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Analysis of Protein Patterns of Cellular and Fluidal Components in the Porcine Follicular Contents

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돼지 난포내 세포 및 난포액 구성분의 단백질상 분석

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적 요

돼지 난포내의 각 구성분들에 대해 10 % SDS-PAGE 와 IEF 를 이용한 이차원 전기영동을 실시하여 세포 및 난포액 구성분의 구조단백질상을 분석하였다. 난자-난구세포 복합체를 호르몬과 15 % 의 FCS 가 포함된 M16 배양액으로 39℃, 5% CO_2 상태에서 35 시간 동안 체외배양하였다. 배양 전후의 난자, 투명대 및 난구세포와 난포 크기별로 회수된 난포액들을 각각 분리 회수하여 구조단백질상을 분석하였으며, Silver 염색과 CBB 염색으로 분석이 가능한 각 구성분의 적정 시료량을 조사하였다.

한편 난포 구성분들에 있어서 난자는 분자량이 25 와 114kd, 난구세포는 20, 33, 58, 78 및 112kd, 투명대는 65kd, 그리고 난포액은 18, 76, 92, 152 및 187kd 단백질을 세포특이단백질로 가지고 있음이 확인되었다. 특히 난자의 경우 성숙에 따라 구조단백질상의 변화가 확인된 반면, 난구세포에서는 차이가 없었다. 또한 난포액은 난포의 크기에 따라서는 단백질상의 차이가 없었으나 호르몬 처리 여부에 따라서는 이차원 전기영동상에서 몇가지 단백질에서 차이가 확인되었고, 난포세포들도 폐쇄 여부에 따라 단백질 조성에 차이를 보였다.

따라서 본 실험에서는 전기영동에 필요한 시료의 양과 준비 방법을 확립하여 각 난포 구성분들의 단백질상 분석에 대한 기초자료를 확립하였으며, 이상의 결과는 앞으로 진행될 단백질의 생합성 분석이나 면역화학학적 분석에 유용하게 이용될 수 있을 것이다.

(key words: porcine, follicular component, protein pattern, SDS-PAGE, IEF)

I. INTRODUCTION

The *in vitro* technique of oocyte maturation has been established with various degree of success. Morphological and cytological events during oocyte maturation have been studied in the pig(Nagai et al., 1984 ; Motlik et al., 1986 ; Grant et al., 1989; Byun et al., 1989; Chung et al., 1991). *In vitro* fertilization of matured oocyte has also been successfully achieved using various culture system(Lu et al., 1988; Mattioli et al., 1989). However, unlike these practical informations, basic knowledge on the components of follicle, the oocyte, cumulus cells and follicular fluid undergoing oocyte maturation is not completely known in a single species of domestic animals.

Porcine oocyte is one of the materials studied extensively in the fields of early fertilization

(Leman and Dziuk, 1971) and immunological studies(Hedrick and Wardrip, 1986; Takagi et al., 1989). However, very little information is available about biochemical nature of the follicular components in the millieu of oocyte maturation. For example, fluidal components of follicle from which the oocyte is collected are significantly different at the levels of hormones(Bellin et al., 1987), protein-carbohydrate complexes(Yanagashita et al., 1981) and biologically active molecules(Sluss et al., 1989) depending on the intrafollicular state. In particular, polypeptides of the each cellular and fluidal component are considered as readily detectable molecules.

However, very little efforts were made to analyse biochemical components in the follicular cells, the oocyte and the follicular fluids.

This experiment describes an effort to establish analytical methods for later use in subsequent experiments. Specific or house-keeping proteins of each components were analysed by the sensitive methods with carefully prepared samples. Furthermore, appropriate amounts of each components were determined in different analytical methods, that will be then very useful for further analyses of synthesizing polypeptides and for immunological studies of the components.

Ⅱ. MATERIALS AND METHODS

1. Collection of cellular and fluidal components

Ovaries were brought to laboratory in saline at 4℃ in a Dewar flask. After washing ovaries several times in chilled saline, follicular content was collected with a 26 gauge needle attached to 1 ml disposable syringe by avoiding blood vessel under a dissecting microscope. The follicular fluids were separately collected from three different size of follicles, i. e., large(>5 mm in diameter), medium(3~5mm in diameter) and small(<3mm in diameter). The content in Eppendorf tube was centrifuged at 5,000rpm for 10min at 4℃. The supernatant was recovered and stored at −20℃ until use. Some fluidal samples were obtained in a similar way from the superovulated gilts with injections of 1,500 IU PMS and hCG. For the comparision of normal to atretic follicles, individual follicles were also isolated from the ovaries. They were torn off to release the content into phosphate buffered saline containing 4mg polyvinylpyrrolidone (Sigma) /ml(PBS+PVP).

