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Isolation and partial characterization of follistatin: A single-chain M_r 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone

(ovary/pituitary/luteinizing hormone/gonadotropins/porcine follicular fluid)

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ABSTRACT **A M_r 35,000 protein with follicle-stimulating hormone release-inhibitory activity was isolated from porcine ovarian follicular fluid by heparin-Sepharose affinity chromatography, gel filtration on Sephacryl S-200, and multiple steps of high-performance liquid chromatography. The isolated molecule is highly enriched in cysteines and is composed of a single polypeptide chain. In addition, it has no sequence homology with the previously characterized follicular fluid inhibins, which are heterodimeric proteins of M_r 32,000 with follicle-stimulating hormone release-inhibiting activity. This protein specifically inhibits the basal secretion of follicle-stimulating hormone, but not that of luteinizing hormone, in the rat anterior pituitary monolayer culture system with a half-maximal effective dose of 2.5–6.0 ng/ml. Another form of the molecule of M_r 32,000 present in much lower concentration in follicular fluid was also isolated. It may differ from the M_r 35,000 form in glycosylation or carboxyl-terminal truncation. We suggest that this compound be called "follistatin" to signify its structural difference from inhibin.**

Secretion of pituitary follicle-stimulating hormone (FSH) is principally regulated by the hypothalamic hypophysiotropic gonadotropin-releasing hormone and gonadal steroids (1). But for many years the existence of a nonsteroidal, water-soluble factor from the gonad that feeds back on the pituitary specifically to suppress the secretion of FSH has been advocated by many researchers (2); the name "inhibin" was coined in 1932 to describe such a factor (3). However, the search for the elusive inhibin did not succeed until 1985, when two closely related glycoproteins of apparent M_r 32,000, named inhibins A and B, were isolated from porcine ovarian follicular fluid (4–6). Subsequently, a similar M_r 32,000 inhibin was also isolated from bovine ovarian follicular fluid (7, 8). Each inhibin was found to be composed of a common M_r 18,000 glycosylated α subunit linked by interchain disulfide bond(s) to either a β_A subunit of M_r 14,700 to yield inhibin A or a β_B subunit of M_r 14,000 to form inhibin B; the two β subunits were closely related but distinct from each other (4). The common α subunit can exist also in a larger molecular form of M_r 44,000 and, in combination with the β subunit of inhibin A, can form an inhibin of M_r 56,000 (9).

Using partial amino acid sequence information, the messages encoding the α, β_A, and β_B subunits of porcine inhibin were cloned from an ovarian cDNA library (10) as were the α and β_A subunits of bovine inhibin (11). DNA sequence analyses showed that all of the subunits were initially synthesized as large precursor proteins with the mature subunits residing at the carboxyl-terminal portion of the precursors. Surprisingly, the two β subunits, besides being homologous to each other, were found to be structurally related to a homodimeric protein, transforming growth factor β, which has totally different biological activities (10). Moreover, in addition to the inhibins, two FSH-releasing proteins of apparent M_r 24,000, named activin A and activin AB, also were isolated from porcine follicular fluid (12–14). The former was characterized as a homodimer composed of two β subunits of inhibin A linked by disulfide bridge(s), while the latter is a heterodimer composed of the β subunits of inhibins A and B.

In our original work on the isolation of the two M_r 32,000 heterodimeric inhibins from porcine ovarian follicular fluid, we had noted a side fraction migrating at the beginning of the first reversed-phase high-performance liquid chromatogram ahead of the activins and inhibins, which also could specifically suppress pituitary FSH secretion in the inhibin bioassay. In this paper we present the purification and isolation of this FSH release-inhibiting protein named "follistatin," present in porcine ovarian follicular fluid, and show that it is a glycoprotein of M_r 35,000 composed of a single polypeptide chain.

MATERIALS AND METHODS

Bioassay. Throughout the purification procedure, the FSH release-inhibiting activity was monitored by an *in vitro* bioassay using immature female rat anterior pituitary monolayer culture as in the isolation of the inhibins (4).

