

Expression and purification of biologically active porcine follicle-stimulating hormone in insect cells bearing a baculovirus vector

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ABSTRACT

Biologically active recombinant porcine FSH (rec-pFSH) free from the cognate pituitary glycoprotein hormone LH was produced. It was synthesized by a baculovirus vector–insect cell system using two cDNAs encoding the glycoprotein α and FSH β subunits. Its antigenicity was the same as that of pFSH prepared from the pituitary. Glycosylation of rec-pFSH was shown by tunicamycin treatment but the molecular mass of each subunit was lower than that of pituitary-derived FSH, because of the absence of trimming of terminal sugars in insect cells. Rec-pFSH was secreted into the culture medium at about 1 mg/l and purified in six fractions, because of the heterogeneity of the sugar group, by S-Sepharose

and concanavalin A–Sepharose column chromatography. The biological activity of rec-pFSH was examined by measuring its effect on progesterone secretion from porcine granulosa cells and germinal vesicle breakdown (GVBD) of porcine oocytes. It showed adequate activity with respect to progesterone secretion, although some fractions rich in the sugar group showed lower activity than that of pituitary-derived FSH. It exhibited higher GVBD activity than that of pituitary-derived FSH at concentrations as low as 1 ng/ml. These results demonstrate that the baculovirus vector–insect cell system can provide biologically active rec-pFSH.

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INTRODUCTION

The life span of the ovarian follicle (maturation, ovulation and luteinization) is modulated by the synergistic action of two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which account for the different reproductive cycle phases (Rao *et al.* 1978, Hsueh *et al.* 1984, Miller 1993, Richards 1994). In the female, FSH is essential for follicular growth, whereas LH induces ovulation from mature follicles and maintains progesterone production by the corpus luteum. In the male, FSH induces growth of the seminiferous tubules and maintains spermatogenesis in the Sertoli cells, whereas LH stimulates

androgen synthesis by Leydig cells and seminiferous tubules. However, some aspects of these hormones remain to be elucidated (Chappel & Howles 1991).

FSH and LH are synthesized and secreted by the same type of cells in the anterior pituitary lobe. They are heterodimers composed of the same α -subunit linked to distinct β -subunits and contain oligosaccharide chains (Pierce & Parsons 1981). Owing to their similar biochemical properties and the presence of multiple isoforms with heterogeneous oligosaccharide moieties, it has proved very difficult to obtain a pure form of either, especially FSH. Preparations of FSH extracted from the pituitary contain small amounts of contaminating

hormones, such as LH, which may produce unexpected activity, as a result of the synergistic actions of FSH and LH, in studies designed to investigate FSH activity, resulting in unclear results. FSH is important not only for human reproduction but also for animal breeding. Recombinant (rec)-FSH is guaranteed to be free from contaminating hormones. Several workers have expressed rec-FSH in a Chinese hamster ovary cell line transfected with the FSH gene or cDNAs encoding the FSH subunits (Keene *et al.* 1989, Van Wezenbeek *et al.* 1990, Mountford *et al.* 1995). Although mammalian cells can add oligosaccharide chains and form the correct bonds between the intramolecular disulphide bridges important for FSH and LH activities (Ryan *et al.* 1987), the yields of recombinant molecules from mammalian expression systems are generally lower than those achieved with bacterial, yeast and insect cell systems. Data demonstrating that a baculovirus vector–insect cell system can express higher yields of recombinant molecules of vertebrates in biologically active forms than mammalian cell lines are accumulating (Lucknow & Summers 1988).

Therefore, in this study, we examined the production of rec-porcine FSH (rec-pFSH) by a baculovirus vector–insect cell system and purified the rec-pFSH. After examination of the expression conditions suitable for mass production, a large amount of rec-pFSH was purified by column chromatography. We demonstrated that the biological activity of the rec-pFSH was equivalent to that of FSH preparations from porcine pituitaries by assaying its effects on progesterone secretion by porcine granulosa cells in culture and germinal vesicle breakdown (GVBD) of porcine oocytes.

MATERIALS AND METHODS

Virus and cells

Autographa californica nuclear polyhedrosis virus (AcNPV) and recombinant virus were grown in monolayers of *Spodoptera frugiperda* (Sf) insect cells by the method of Brown & Faulkner (1977). Sf cells were maintained in Grace's insect medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% (v/v) fetal calf serum (Filtron, Victoria, Australia), 0.26% (w/v) Bacto tryptose broth (Difco Laboratories, Detroit, MI, USA) and 50 µg/ml kanamycin. In order to obtain the dimeric form of FSH, *Trichoplusia ni* 5B1-4 (Tn5) cells (Invitrogen, San Diego, CA, USA) were grown in serum-free medium SF900II

(Gibco Laboratories) used for propagation of the recombinant virus.