After examining the contents, they were divided into two groups, cellular association of the OCCs and translucence of the fluid. If the OCC was very tight, and follicular cells appeared shining under a dissecting microscope, the follicle containing the OCC was considered as normal. When the fluidal component showed precipitant, disorganized OCC, and free follicular cells, the follicle was considerd as atretic. They were collected separately as described above.

The oocytes and cumulus cells were isolated from the OCCs immediately after collection and at the end of culture *in vitro* in M16+15% FCS+10IU PMS+10IU hCG(M16+FCS+Gn) at 39℃ in an atmosphere of 5% CO_2 in air for 35 h. The oocytes and cumulus cells were washed in PBS+PVP, centrifuged to remove remaining proteins, and recovered in a small amount of PBS+PVP.

The zonae pellucidae(ZP) were obtained from the frozen-thawed ovaries. The zonae were punctured by the repeated pipetting of cumulus-free oocytes with a finely drawn pasteur pipette in 300 IU hyaluronidase in M2 prior to washing in PBS+PVP and centrifugation. They were stored at −20℃ until use.

2. Sample preparation for SDS-PAGE and IEF

Chemicals required for electrophoresis were purchased from Bio-Rad(Richmond, CA, U.S.A.). Follicular fluid samples were mixed with the same volume of cold acetone. They were vigorously vortexed and centrifuged at 15,000rpm for 5min at 4℃. The supernatant was discarded, and the pellet was resuspended in cold acetone. This procedure was repeated three times. After the final washing, the pellet was dried at 40℃ overnight to evaporate the remaining acetone. The powder was lysed in 20㎕ of sodium dodecylsulfate(SDS)-sample buffer containing 4%(w/v) SDS, 20%(w/v) glycerol, 10%(w/v) β-mercaptoethanol, 1 %(w/v) bromophenol blue and 0.125 M tris-HCl(pH 6. 8) by standing for 1 h at room temperature.

The oocytes and cumulus cells were repeatedly frozen and thawed thoroughly by the use of liquid nitrogen(LN_2), then lysed in SDS-sample buffer. They were stored at −20℃ until use. For isoelectrofocusing(IEF) samples, the acetone powder of follicular fluid and follicular cells were separately lysed in 30㎕ of lysis buffer containing 9.5M urea, 2%(w/v) Nonidet-P40 (NP-40), 2% ampholines(Ampholyte, Bio-Rad Co., pH 3.5~10:pH 5~7=4:1) and 5% β-mercaptoethanol. These samples were used for two dimensional(2-D) electrophoresis combining IEF and SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3. Analysis of specific proteins by electrophoresis

SDS-PAGE was carried out according to Laemmli(1970). Samples were loaded on 10% SDS polyacrylamide gels, 0.75mm thick. Samples of the oocytes, cumulus cells and follicular fluid were electrophoresed at a constant current of 8mA per gel for approximately 7h under a cooling device. Molecular weight standards used were described in previously(Byun et al., 1991).

Two-D electrophoresis was performed as described in the report of O'Farrell(1975). Briefly, 4% polyacrylamide gels containing 9M urea, 2% NP-40, and 2% Ampholyte were formed in glass tubes with 130mm long, 2.5mm in diameter. After pre-run for 75min, sample were loaded in the tube gels and focused for 12h at 400V constant voltage, then further focusing for 1h at 800V constant voltage. The gels were removed by gentle pressure after freezing and thawing, and equilibrated in SDS-sample buffer without bromophenol blue for 1 h on gentle rocking device. The tube gels were electrophoresed on 10% SDS-PAGE gel under similar condition for SDS-PAGE analysis.

4. Visualization of separated proteins

After electrophoresis gels were stained as described previously(Byun et al., 1991). For sensitive visualization, a silver staining method was employed. The silver staining was performed according to the method of Switzer et al.(1979). As a faithful way of visualization, gels were stained with CBB, followed by silver staining. Gels were photographed on PAN F film(ASA 50, Ilford).

