Eight protein spots which were increased on cystic FF were excised from the silver stained gels and subjected to mass spectrometry analysis and protein identification.

Details of each experimental process are given in the following section.

Enzyme immunoassay of progesterone and estradiol-17β in follicular fluid

The enzyme immunoassay procedure for progesterone and estradiol-17β was followed as described previously [20]. FF diluted with H_2O was extracted with petroleum ether for progesterone or diethylether for estradiol-17β. The decanted ether phase was dried in a glass tube and a buffer (0.05 mol/L boric acid, 0.2% BSA) was added. These reconstituted samples were placed into wells of a microtiterplate that was coated previously with an anti-rabbit IgG antibody (ICN, Pharmaceuticals Inc., USA) followed by the addition of an anti-progesterone antibody and Horseradish peroxidase (HRP) conjugated progesterone for progesterone, and an anti-estradiol-17β antibody (Kambegawa Institute, Tokyo, Japan) and HRP conjugated estradiol-17β (Kambegawa Inst.) for estradiol-17. After 2-h incubation plates were washed and a 3,3',5,5'-Tetramethylbenzidine solution was applied to the substrate followed by reading optical density measurement at a wavelength of 450 nm.

Protein sample preparation

Initially, the total protein content in FF was determined using a commercial protein assay kit (DC Protein Assay, Bio-Rad laboratories Inc., Hercules, USA). After measuring the protein content, high abundant proteins, albumin and immunoglobulin G (IgG) were removed from FF using an Aurum serum protein mini kit (Bio-Rad laboratories, Inc., Hercules, USA) to enable the visualization of low abundant proteins. Then, proteins in depleted FF samples were concentrated by centrifugation using a 3 kDa cut off cellulose membrane filter unit (Microcon YM-3, Millipore, Bedford, USA). Finally, impurities such as salts, lipids, detergent or nucleic acid were removed from concentrated samples using a 2-D Clean-Up Kit (Amersham Biosciences, San Francisco, USA).

2-D PAGE

Fifty μg of protein sample was dissolved in a sample buffer (8 M urea, 0.5% ampholine ph3.5-10, 0.5% Triton X-100, 10 mM dithiothreitol (DTT) and Orange G). The sample buffer, including 50 μg protein, was absorbed into an immobilized pH gradient (IPG) strip (Immobiline Dry Strip, 11 cm, pH range 3–10, Amersham Biosciences, Uppsala, Sweden) overnight. The rehydrated strip was subjected to isoelectric focusing on a MultiPhor II electrophoresis chamber (Amersham Biosciences, Uppsala, Sweden) for a total of 22,651 Vh at 15°C. The focused IPG strip was equilibrated in sodium dodecyl sulphate (SDS) buffer (30% glycerol, 1.0% SDS and 6 M urea in 50 mM Tris-HCl). The first equilibration step was carried out in SDS equilibration buffer with 16 nM DTT, and the second portion of the SDS equilibration buffer contained 240 mM iodoacetamide and bromophenol blue. Both equilibration steps lasted 15 min at room temperature. The equilibrated IPG strip was placed onto an 8–18% gradient polyacrylamide gradient gel (ExcelGel SDS, Amersham Biosciences, Uppsala, Sweden) for second dimensional SDS-PAGE. The SDS-PAGE was performed at 20 mA for 30 min, 50 mA for 5 min and 50 mA for 70 min at 15°C with power supply limitation of 600 V and 30 W using a MultiPhor II electrophoresis chamber. After separation in SDS-PAGE, the proteins on the gel were fixed and visualized by silver staining using a Silver Stain Plus Kit (Bio-Rad laboratories Inc., Hercules, USA).

Mass spectrometry analysis and protein identification

The spot was excised from the silver stained gel and then digested with trypsin using a previously described method [21]. For in-gel digestion, the pieces of gel were immersed in 50% acetonitrile solution for 10 min and this process was repeated several times. After washing with acetonitrile, acetonitrile was removed and the gel pieces were dried in a vacuum centrifuge for 10 min. The gel pieces were shaken with 100 μL of reducing solution (10 mM DTT and 25 mM ammonium bicarbonate) for 60 min at 56°C. Subsequently, the gel pieces were shaken under dark conditions with 100μL of an alkylating solution (55 mM iodoacetamide and 25 mM ammonium bicarbonate) for 45 min. The gel pieces were shaken with 50% acetonitrile and 25 mM ammonium bicarbonate for 10 min twice. The solvent was removed, and the gel pieces were dried again. The gel pieces were then immersed in a digestion solution containing 12.5 μg/mL sequencing grade modified trypsin and 25 mM ammonium bicarbonate and incubated on ice for 10 min. After removing unabsorbed solution, the gel pieces were incubated for 16 h at 37°C. Digested peptides were extracted twice from gel pieces with 50 μL of 50% acetonitrile and 0.1% trifluoroacetic acid. The pooled supernatants were dried in a Speed Vac, and the peptides were dissolved in 10 uL of 50% acetonitrile with0.1% trifluoroacetic acid. MALDI-TOF MS was performed on an Ultraflex time-of-flight instrument (Bruker Daltonics, Billerica, USA) equipped with a nitrogen laser operating at 337 nm. All MALDI-TOF results were obtained in the reflector-positive mode using α-cyano-4-hydroxycinnamic acid (saturated solution in 50 % acetonitrile with 0.1% trifluoroacetic acid) as the matrix. Analytes were prepared by mixing 0.5 μL of peptide sample with 0.5 μL of matrix soln. on a MALDI plate and allowed to air dry at room temperature in a hood before inserting then into the spectrometer. Mass spectra were calibrated with angiotensin II (1046.54 Da),