Construction of transfer vectors and selection of recombinant viruses

All the basic procedures involved in the recombinant technique were standard protocols (Maniatis *et al.* 1989). In order to express FSH α cDNA exclusively, a 470 bp EcoRI fragment containing the entire open reading frame (363 bp) was obtained from clone α -49 (Hirai *et al.* 1989), its end was filled with the Klenow enzyme and the resulting fragment was inserted into the unique SmaI site of the transfer vector pAcYM1 (Matsuura *et al.* 1987). In order to express β cDNA, a 436 bp EcoRI fragment containing the entire open reading frame (390 bp) was obtained from clone FSH β , which was constructed by carrying out PCR using two primers encoding the initiation site CTCGAGTCGACT-CATGAAGTCGCTGCAGTTTTGCTTCCT-ATTCT and termination site AGCATGAAAT-GTCCACTGCT designed according to the sequences reported in previous papers (Kato 1988, Hirai *et al.* 1990). This fragment was inserted into pAcYM1 as described above for the α -subunit cDNA. The resulting plasmids were designated pAcYM1 α and pAcYM1 β respectively (Fig. 1). To construct a dual-expression vector with α - and β -subunit cDNA in a single vector, both pAcYM1 α and pAcYM1 β were digested with BamHI, and the two fragments were inserted stepwise into the unique BglII and BamHI sites respectively of the transfer vector pAcUW3 (Weyer & Possee 1991). In the resulting clone, pAcUW3 $\alpha\beta$, the α - and β -subunit cDNAs were localized downstream from the P10 and polyhedrin promoters respectively as described in the legend to Fig. 1.

Co-transfection and tunicamycin treatment

Sf cells were co-transfected with a mixture of purified infectious AcNPV DNA and transfer vector DNAs (pAcYM1 α , pAcYM1 β and pAcUW3 $\alpha\beta$), as described previously (Matsuura *et al.* 1986). After incubation for 4 days, each culture supernatant was harvested and subjected to the plaque assay. Plaques without inclusion bodies were picked up and subjected to purification by two cycles of plaque isolation, and the resulting recombinant baculoviruses were named AcNPV α , AcNPV β and AcNPV $\alpha\beta$ respectively. These recombinant baculoviruses were allowed to infect Sf or Tn5 cells at a multiplicity of 10 plaque forming units (pfu)/cell and then incubated at 27 °C for 2 days. The medium was changed for

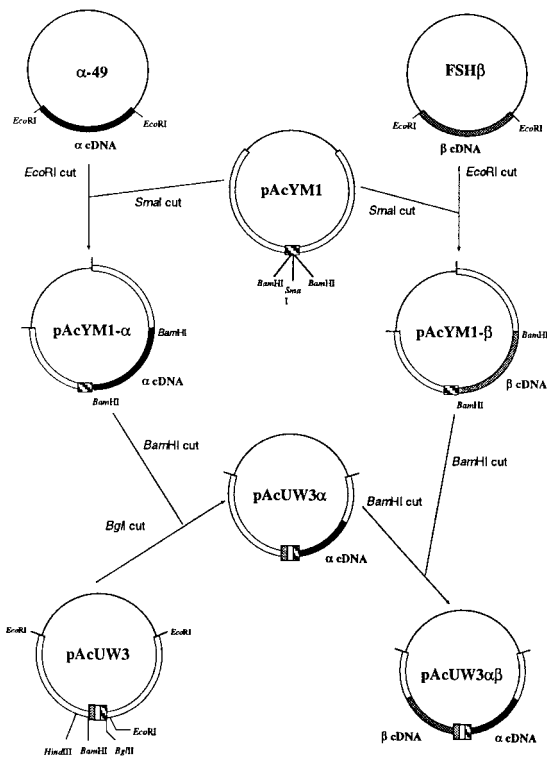


FIGURE 1. Construction of expression vectors. Porcine FSH α - and β -subunits were ligated separately in the expression vector pAcYM1 and then both ligated in an expression vector with a dual promoter. The solid and hatched squares indicate the polyhedrin and P10 promoters respectively.

fresh of the same composition, and, after incubation for a further 4 days, the cells were harvested.

To investigate the presence of carbohydrates in rec-pFSH, Sf cells were infected with either AcNPV α or AcNPV β at a multiplicity of 10 pfu/cell. After incubation for 1 h at room temperature, each inoculum was replaced with Grace's medium with or without 5 μ g/ml tunicamycin (Boehringer-Mannheim GmbH, Mannheim, Germany), an inhibitor of N-linked glycosylation. Medium was collected as described above.

Immunoblotting

Aliquots of infected cells were boiled for 10 min in sample buffer (2% (w/v) SDS, 5% (v/v) β -mercaptoethanol, 10% (v/v) glycerol and 62.5 mM Tris-HCl, pH 6.8), and subjected to electrophoresis in 20% (w/v) polyacrylamide gels, as described by Laemmli (1970). After electrophoresis, the proteins were transferred to polyvinylidene difluoride filters

(Immobilon; Millipore, Bedford, MA, USA) using a semi-dry transblot apparatus (Sartorius GmbH, Göttingen, Germany). The filters were blocked with 3% (w/v) skimmed milk in PBS and incubated with either rabbit anti-FSH α peptide of the sequence 37–53 (FSRAYPTPARSKKTMLV) (50:1 dilution) (Tanaka *et al.* 1997) or anti-rat FSH β serum (NIH; AFP-856-1) at room temperature for 1 h. After a wash with PBS, the filters were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Organon Teknika, West Chester, MD, USA) and the protein bands that reacted with this antibody were visualized by incubation of the filters in solution comprising 0.5 mg/ml diaminobenzidine and 0.05% (w/w) H₂O₂.