5. Determination of relative molecular weight

The molecular weight of the resolved proteins were determined by the instruction provided by the company(Sigma). Rf values and the standard curves were plotted, and relative molecular weight of resolved proteins were determined on the standard curve.

Ⅲ. RESULTS AND DISCUSSIONS

1. Appropriate amount of samples for analysis

In preliminary studies the amounts of each sample were determined to give correct resolution of protein bands with different staining methods. Two μl of follicular fluid, 6×10^6 cumulus cells and 50 oocytes gave proper detection of protein bands in SDS polyacrylamide gel when CBB staining was used. With silver staining, similar resolution could be obtained with much less amount of sample. Amounts of sample for IEF were also determined(Table 1).

The number of the oocytes varied in different experiments. The fact is known that cytoplasm of porcine oocyte is not so easily dissolved even in the lysis buffer. Therefore, it should be important to dissolve the sample completely to obtain complete release of protein components from the cell. It was found that repeated freezing and thawing of the samples in LN_2 was satisfactory. The average number of cumulus cells was 1.2×10^5 cells per oocyte. For maximum resolution of protein bands about 10 oocytes were required when silver staining was employed (Table 1).

2. Protein patterns of follicular components

The resolved proteins of each cellular component, i. e. the oocytes, cumulus cells and the ZP were visualized with CBB staining(Fig. 1a). Under the experimental condition more bands could be seen in cumulus cells than those of the oocytes(Fig. 1a lanes 2 and 4). With silver staining, more clear resolution was achieved and similar number of bands were found (Fig. 1b lanes 1 and 3). The ZP were resolved as a protein band at 62 kd when CBB staining was applied (Fig. 1a lane 5). However, several minor bands were also found with sensitive silver staining, although the complete removal of cumulus cells in the preparation of the ZP was achieved at least under a dissecting microscope (Fig. 1b lane 4).

The minor bands probably orginated from the remnants which survived through the mechanical and enzymatic removal. The electron microscopic analysis demonstrated that the remnants of the cumulus cells were studied in the ZP (Dunbar et al., 1980). Therefore, the oocyte for protein analysis was denuded completely with acid Tyrode's solution (pH 2.5) to eliminate the possible contamination.

The protein patterns of zona-free oocytes were shown with CBB staining(Fig. 1a lane 4) and silver staining(Fig. 1b lane 3). The protein patterns of cumulus cells and the oocyte

Table 1. Determination of appropriate amounts of samples for the analysis of follicular components[1]

Electrophoresis	Methods of staining	Volume of follicular fluid(μl)	No. of cumulus cells	No. of the oocytes	No. of the zonae pellucidae
SDS-PAGE[2]	Cbb	2.0	6×10^6	50	–
	Silver	0.1	1×10^6	10	30
IEF[3] for 2-D	CBB	8.0	–	–	–
	Silver	–	4×10^7	300	–

1. Abbreviations are SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; IEF, isoelectrofocusing; 2-D, two-dimensional electrophoresis and CBB, Coomasis Brillant Blue.
2. Gel dimension: 10% polyacrylamide, 0.75mm thick.
3. Gel dimension: 4% polyacrylamide, 2mm in diameter, 130mm long.

were remarkably similar, thus suggesting that the similar house-keeping proteins for general cell activity and structure were abundant.

The cell-specific protein may be seen, i. e., 25 and 114 kd in the oocyte and 20, 29, 33, 56, and 78 kd in cumulus cells(Fig. 2 and Table 2). Five bands, i. e., 36, 44, 60, 92 and 220 kd were also commonly found in 3 different components of the follicle(Fig. 1). These proteins may be useful for the identification of each components in a follicle for further analysis or immunological studies. In particular, ZP proteins have been favorate materials for the study of gamete interaction and for the preparation of antigens.

These proteins are known to be species-specific, and extensively studied (Gwatkin et al., 1980; Dunbar et al., 1980; Hedrick and Wardrip, 1987; Shabanowitz and O'Rand, 1988). But it is well described that ZP protein pattern is exhibited variously according to the methods used to solubilize it prior to electrophoresis (Dunbar et al., 1980).