Reversed-Phase HPLC. The reversed-phase HPLC system used for the purification of the follistatin consisted of a Beckman series 322 gradient liquid chromatography system equipped with a Spectroflow 757 UV detector (Kratos, Ramsey, NJ), a Soltec 220 recorder (Soltec, Sun Valley, CA), and a Redirac 2112 fraction collector (LKB). Two reversed-phase HPLC solvent systems were used. In the triethylammonium phosphate (Et_3NHP_i) system, solvent A consists of 0.25 M Et_3NHP_6 (pH 3); solvent B is 80% (vol/vol) acetonitrile in solvent A. In the CF_3COOH system, solvent A contains 1 ml of CF_3COOH in 999 ml of water; solvent B is 1 ml of CF_3COOH in 199 ml of water and 800 ml of acetonitrile. Other reversed-phase HPLC conditions are given in the legends to Figs. 1 and 2.

Anion-Exchange HPLC. The anion-exchange HPLC system used for the purification consisted of a Beckman series 332 gradient liquid chromatography system equipped with a Beckman 160 UV detector, a Soltec 1242 recorder, and a Redirac 2112 fraction collector. The solvent system used for the anion-exchange HPLC consisted of buffer A, which is

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Abbreviations: FSH, follicle-stimulating hormone; Et_3NHP_i, triethylammonium phosphate.

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0.01 M Na_2HPO_4 (pH 6.5), and buffer B, which is 1 M NaCl in buffer A.

Initial Purification. The starting material for the purification of the follistatin was a side fraction derived from the purification of the porcine ovarian inhibins and activins (4, 12, 14). Briefly, porcine follicular fluid was processed batchwise through a heparin-Sepharose affinity chromatography column, and the adsorbed protein was further size-fractionated on a Sephacryl S-200 column in acidic medium. Fractions containing the M_r 32,000 inhibins and FSH-releasing activities from the column (fractions 35–43 in figure 1a of ref. 4) were pooled and lyophilized. The lyophilized material (≈40 mg) was dissolved in 40 ml of 0.2 M acetic acid and filtered through a Millex-HA 0.45-μm filter (Millipore). The filtrate was applied directly to a 10 × 250 mm Vydac 5-μm C_4 column (The Separation Group, Hesperia, CA). After all of the filtrate had been loaded, the column was washed with solvent A until the UV absorption reached baseline. The inhibins and activins were separated with a linear gradient of 27–36% acetonitrile in the Et_3NHP_i solvent system in 90 min at a flow of 3 ml/min. Three zones of FSH release-inhibiting activity corresponding to inhibins A and B and follistatin were detected, as were two zones of FSH-releasing activity as shown in Fig. 1*a*. Inhibins A and B, as well as the FSH-releasing substances (activin A and activin AB) have been purified to homogeneity and their primary structures characterized as reported (4, 10, 12, 14). The remaining FSH release-inhibiting zone migrating ahead of the activins and inhibins on the HPLC column was saved for the present work.

$NaDodSO_4$/PAGE. The follistatin isolated from the final reversed-phase HPLC purification was analyzed in a 1-mm thick, 10% acrylamide gel by the method of Laemmli (15). The protein band was revealed by Coomassie blue reagent (Bio-Rad). The following molecular weight standards were