Quantification of FSH

Time-resolved fluoroimmunoassay (TR-FIA) of FSH was performed as described in a previous paper (Iwasawa *et al.* 1994). Porcine pituitary-derived FSH NIH porcine-pFSH-I-1 of radioiodination grade, 20 μ g in 20 μ l 0.05 M sodium carbonate-bicarbonate buffer, pH 9.8) was labeled with a 30-fold molar excess of Eu-labeling reagent (Eu chelate of N₁-(*p*-isothiocyanatobenzyl)-diethylenetriamine-N₁,N₂,N₃,N₄-tetra-acetic acid (Pharmacia, Uppsala, Sweden)) for 4 h at room temperature and the Eu-labeled FSH was separated on a Sephacryl S-100 HR column (1 \times 18 cm; Pharmacia). Ninety-six-well titer plates were immobilized with HCl-treated anti-rabbit IgG before the experiment and 50 μ l standard or sample and 100 μ l antiserum in assay buffer (0.5% (w/v) BSA, 0.05 M Tris-HCl, pH 7.75; Pharmacia) were added to each well and incubated with shaking at room temperature for 1.5 h. Next, 100 μ l Eu-labeled FSH (20 ng/ml) were added to each well, followed by shaking for 3.5 h at room temperature. Each well was washed and 200 μ l enhancement solution (Pharmacia) were added and mixed to enhance the Eu-fluorescence, which was measured with a time-resolved fluorometer (Arcus 1230; Pharmacia).

Purification of rec-pFSH

Culture medium from cells infected with the dual-expression vector AcNPV $\alpha\beta$ was filtered with UF-3000ps (Tosho, Tokyo, Japan) to remove the viruses and applied to an S-Sepharose column 26/10 (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.0. After loading and washing of the column with this buffer, the proteins were eluted with a linear gradient of NaCl (0–0.5 M) in this buffer and fractionated (6 ml/tube). Fractions containing FSH, detected by TR-FIA, were dialyzed against a buffer comprising 20 mM Tris-HCl, pH 7.4, containing

0.5 M NaCl, pooled and separated on a concanavalin A (ConA)-Sepharose column (1.0 × 4.5 cm; Pharmacia) with a gradient of methyl- α -glucose (0.0–5 M) in this buffer. Fractions of volume 1 ml were collected and an aliquot of each was subjected to TR-FIA to measure the rec-pFSH content.

Isolation of granulosa cells and measurement of progesterone secretion

Porcine granulosa cells from follicles 2–5 mm in diameter obtained from the ovaries of maturing gilts were aspirated through an 18-gauge needle into a disposable syringe, agitated mechanically with a Pasteur pipette to remove the oocytes, filtered through polyester mesh, and washed with Dulbecco's modified Eagle's and Ham's F-12 media (1:1, v/v) supplemented with 10% (v/v) calf serum and 20 μ g/ml gentamicin. Cell viability was determined by trypan blue exclusion (about 30%). Granulosa cells (3×10^5 /ml) were incubated in 48-well culture plates (Costar, Cambridge, MA, USA) for up to 72 h (5% CO₂ in air at 37.5 °C). The medium was changed 48 h after the start of the culture, when rec-pFSH or pFSH (USDA-pFSH-B-1) was added to a concentration of 10 ng/ml, and the culture medium was collected 24 h after this change and stored at –20 °C until the assay was performed.

Progesterone concentrations in the cell culture medium were determined directly by an enzyme immunoassay, as described previously (Okuda *et al.* 1997). The significance of values was analyzed by Duncan's new multiple range test.

Assessment of oocyte maturation

Oocytes with compact cumuli attached to a piece of parietal granulosa cells from prepubertal gilts were prepared as described by Motlik *et al.* (1991), and maturation of about 10 oocytes in 0.5 ml culture medium was assessed. The medium consisted of TCM-199 (Gibco BRL, Grand Island, NY, USA) buffered with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Gibco) supplemented with 10% (v/v) fetal calf serum, 0.2 mg/ml sodium pyruvate (Wako Pure Chemical Ind. Ltd, Osaka, Japan), 100 iu/ml penicillin (Meiji Conf. Co., Tokyo, Japan) and 100 iu/ml streptomycin (Meiji Conf. Co.). The required amounts of rec-pFSH were added to the cultures and the oocytes were incubated at 38.5 °C under an atmosphere of 5% CO₂ in air for 24–25 h, after which the extent of cumulus cell expansion was observed. After the granulosa cells had been removed with 1% (w/v) hyaluronidase, the oocytes were mounted on glass