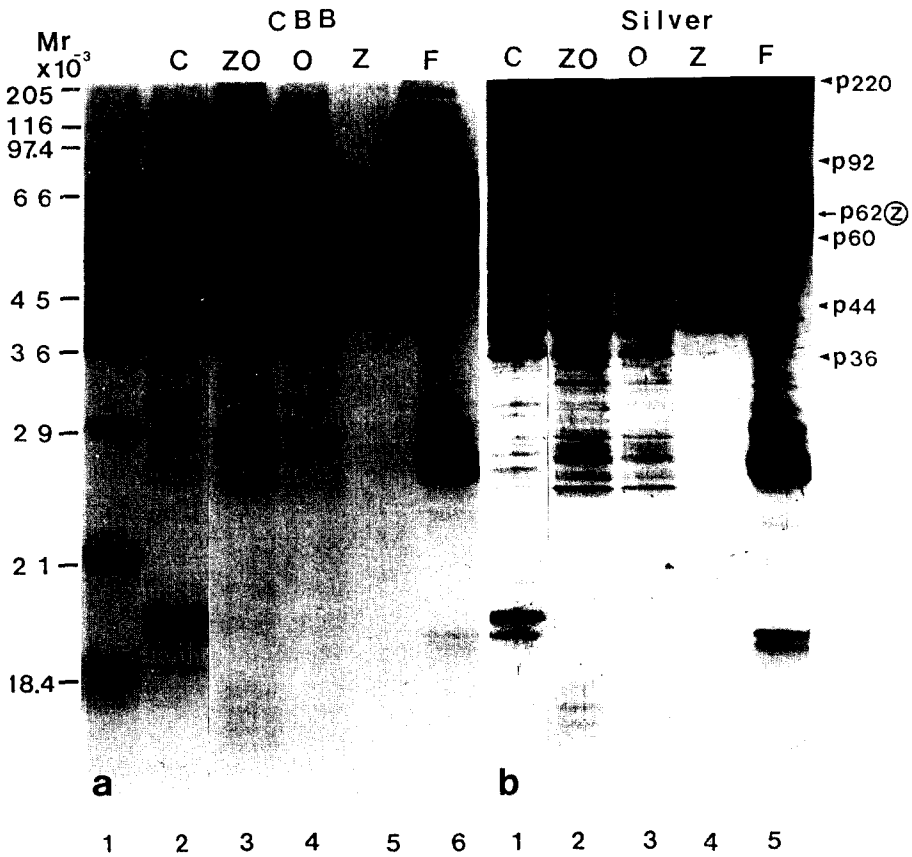

Fig. 1. Electrophoretic patterns of follicular components of porcine follicles in SDS-PAGE gel. Representative protein patterns were visualized in a gel stained with CBB(a), and the identical gel was further stained with a sensitive silver staining method(b). The protein marked on the right are some of specific or common protein bands. Abbreviations are C. cumulus cells; ZO, zona-intact oocyte; O, zona-free oocytes; Z, zonapellucida and F, follicular fluids.

