Y.H. Lin, M.L. Lai and Y.H. Chen, Developmental profile of a caltrin-like protease inhibitor, P12, in mouse seminal vesicle and characterization of its binding sites on sperm surface, *Biol. Reprod.* **59** (1998), pp. 1498–1505.
2. C.W. Luo, H.J. Lin and Y.H. Chen, A novel heat-labile phospholipid-binding protein, SVS VII, in mouse seminal vesicle as a sperm motility enhancer, *J. Biol. Chem.* **276** (2001), pp. 6913–6921


Publication


1 Lin HJ, Lin CH, Tseng HC and **Chen YH** (2006), "Detecting disulfide crosslinks of high-molecular weight complexes in mouse SVS by diagonal electrophoresis." *Anal. Biochem.*, 352,296-298

2 Lin HJ, Luo CW and **Chen YH** (2006), Epitope Topology and Removal of Mouse Acrosomal Plasma Membrane by P12-targeted Immunoaggregation. Biochem.Biophys.Res.Comm. 349, 284-288

3 Huang YH, Chen YH et al (2006) "Suppression effect of seminal vesicle autoantigen on platelet-activating –factor-induced mouse sperm capacitation ' J. Cellular Biochem, in press

4 Tang JB and **Chen YH**(2006) "Identification of a tyrosine-phosphorylated CCCTC-binding nuclear factor in capacitated mouse spermatozoa" Proteome. 6, 4800-4807