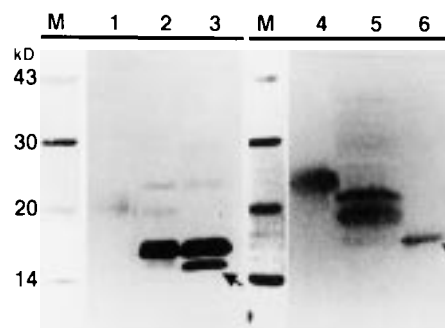


FIGURE 2. PAGE analysis of expressed rec-pFSH and its subunits. The constructed expression vectors were expressed in the insect cells after individual transfection or co-transfection. Pituitary FSH (lanes 1 and 4) and lysates prepared from cells cultured in the absence (lanes 2 and 5) and presence (lanes 3 and 6) of tunicamycin were subjected to SDS-PAGE followed by Western blotting using specific antibodies against the FSH α - (lanes 1–3) and β - (lanes 4–6) subunits. M indicates molecular size markers.

slides, fixed in acid ethanol and stained with orcein. The GVBD frequency was observed in triplicate experiments and the data were compared using the χ^2 test. $P < 0.05$ was considered significant.

RESULTS

Expression of rec-pFSH in insect cells

AcNPV α and AcNPV β were expressed independently after infection of Sf cells with 10 pfu/cell followed by incubation for 4 days; the cell lysates were prepared and analyzed by SDS-PAGE (Fig. 2). Although dye staining revealed a large number of proteins (data not shown), Western blotting with specific antisera against the FSH α - and β -subunits revealed specific positive bands. These antisera did not cross-react with the lysates of cells carrying the other expression vector. The finding that more than one band from each lysate reacted with the anti-FSH α - and β -subunit sera (Fig. 2, lanes 2 and 5) suggested that the recombinant subunits were heterogeneous, presumably because of incomplete glycosylation in the insect cells. In order to explore this further, the expression experiment was carried out using culture medium containing 5 μ g/ml tunicamycin. This treatment changed the composition of the immunoreactive bands demonstrated by Western blotting (Fig. 2, lanes 3 and 6): the molecular mass of most of the β -subunit decreased to about 20 kDa as a single band, whereas only part of the α -subunit decreased to about 15 kDa,

possibly because the tunicamycin concentration was not high enough. Furthermore, the molecular masses of the recombinant subunit molecules were lower than those of the corresponding pituitary-derived FSH subunits.

In order to obtain FSH from recombinant α - and β -subunits, we attempted to reconstitute FSH *in vitro*. Cells infected with AcNPV α and AcNPV β were harvested by low-speed centrifugation, mixed and lysed with 6 M urea at acid pH; this was followed by reconstitution with dialysis against neutral buffer. Western blotting analysis after SDS-PAGE showed that the α -subunit was recovered mainly from the insoluble material with very little in the soluble fraction (data not shown). In contrast, the β -subunit was found to be present predominantly in the soluble fraction (data not shown). As the TR-FIA for FSH detects only the dimer of the α - and β -subunits, the assay of the reconstituted product showed that only an extremely small amount of FSH was obtained (134 ng/10⁷ cells), indicating that *in vitro* reconstitution of rec-pFSH is not suitable for large-scale production of FSH.

Expression after co-transfection with two subunit vectors and transfection with a dual-expression vector

Co-expression of α - and β -subunit vectors and expression of a dual-expression vector were examined. Co-expression of these two vectors in Sf cells yielded immunoreactive rec-pFSH in the culture medium, suggesting that construction of the dimeric form and its secretion was carried out successfully *in vivo*. However, the amount produced was very low, 35 ng/ml. Several repeated expression experiments improved the yield somewhat, but it varied from 35 to 113 ng/ml (Table 1). Expression in Tn5 cells instead of Sf cells increased production over 10-fold, but the expression levels were variable, which might have been due to uneven infection by the recombinant viruses. In an attempt to resolve this problem, we finally constructed a vector carrying the α - and β -subunits at two separate promoters in a single vector (pAcUW3 $\alpha\beta$) using two expression vectors (pAcNV α and pAcNV β) and found that the dimer form of FSH was expressed in Tn5 cells carrying the dual-expression vector. The amount expressed was about 2 μ g/ml, roughly the same order of magnitude as that achieved with co-expression (Table 1). In repeated expression experiments, the amounts ranged from 1 to 2 μ g/ml. Consequently, we used this construct and Tn5 cells for large-scale expression to produce rec-pFSH.