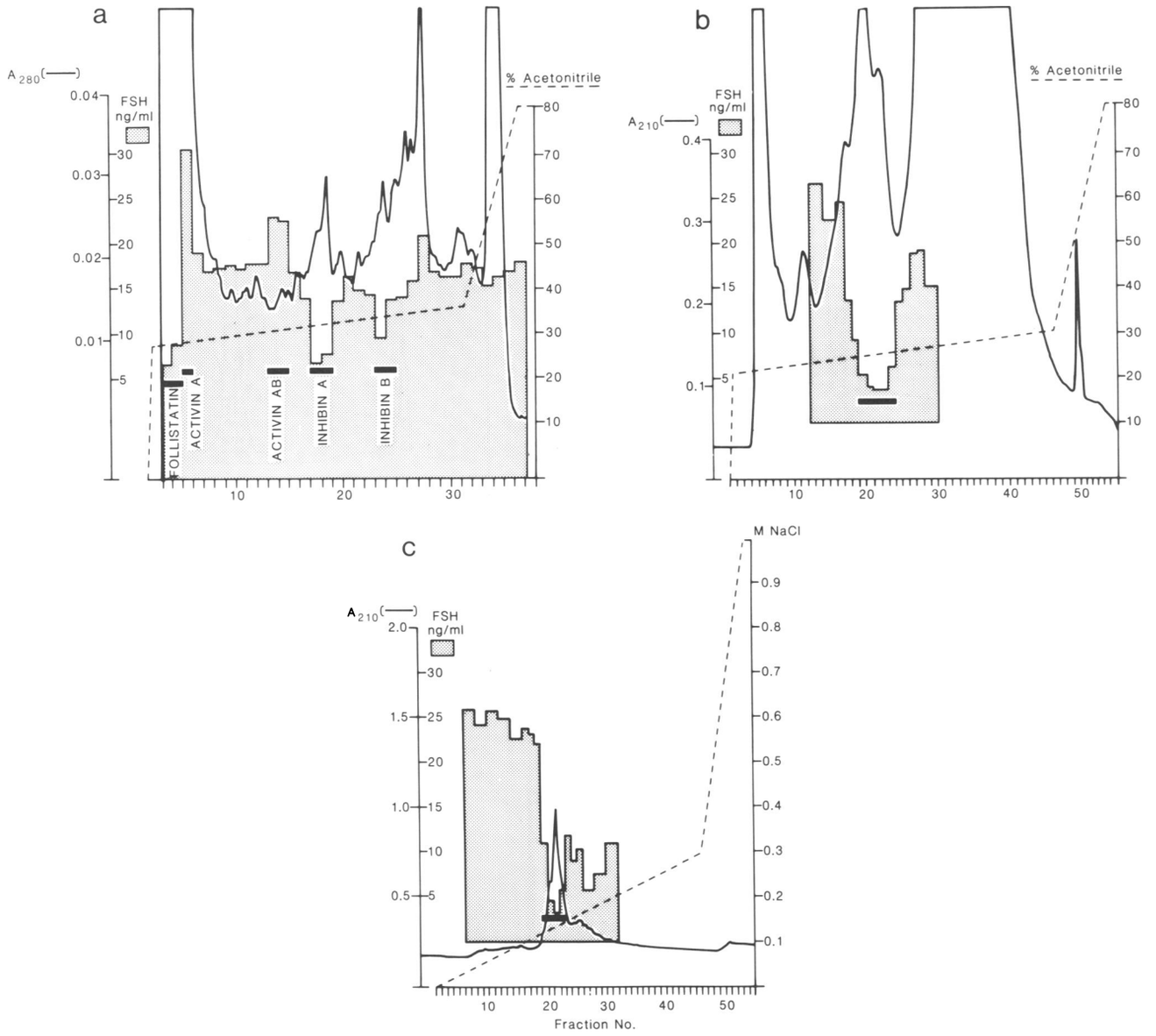

FIG. 1. (*a*) Reversed-phase HPLC purification of the FSH release-inhibiting as well as FSH-releasing proteins recovered from gel filtration. Three zones of FSH release-inhibiting activity (inhibins A, B, and follistatin) and two zones of FSH-releasing activity (activins A and AB), indicated by the solid bars, were recovered. (*b*) Reversed-phase HPLC purification of follistatin. Active fractions 3 and 4 in *a* were pooled and, after dilution with 0.2 M acetic acid, applied directly onto a Vydac C_4 column and eluted with the indicated gradient of acetonitrile in the CF_3COOH system at 3 ml/min. (*c*) The active material (denoted by solid bar in *b*) was pooled and, after removal of the acetonitrile and adjustment to pH 6.5, was applied to a Spherogel-TSK DEAE-5PW column. The activity was separated by the indicated gradient of NaCl in sodium phosphate buffer.

used to calibrate the gel: bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), and α chymotrypsinogen (M_r 25,700). The purified protein was analyzed under nonreducing and reducing conditions as before (4).

Amino Acid Analyses and Microsequencing. Amino acid analyses and amino-terminal sequence determination of follistatin were performed as described (16, 17).

Concanavalin A-Sepharose 4B Affinity Chromatography. Approximately 1 μg of the purified M_r 35,000 follistatin was iodinated with lactoperoxidase (Calbiochem), and the labeled protein was purified on a 0.7 × 50 cm Sephadex G-50 column with 0.01 M phosphate buffer (pH 7.5). The labeled follistatin was loaded onto a 1-ml concanavalin A-coupled Sepharose 4B column (Sigma) and washed successively with the loading buffer, 0.2 M and 1.0 M methyl α-D-mannopyranoside in loading buffer, and 0.1 M acetic acid as described (18).