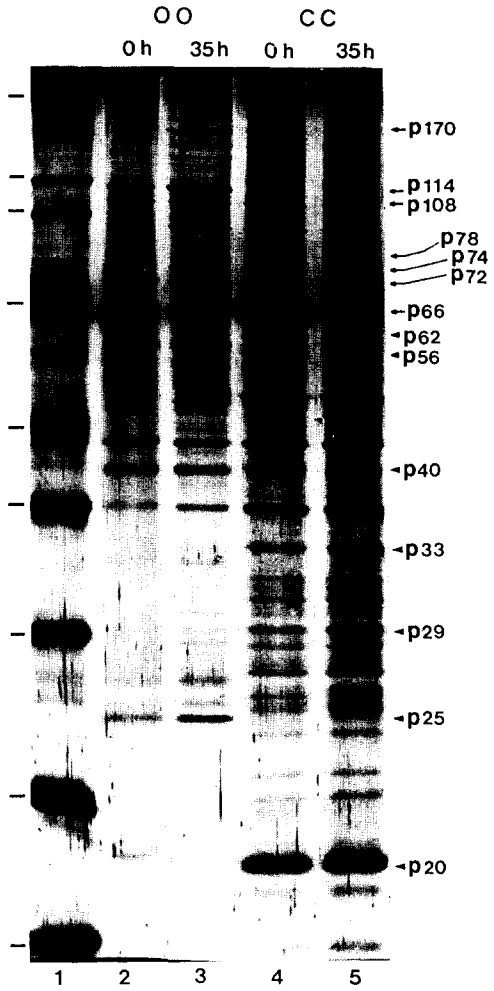

Fig. 2. Electrophoretic patterns of cellular components in SDS-PAGE gel. The gel was stained by silver staining method. Comparative cell-specific protein patterns between the oocytes(25 and 114 kd) and cumulus cells(20, 29, 33, 56 and 78 kd) were clearly seen, but 7 bands, i. e. 40, 62, 66, 72, 74, 108 and 170 kd were seen commonly. Also the difference of protein patterns in matured oocyte could be found. The numbers on the right are some of the specific bands showing differences. See Fig. 1 for details of molecular standard. Abbreviations are OO, oocyte and CC, cumulus cells.

The fluidal component also showed various proteins, which were either present or absent in cellular components. However, the major band was albumin as expected(Fig. 1a lane 6 and 1b lane 5). The proteins found in cellular and fluidal components are summarized(Table 2). This basic data will be useful for further biochemical analysis.

3. Comparison of protein patterns in immature and mature oocytes

Once the protein patterns of each components being established, it was interesting to know whether there are any differences in the cellular components of immature and mature oocytes. Mature oocytes were prepared at the end of 35 h culture. Although the number of the oocyte was identical in different lanes, the amount of proteins was much higher in the mature oocyte, as judged by the stained protein(Fig. 2 lane 2 and 3). This may account for the different protein composition between immature and mature oocytes. However, it did not eliminate possible properties of different cytoplasm, thus causing different degree of dissolution.

No specific alteration was found in the cumulus cells from mature and immature oocytes (Fig. 2 lane 4 and 5). Therefore, it appears that no significant changes would occur in the house-keeping proteins of the oocyte and cumulus cells during oocyte maturation *in vitro*.

4. Fluidal protein patterns in different sources of follicles

Follicular fluids were obtained from 3 different sizes of follicles, and either from follicles of pig ovaries of slaughterhouse or gonadotrophin-treated pig ovaries. The fluidal proteins were analysed to evaluate any visible differences in various sources of follicles. No clear

Table 2. List of proteins with CBB and silver staining in follicular components

Follicular compounds	No. of protein bands	Specific protein bands	Common protein bands to[1]			
			O&C	C&F	O&F	All
oocytes	14	p25, p66,	p40			
		p72, p114	p62		p36	
Cumulus cells	19	p20, p29	p108		p44	
		p33, p56, p78	p170	p26		p60
Follicular fluid	12	p18.5, p19, p76,		p50		p92
		p152, p187				p220
Zona pellucida	1	p62				

1. Abbreviations are O, oocytes; C, cumulus cells and F, follicular fluid.

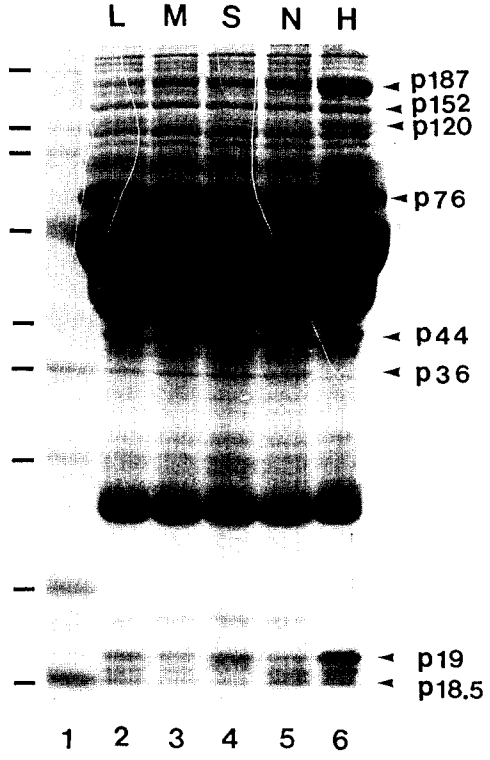

Fig. 3. Electrophoretic patterns of proteins found in follicular fluid collected from different conditions. Follicular fluids were collected from large size of follicles(L), medium-sized follicles(M), and small size of follicles(S), respectively. Follicular fluids from the ovary recovered from slaughterhouse(N) and superovulated ovary(H) were also compared. See Fig. 1 for detail of molecular weight standard.

difference was found except for a few qualitative differences. For example, 44kd protein was not detectable in the medium-sized follicle of ovaries from slaughter house. And another protein 36 kd was absent, but 120 kd was found only from the follicular fluid of gonadotropin-treated pig.