TABLE 1. Amount of recombinant porcine FSH expressed in insect cells

Host cells	FSH concentration (μ g/ml)
SF21	
Experiment 1	0.113
Experiment 2	0.035
Tn5	
Co-expression pAc- α + pAc- β	1.24
Experiment 1	1.48
Experiment 2	4.23
Dual-expression vector UW- $\alpha\beta$	2.25

Purification of rec-pFSH

Culture medium containing rec-pFSH (about 2 mg from 2 litres) was loaded on to an S-Sepharose column (Fig. 3a). Although the rec-pFSH expressed was presumed to lack sialic acid groups, as insect cells do not glycosylate sialic acid, a cation exchanger of S-Sepharose resin was used. Most of the proteins in the lysate were not adsorbed and about 85% of rec-pFSH was retained and eluted with an NaCl gradient in several fractions (22–45). The immunoreactive fractions were combined and rec-pFSH was separated on a ConA-Sepharose column (Fig. 3b) with a linear gradient of methyl- α -glucose, which yielded several rec-pFSH-containing fractions (peaks 1–6, mainly in peaks 2 and 4), which were collected separately for further analysis. The total yield of immunoreactive FSH was about 770 μ g.

SDS-PAGE and Western blotting analyses

The proteins separated by column chromatography were analyzed by SDS-PAGE and Western blotting (Fig. 4). The first chromatographic separation by S-Sepharose removed numerous proteins, but a large number remained (Fig. 4, lane S). Further purification on a ConA-Sepharose column removed the non-glycosylated proteins and provided rec-pFSH that was predominantly homogeneous from the pooled fractions, except for a small amount of contaminating adsorbed proteins that were present in and eluted around peak 1. Western blotting showed that all the fractions consisted of α - and β -subunits (Fig. 4b and c). The molecular masses of the α - and β -subunits of the purified rec-pFSH were slightly lower than those of pituitary-derived FSH, a similar result to that with independent

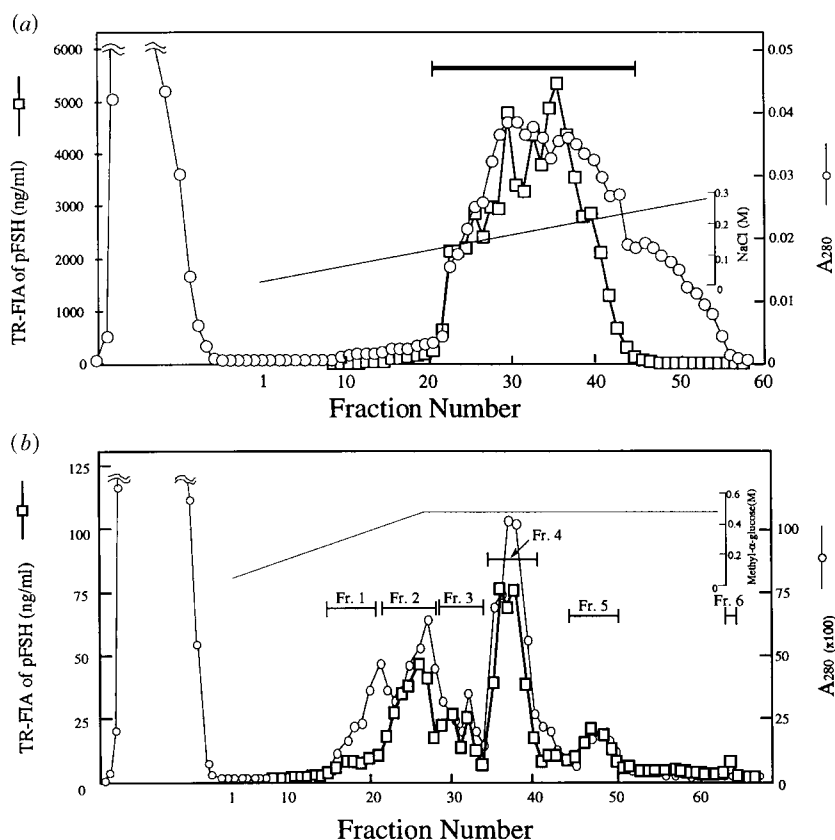


FIGURE 3. Purification of rec-pFSH. (a) The rec-pFSH in culture medium was separated on an S-Sepharose column with a linear gradient of NaCl. (b) Partially purified rec-pFSH, which is indicated by the solid bar in (a), was separated on a ConA-Sepharose column with a linear gradient of methyl- α -glucose.

expression of the subunit vectors (Fig. 2). The fractions thus separated were subjected to the following biological assays for FSH.

Antigenicity of rec-pFSH

The antigenicity of rec-pFSH is important for the evaluation of the structural information of the recombinant protein as well as for measuring its concentration. The TR-FIA for pFSH demonstrated that the antigenicities of rec-pFSH and pituitary-derived FSH did not differ, as their competition curves were parallel (Fig. 5).