RESULTS

For the purification of follistatin, fractions 3 and 4 in Fig. 1*a*, saved from the previous purification of the inhibins and activins, were pooled, mixed with an equal volume of 0.2 M acetic acid, and applied directly onto a 10 × 250 mm Vydac 5-μm C_4 column as before (4). The active material was eluted from the column with a linear gradient of 21–30% acetonitrile in the CF_3COOH system in 90 min at a flow of 3 ml/min as shown in Fig. 1*b*. Fractions with FSH release-inhibiting activity (fractions 19–23) detected by the bioassay were pooled and, after the acetonitrile was removed in a Speed-Vac concentrator (Savant) and the pH was adjusted to 6.5 with 0.1 M ammonium hydroxide, were pumped directly onto a 7.5 × 75 mm Spherogel-TSK 10-μm DEAE-5PW column (Toyo Soda, Tokyo). After loading, the column was washed with buffer A until the UV absorption reached baseline. The follistatin activity was separated with a linear gradient of 0–0.3 M NaCl in 90 min at a flow rate of 1 ml/min as shown in Fig. 1*c*. The active material eluted in fractions 19–22 was pooled and, after dilution to 4 times its original volume with 0.2 M acetic acid, was further purified on a 10 × 250 mm Vydac 5-μm C_4 column and eluted with a linear gradient of 21–30% acetonitrile in the CF_3COOH solvent system in 90