The quantities of some protein bands, such as 19 and 187 kd may be different(Fig. 3). This results by no means suggest that no differences were found in different sources of follicular fluid. It has been suggested that the total protein concentration is similar in small and large follicle(McGaughey, 1975). Probably the limitation of SDS-PAGE may be one of the major factors. Other techniques such as IEF may be more sensitive to detect such minor differences as shown by others(Manabe et al., 1982).

The fluidal and cellular proteins were more closely compared in normal and atretic follicles, and follicles from slaughter house and gonadotrophin-treated pig, respectively by 2-D electrophoresis to demonstrate any minor changes

undetected in SDS-PAGE analysis(Fig. 4 and 5). By 2-D electrophoresis it has been revealed that the serum and follicular fluid were similar in protein composition(Dunbar et al., 1980).

Also, Andersen et al. (1976) has suggested that the proteins in follicular fluid from bovine follicles have a minimum 40 individual proteins distinguishably. General distribution of protein spots was quite similar both in normal and atretic follicle(Fig. 4). However, a few additional protein spots were found only either in normal or in atretic follicle. Therefore, some of the protein components may differ in the fluidal environment, thus suggesting that different state of follicles could influence the oocyte by surrounding it. The patterns of protein spots were slightly different between the follicles with different sources. Andersen et al. (1976) found that the protein composition in healthy growing follicles differs from that of atretic and cystic follicles.

The similar bands with specific molecular weight were even differently separated with 2-D analysis, thus showing the details of the protein composition in a particular band found in SDS-PAGE analysis. Along with the histological evidence(Byun et al., 1990) this biochemical differences found here open a way to study the follicular environment more closely. The proteins specific in certain type of follicles may be separated from the gel, and could be used as antigens for the marker for selection or determination of follicular state.

The information of general protein patterns

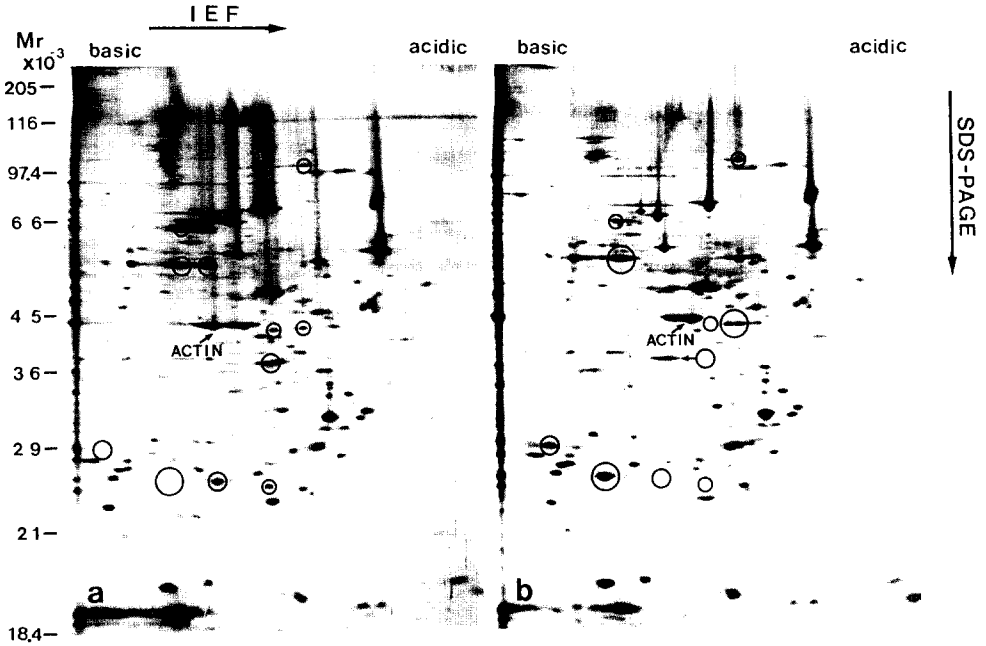

Fig. 4. Two-D electrophoretic patterns of polypeptide spots found in porcine follicular cells collected from atretic(a) and normal follicles(b), respectively. Two gels were electrophoresed under an identical condition. Some of landmark protein(actin, 44 kd) and proteins showing differences were marked.