Effect of rec-pFSH on progesterone secretion by cultured porcine granulosa cells

Pituitary-derived FSH (10 ng/ml) distinctly stimulated progesterone secretion by cultured porcine granulosa cells (Fig. 6). Under the same assay

conditions, all the rec-pFSH-containing fractions tested exhibited clear stimulatory effects on progesterone secretion that were significantly different from the control values ($P < 0.01$). Although the potency of the S-Sepharose crude fraction was significantly lower than that of pituitary-derived FSH, fraction 1 was more potent than pituitary-derived FSH ($P < 0.01$). One of the major fractions, 2, showed almost the same activity as pFSH, but those of fractions 3, 4 and 5 were somewhat lower. Fraction 6, which had the highest affinity for ConA-Sepharose resin, exhibited the lowest activity (about 50% of the control value).

Effects of rec-pFSH on cumulus expansion and GVBD progression

Expansion of porcine cumulus cells (GV) and GVBD were examined. Oocytes treated with all the rec-pFSH fractions and pituitary-derived FSH

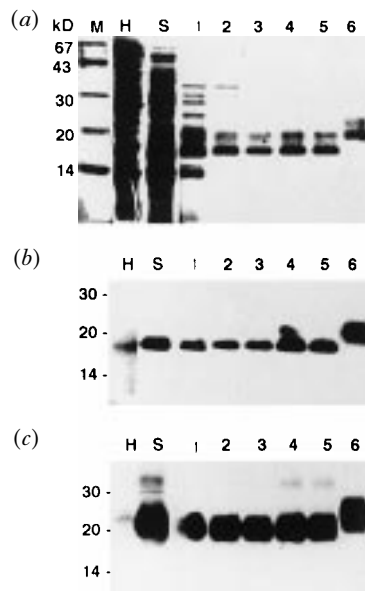


FIGURE 4. PAGE analysis of rec-pFSH. (a) SDS-PAGE of crude, S-Sepharose and ConA-Sepharose rec-pFSH fractions was performed, followed by staining with a silver staining kit (Daiichi Pure Chemical, Tokyo, Japan). (b) After separation on SDS gel and blotting on a nitrocellulose filter, the filter was allowed to react with an anti-FSH α -subunit antibody. (c) A filter prepared as described for (b) was allowed to react with an anti-FSH β -subunit antibody. Lane H, cell lysate of AcBPV $\alpha\beta$ -infected cells; lane S, pooled fraction after S-Sepharose column chromatography; lanes 1–5, ConA-Sepharose column fractions 1–5; lane 6, pituitary-derived FSH. Each lane except H (100 ng) was loaded with 200 ng FSH.

underwent GVBD, although oocytes incubated without FSH stopped at the GV stage (Table 2). In addition, the expansion of cumulus cells was observed in oocytes treated with rec-pFSH and pituitary-derived FSH but not in oocytes cultured without FSH. The percentage of oocytes undergoing GVBD in the presence of 10 ng/ml rec-pFSH was almost the same as that in the presence of pituitary-derived FSH, except for fractions 1 and 6 from the ConA-Sepharose column, which showed slightly lower values. When the concentration of FSH was reduced to 1 ng/ml, the GVBD values for all the fractions decreased, but, except for that of fraction 3, they were higher than that for pituitary-derived FSH (22%). As shown in Fig. 7, GVBD occurred in the presence of rec-pFSH.

DISCUSSION

The expression of biologically active porcine FSH in insect cells carrying a baculovirus vector

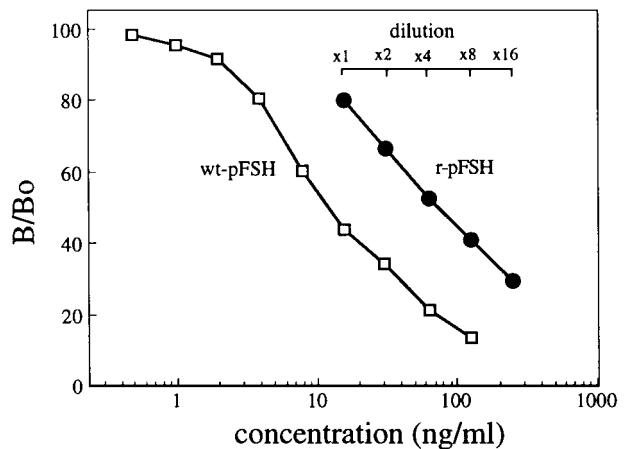


FIGURE 5. TR-FIA of FSH. Samples of crude rec-pFSH (r-pFSH) were diluted as indicated and their concentrations were measured. wt, wild-type.

and the purification of this FSH are described. The expression vector constructed with pFSH α - and β -subunits in a dual promoter vector resulted in the successful production and secretion of rec-pFSH *in vivo*, whereas the independent expression of α - and β -subunits followed by reconstitution *in vitro* did not yield adequate FSH. The antigenicity of rec-pFSH did not differ from that of pituitary-derived FSH. The rec-pFSHs produced by large-scale expression were separated as a predominantly homogeneous form by two column chromatographic steps. However, rec-pFSH was recovered in several fractions, possibly because of the heterogeneities of the carbohydrate moieties. Nevertheless, assays of progesterone production by porcine granulosa cells and GVBD of porcine oocytes revealed that all the rec-pFSH fractions expressed in insect cells possessed biological activity equivalent to the pituitary-derived hormone.