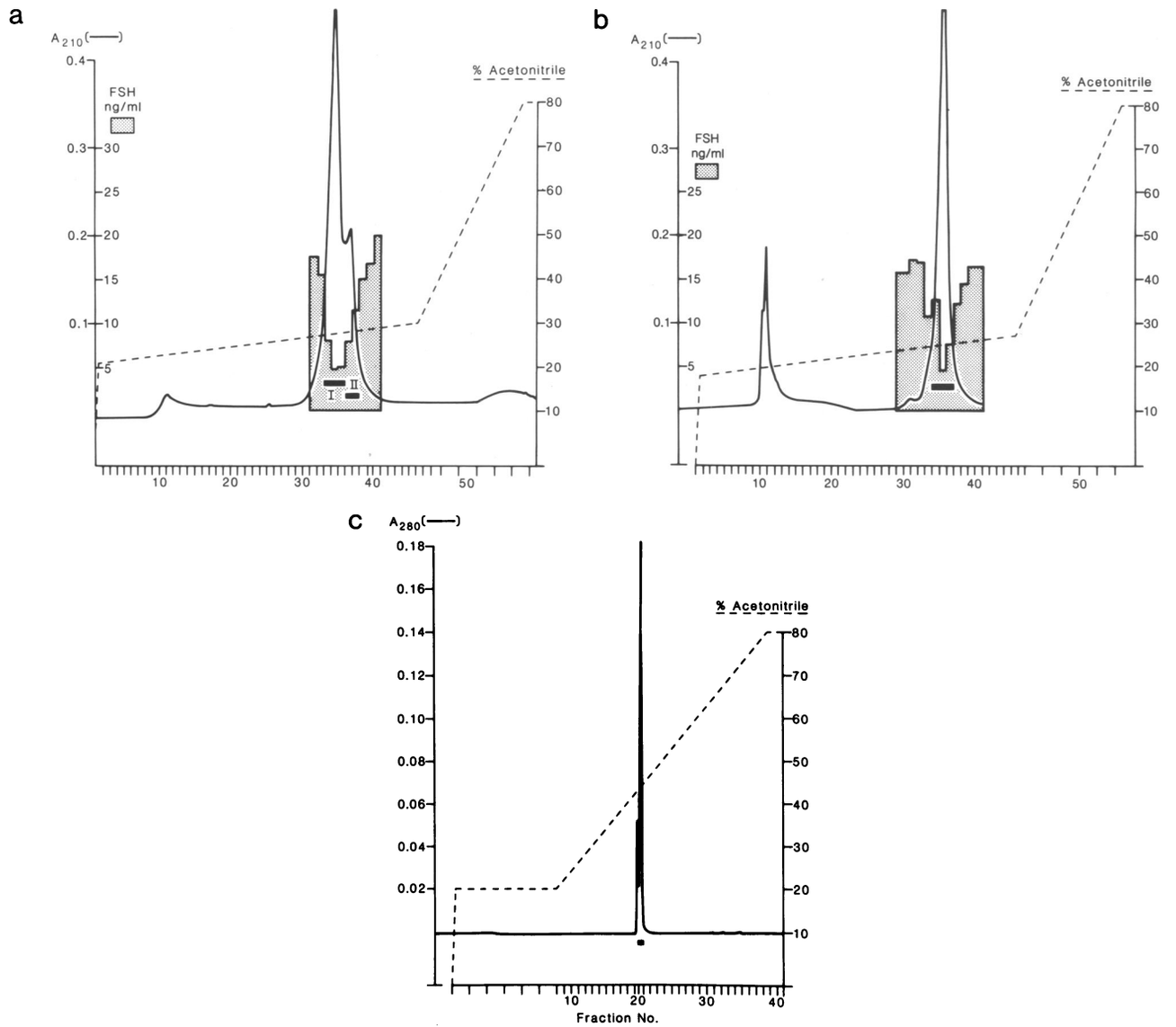

FIG. 2. Reversed-phase HPLC purification of follistatin. (*a*) Active fractions (solid bar in Fig. 1*c*) were pooled and after dilution were applied directly onto a Vydac C_4 column and eluted with the indicated gradient of acetonitrile in the CF_3COOH system at 1 ml/min. (*b*) The active material (denoted by solid bar I in *a*) was pooled, diluted, and chromatographed on a Vydac phenyl column with the indicated gradient of acetonitrile in the Et_3NHP_i system at 1 ml/min. (*c*) The material denoted by the solid bar in *b* was pooled and concentrated by chromatography on an Aquapore RP-300 column with the indicated gradient of acetonitrile in the CF_3COOH system at 0.5 ml/min.

min at a flow of 1 ml/min as shown in Fig. 2*a*. The active material eluted in fractions 33–35 (peak I) was pooled and diluted to twice its original volume and rechromatographed on a 10 × 250 mm Vydac 5-μm phenyl column with a linear gradient of 18–27% acetonitrile in the Et_3NHP_i system in 90 min at a flow rate of 1 ml/min as shown in Fig. 2*b*. Finally, the active material in fractions 34–36 was pooled and, after dilution with 0.2 M acetic acid, was concentrated by chromatography on a 4.6 × 250 mm Aquapore 10-μm RP-300 column with a linear gradient of 20–80% acetonitrile in the CF_3COOH system in 60 min at a flow rate of 0.5 ml/min as shown in Fig. 2*c*. Altogether, ≈400 μg of follistatin was isolated from 18 liters of follicular fluid.

The purified protein showed half-maximal inhibition of FSH release at a concentration of 2.5–6.0 ng/ml (0.07–0.17 nM). It had no effect on the secretion of luteinizing hormone, growth hormone, prolactin, or thyroid-stimulating hormone.

Amino acid analyses showed that follistatin, like inhibins A and B, is highly enriched in cysteines (Table 1). On $NaDodSO_4$/PAGE under nonreducing conditions, follistatin showed a single band migrating at an apparent M_r of 35,000 as shown in Fig. 3*a*. In contrast to inhibins A and B, follistatin exhibited a single band that migrated at an apparent M_r of 42,000 under reducing conditions (Fig. 3*b*), indicating that it is composed of a single polypeptide chain. Microsequence analyses of follistatin revealed its amino terminus to be Gly-Asn-Cys-Trp-Leu-Arg-Gln-Ala. In addition, 63% of the labeled follistatin was retained on the concanavalin A-Sepharose 4B affinity column, which could be displaced with 0.2 M methyl α-D-mannopyranoside, indicating that follistatin is glycosylated.

Using similar reversed-phase HPLC conditions (data not shown), a M_r 32,000 form (Fig. 4*a*) of follistatin with an identical amino-terminal sequence as that of the M_r 35,000 follistatin was isolated from fractions 36 and 37 of Fig. 2*a* (peak II). Its amino acid composition is related to the M_r 35,000 form (Table 1), and upon reduction, this protein migrated as a single band at an apparent M_r of 40,000 on $NaDodSO_4$/PAGE (Fig. 4*b*).