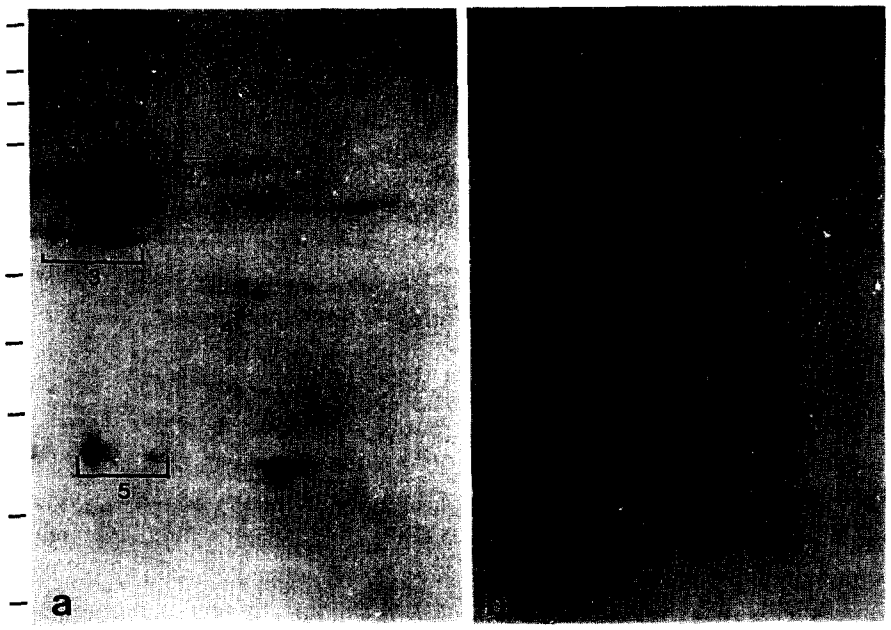

Fig. 5. Two-D electrophoretic pattern of follicular fluid collected from follicles of the ovary recovered from slaughterhouse(a) and superovulated ovary (b), respectively. The protein spots with numbers were marked for comparisons. The gel was stained with CBB. See Fig. 4 for detail of molecular weight standard.

may provide a standard for the identification and characterization of specific proteins of fluidal components. The established sample preparation and analytical method will further provide important tools when they are combined with other approaches such as radiolabelling of oocyte surface membrane and quantitative analysis of protein contents.

IV. SUMMARY

The polypeptide patterns of cellular and follicular components were analysed by SDS -PAGE and two dimensional(2-D) electrophoresis combined with isoelectric focusing (IEF) to establish protein profiles in each of the components in porcine follicles.

Oocyte-cumulus complexes were cultured in M16+FCS+Gn at 39 in an atmosphere of 5% CO_2 in air for 35 h. At the end of the culture, the zona-free oocyte, ZP alone and cumulus cells were prepared and analysed either on 10% SDS-PAGE for the protein profile at the first dimensional gel or 2-D protein pattern. The amounts of each samples were determined for the visualization with Coomasie brilliant blue (CBB) or silver staining, thus giving useful information for the identification of specific proteins in the components or appropriate amount of samples for proper visualization.

Oocyte showed 25 and 114 kd major protein band. Other minor components were additionally

visualized with CBB on the same gel after silver staining procedure. Cumulus cells also showed specific proteins which is not present in the oocytes. The number of cumulus cell was proper to give major bands with CBB and additional minor bands with silver staining. To establish the degree of contamination from the remnant of the corona radiata to the ZP, zonae were differently prepared or analysed by SDS-PAGE.

The preparation of the ZP in this study did not showed any contamination judged by the protein profile of the components. Also follicular fluid showed its specific protein profile without any significant differences among the different sizes of follicles. The established protein profile of each follicular component should be helpful for the identification and elimination of contaminated components, i. e., antigen preparation or immunological studies.

The results also suggest that the preparation of each components in the study was appropriate and can be used for a further sensitive biochemical analysis in mammalian oocytes and early embryos.

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