It is known that FSH alone can stimulate follicular growth and that LH activity is required to produce androgen, the precursor of estradiol and substrate for aromatase induced by FSH. In order to examine these sequential events during the reproductive cycle, a preparation of pure FSH is essential. Recombinant techniques guarantee production of FSH without concomitant LH, whereas conventional methods of purifying FSH by for example column chromatography cannot avoid some contamination with the cognate hormone LH. In contrast, the recombinant technique enables us not only to obtain FSH free from LH, but also to produce it on a large scale. The baculovirus vector recombinant system is known to yield high expression levels as well as providing bioactive forms of eukaryotic molecules (Lucknow &

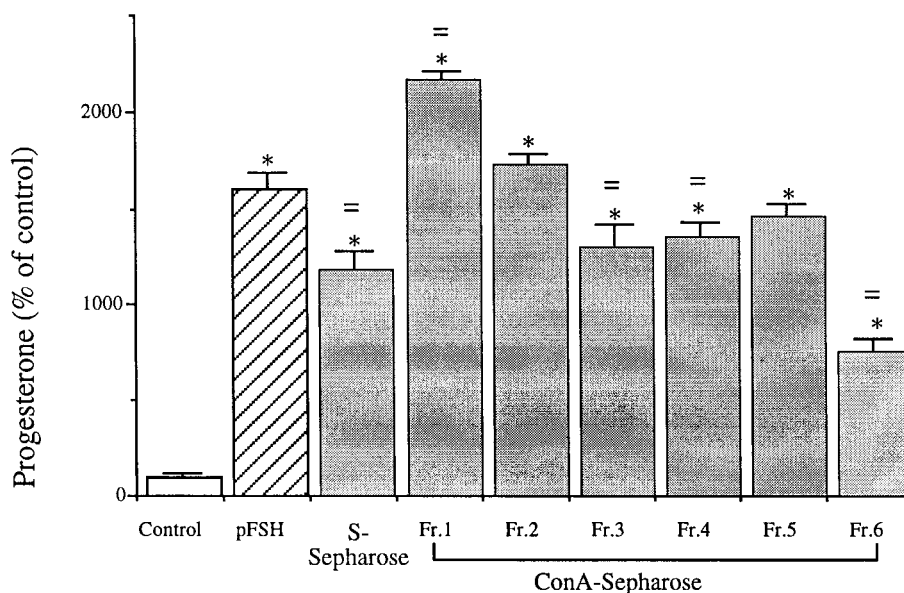


FIGURE 6. Effect of rec-pFSH on progesterone secretion by cultured porcine granulosa cells. Granulosa cells (3×10^5 /ml) were incubated for up to 72 h and when the medium was changed 48 h after the start of the culture, rec-pFSH or pFSH was added to a concentration of 10 ng/ml. The mean \pm s.d. concentration of progesterone in the control medium was 153.9 ± 11.9 ng/ml ($n=6$). Asterisks indicate that the effects on progesterone secretion were significant compared with the control value ($P<0.01$) and the symbol = indicates a significant difference between the effects of pFSH and each rec-pFSH fraction ($P<0.01$).

Summers 1988). In our present repeated expression study, the amount of rec-pFSH expressed ranged from 1 to 2 μ g/ml, which is significantly higher than that of ovine FSH (less than 100 ng/ml) in Chinese hamster ovary cells (Mountford *et al.* 1995). Nevertheless, this yield was not as high as expected, although it is superior by severalfold to previous yields from mammalian cell systems (Keene *et al.* 1989, De Boer & Mannaerts 1990, Hård *et al.* 1990, Mountford *et al.* 1995). It may be worthwhile reconstructing the upstream region of the transcription initiation site in future, as it was found to affect the expression level (Lucknow & Summers 1988).

Expression of recombinant proteins in insect cells carrying a baculovirus vector has been demonstrated to raise biologically active molecules that induce secretion by cells, targeting of the nucleus or cell surface, assembly of oligomeric complexes including disulphide bridges, proteolytic modification and phosphorylation (Lucknow & Summers 1988). However, insect cells can carry out N-glycosylation by adding a high molecular mass mannose-type polysaccharide, but have only limited ability to trim the terminal sugars to resemble the authentic proteins (Hsieh & Robbins 1984). FSH is

a member of the glycoprotein hormone family, as are the other pituitary hormones, LH and thyroid hormone stimulating hormone. They all have complex and branched carbohydrate chains, which play roles in several important biological functions, such as receptor binding (Galway *et al.* 1990), stability (Kaetzel *et al.* 1989), clearance (Vaitukaitis *et al.* 1976) and biological activity (Wilson *et al.* 1990). Isoelectric focusing of pituitary FSH showed that it was distributed at several positions in the low pH range 3.6–6.0, indicating that the heterogeneous FSH contents change during the estrous cycle (Blum & Gupta 1985, Blum *et al.* 1985), which is attributable primarily to the heterogeneous sialic acid contents of the sugar chain termini. Separation of rec-pFSH on S-Sepharose indicated that rec-pFSH is a basic protein that contains no sialic acid, and Western blotting indicated that the glycosylation of rec-pFSH is heterogeneous (Fig. 2), as did the fractionation by ConA-Sepharose column chromatography. The different glycosylation status of rec-pFSH from that of pituitary FSH, however, did not result in any loss of bioactivity *in vitro* (Fig. 6, Table 2). It is noteworthy that fraction 1 from ConA-Sepharose chromatography of rec-pFSH was most potent at