Table 1. Amino acid composition of purified follistatin from porcine follicular fluid

Amino acid	M_r 35,000 form	M_r 32,000 form
Asx	34.1 ± 0.2	28.4 ± 0.0
Thr	17.7 ± 0.3	15.9 ± 0.0
Ser	25.6 ± 0.2	22.2 ± 0.2
Glx	36.7 ± 0.1	31.1 ± 0.1
Gly	23.3 ± 0.2	24.5 ± 0.0
Ala	17.1 ± 0.2	15.6 ± 0.0
Val	15.5 ± 0.3	14.0 ± 0.0
Met	3.2 ± 0.1	3.4 ± 0.0
Ile	10.1 ± 0.1	7.5 ± 0.1
Leu	20.9 ± 0.2	19.1 ± 0.0
Tyr	9.5 ± 0.1	8.8 ± 0.1
Phe	5.0 ± 0.1	4.6 ± 0.0
His	2.2 ± 0.0	2.0 ± 0.0
Trp	5.8 ± 0.1	5.6 ± 0.1
Lys	26.2 ± 0.0	25.2 ± 0.1
Arg	12.7 ± 0.1	13.7 ± 0.1
Cys*	35.9 ± 0.2	33.5 ± 0.1
Pro	13.5 ± 0.3	13.0 ± 0.1

Values represent means ± SD of two analyses, normalized to a protein of 35,000 daltons for the M_r 35,000 form of follistatin and a protein of 32,000 daltons for the M_r 32,000 form.
*Determined as cysteic acid after performic acid oxidation.

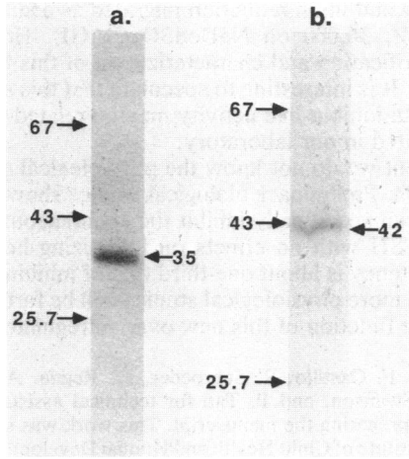

FIG. 3. $NaDodSO_4$/PAGE analysis of the purified M_r 35,000 follistatin under nonreducing conditions (*a*) and under reducing conditions (*b*). Positions of the molecular weight standards (shown × 10^{-3}) are indicated at the left.

DISCUSSION

Two novel proteins with FSH release-inhibiting activity were purified from porcine follicular fluid by using essentially the same procedures employed for the isolation of the inhibins and activins (4, 12, 14). The protein obtained in much higher yield has an apparent M_r of 35,000, whereas the other has an apparent M_r of 32,000. Both proteins were found to have an identical amino terminus and are composed of a single polypeptide chain. In view of the similar amino acid compositions and identical amino termini, the two forms of follistatin are probably related, and their difference in molecular size might be due to variation in glycosylation or carboxyl-terminal truncation.

Aside from the high content of cysteines, there is no relation in amino acid composition between follistatin and inhibins A and B. In addition, follistatin is a monomeric protein, whereas inhibins A and B and all higher molecular weight inhibins reported thus far are heterodimers (9, 19).

In a recent publication, Leversha *et al.* (20) reported the copurification of a M_r 29,000–30,000 single-chain protein together with inhibin A from ovine follicular fluid. They demonstrated that this protein also has FSH release-inhibit-

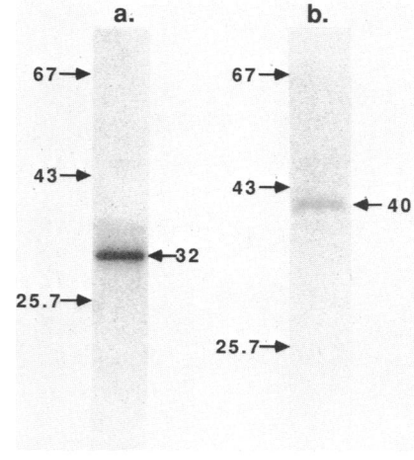

FIG. 4. $NaDodSO_4$/PAGE analysis of the purified M_r 32,000 follistatin under nonreducing conditions (*a*) and under reducing conditions (*b*). Positions of the molecular weight standards (shown × 10^{-3}) are indicated at the left.

ing activity and upon reduction migrated as a single band of apparent M_r 36,000 on NaDodSO$_4$/PAGE. However, no further purification and characterization of this protein was attempted. It is interesting to speculate that this single-chain protein with inhibin-like activity may be related to the follistatin isolated in our laboratory.

At present we do not know the physiological significance of follistatin. Preliminary biological studies showed that this molecule can specifically inhibit the spontaneous release of pituitary FSH with no effects on luteinizing hormone. Its relative potency is about one-third that of inhibin A (4). Undoubtedly, more physiological studies will be forthcoming to unravel the function of this new ovarian regulatory protein.

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