TABLE 2. Assays for GVBD and GV of porcine follicular oocyte cultures

Concentration of FSH (ng/ml)	Source of FSH	Number of oocytes examined	Number (%)	Number (%)
			of oocytes undergoing GVBD	of oocytes at GV stage
—	—	30	4 (13)	26 (87)
10	pFSH	29	23 (79)	6 (21)
	S-Sepharose	30	21 (70)	9 (30)
	ConA-Fr. 1	27	15 (56)	12 (44)
	ConA-Fr. 2	28	20 (71)	8 (29)
	ConA-Fr. 3	29	20 (69)	9 (31)
	ConA-Fr. 4	28	20 (71)	8 (29)
	ConA-Fr. 5	28	22 (79)	6 (21)
ConA-Fr. 6	30	17 (57)	13 (43)	
—	—	32	1 (3)	31 (97)
1	pFSH	32	7 (22)	25 (78)
	S-Sepharose	32	13 (41)	19 (59)
	ConA-Fr. 1	32	12 (38)	20 (62)
	ConA-Fr. 2	34	14 (41)	20 (59)
	ConA-Fr. 3	32	6 (19)	26 (81)
	ConA-Fr. 4	31	10 (32)	21 (68)
	ConA-Fr. 5	32	16 (50)	16 (50)
ConA-Fr. 6	30	16 (53)	14 (47)	

ConA-Fr. 1 etc. means fraction 1 from ConA-Sepharose chromatography. pFSH means pituitary-derived FSH.

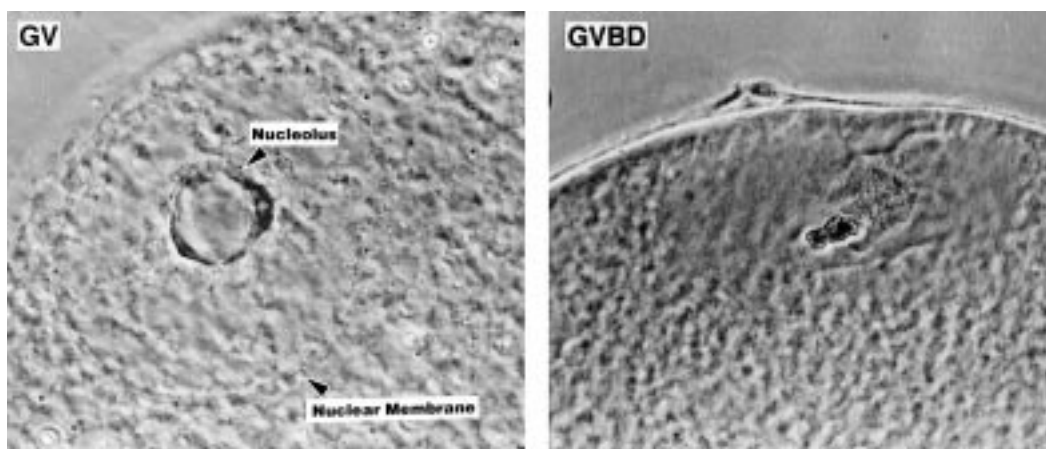


FIGURE 7. Micrograph of GVBD. Oocytes before (GV) and after (GVBD) treatment with 10 ng/ml rec-pFSH (fraction 2 from ConA-Sepharose chromatography) are shown. The nucleolus and nuclear membrane are indicated.

10 ng/ml in the assay of progesterone secretion (Fig. 6), whereas it showed the lowest value of GVBD at the same concentration (Table 2). An explanation of this discrepancy is that the carbohydrate chain bestows different potencies on the

different functions of FSH. This subject will be investigated further in the future.

It is known that removal of sialic acid from FSH reduces the half-life of the circulating hormone (Vaitukaitis *et al.* 1976), but does not change its

receptor-binding activity (Reichert & Bhalla 1974). Although it is essential to examine rec-pFSH activity *in vivo*, we observed that administration of rec-pFSH to rodents induced significant ovary and uterine augmentation in rats, follicular growth in female rats and ovulation in mice primed with human choriogonadotropin (Inaba *et al.* 1997a). We also observed testosterone production in mouse Leydig cells and estradiol-17 β production in immature rat Sertoli cells (Inaba *et al.* 1997a) as well as activation of tissue-type plasminogen activator in the cultured rat granulosa cells (Inaba *et al.* 1997b).

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