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PURIFICATION AND PARTIAL CHARACTERIZATION OF INHIBIN FROM PORCINE FOLLICULAR FLUID

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Inhibin, a protein of gonadal origin that suppresses the basal secretion of follicle stimulating hormone by anterior pituitary cells has been purified from porcine follicular fluid. Using several RP-HPLC steps and gel filtration under denaturing conditions, we obtained a fraction approximately ten thousand fold purified which showed one band on SDS PAGE and in the same experiment two bands after reduction (MW ca 14K and ca 18K) suggesting a molecular weight of 32K for inhibin. Edman degradation of isolated inhibin and carboxymethylated chain A indicated that the first 6 residues were H-Ser-Thr-Ala-Pro-Leu-Pro-; by subtraction, the first 3 residues of chain B could be deduced to be H-Gly-Leu-Glu-. EC_{50} was ca 0.3 ng/ml or 10 pM in our *in vitro* pituitary cell culture assay. Antibodies to residues 1-6 were raised which could immunoneutralize purified inhibin activity in an *in vitro* assay.

The existence of water soluble substances of gonadal origin that can selectively suppress FSH secretion has been suspected for more than fifty years (1). The quest to fully characterize these molecules referred to as inhibins (INs) has not succeeded to date (2-4). Recently, Robertson et al. (5) reported the isolation of IN from bovine follicular fluid; this entity was purified over 3,000 fold and had an apparent molecular weight of 56K. Under reducing conditions it could be dissociated into two subunits of MW 14K and 44K the N-termini of which were H-Tyr-Leu-Glu- and H-Asn-Ala-Val-. We reported on a purified fraction from pFF which had an apparent MW significantly smaller (6) using gel permeation chromatography. Miyamoto et al. (7) described the isolation of a 32K IN from pFF and, using SDS-PAGE, postulated the existence of

Abbreviations: IN, Inhibin; FSH, follicle stimulating hormone; SDS PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high performance liquid chromatography; FPLC, fast protein liquid chromatography; TFA, trifluoroacetic acid; MW, molecular weight; pFF, porcine follicular fluid; K, 1,000; NRS, normal rabbit serum; DMS, dimethylsulfide; TG, thioglycol; TEAP, triethyl ammonium phosphate; BDB, bisdiazotized benzidine; h(r)GRF, human (rat) growth hormone releasing factor.

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another three IN-like substances of MW 100K, 80K and 55K. We report here the purification and partial biochemical and biological characterization of a highly potent 32K heterologous dimeric form of IN.

MATERIALS AND METHODS

Starting material was non-charcoal treated pFF made available to us by the Contraceptive Development Branch of NICHD. It was thawed and centrifuged at 700 x g for 5 minutes in order to separate cell debris. The crude supernatant averaged 65 mg protein/milliliter follicular fluid.

In vitro IN assay was based upon the ability of IN to lower basal FSH secretion by cultured rat anterior pituitary cells (8,9). Cells were enzymatically dispersed and plated as previously described (8,10). After washing the cells 3 times, treatments were added and allowed to remain on the cells for 48-72 hrs at which time the fluids were removed and tested for FSH using an RIA kit provided by the National Hormone and Pituitary Program of NIADDK. Parallel dose response curves (3-5 points) gave relative potencies versus an internal standard of crude pFF.

Protein Precipitation

a) Organic solvent precipitation

The IN crude supernatant (3000 ml) was brought up to six liters with TEAP 5.0. n-Propanol (4 liters) was slowly added to the solution while stirring; a precipitate was generated and centrifuged at 1700 x g for ten minutes. The pellet was then washed three times with 40% propanol/60% TEAP 5.0 buffer and supernatants were pooled. The supernatant was then diluted to 10% n-propanol with deionized water, filtered through a 5 μm membrane, loaded onto a Waters preparative HPLC using a Vydac C_4 cartridge and eluted as described in Table 1, Step 1. Recovery of bioactivity was approximately 10%.

b) Ammonium Sulfate Precipitation

To the IN crude supernatant (6050 ml) was added dropwise over 2-3 hours an equal volume of Schwartz/Mann ultrapure 100% saturated ammonium sulfate adjusted to pH 7.8 with ammonium hydroxide. The mixture was centrifuged at 8200 x g for 30 min. Supernatant and precipitate were separated; the precipitate was resuspended in 10 mM Tris, pH 7.8, dialyzed using tubing with MW cut off ~1000 against 10 mM Tris pH 7.8 and tested for IN activity and was found to still contain some of the initial IN activity. The supernatant was ultrafiltered and concentrated using a Millipore Pellicon Cassette system with 5 square feet of filters of 10,000 MW cut off at a rate of 300 ml/min using a peristaltic pump with back pressure applied until filtration rate was 20 ml/min. The retentate was washed several times with 10mM Hepes and 0.05% dimethyl sulfide pH 7.5. The final retentate was collected (6,050 ml-eq in 2.24 l). Overall recovery of biological activity was approximately 13%. Protein concentration (determined by amino acid analysis after acid hydrolysis) was 10 mg/ml-eq. All filtrates from this ultrafiltration step were tested for IN bioactivity after dialysis against 10 mM Tris pH 7.8 using tubing with MW cut off ~1000 and were found inactive.

Preparative, semipreparative and analytical reversed phase HPLC was carried out as described in references 6, 11 and 12. Conditions are reported in Table 1. Steps 5 and 8 (Table 1) were gel permeation separations carried out under denaturing conditions on FPLC.

Large scale gel permeation was applied to the active zone generated by preparative HPLC after ammonium sulfate precipitation. Active fractions were pooled, lyophilized, and resuspended in column eluent. Column eluent was 6M guanidine·HCl, 0.1 M ammonium acetate, 0.05% dimethylsulfide pH 4.75 in 18 mΩ deionized H_2O. Eluent was 0.22 μm filtered and degassed before use. Large scale gel permeation chromatography was carried out using a Pharmacia Bioprocess column, 25.2 x 120 cm, packed with 104 cm Sepharose CL-6B, V_t = 52 l. Flow rate was 1100 ml/hr.

SDS polyacrylamide gel electrophoresis. IN fraction from step 9 (1 μg) was exposed to 2% sodium dodecyl sulfate (SDS) with and without 5% thioglycol at neutral pH in a boiling water bath for 2-3 minutes; both aliquots were subsequently applied to a slab gel and subjected to SDS-PAGE as described by Laemmli (13). Protein bands were located by silver staining.

Amino Acid Composition/Peptide Concentration was determined by amino acid analysis after acid hydrolysis in 4M methane sulfonic acid and 0.2% tryptamine at 110°C for 24 hrs (14). Because of the presence in some hydrolysates of contaminants, possibly amino sugars, which interferred with the quantitation of norleucine, we used 4 fluoro phenylalanine as internal standard instead of norleucine (J. Spiess, et al. unpublished). The amino acid analyzer was a Beckman Model 121M with ninhydrin post column derivatization.

Edman Degradation was achieved in an Applied Biosystem gas phase protein sequencer model 470A. PTH amino acids were determined with a Dupont PTH column (Woo, W. and Spiess, J. unpublished).

Peptide Synthesis. H-Ser-Thr-Ala-Pro-Leu-Pro-Gly-Tyr-OH [IN(1-6)-Gly-Tyr] was synthesized using the solid phase approach according to previously reported protocols (15). Purification was achieved using preparative HPLC (11). Amino acid composition gave the expected ratio; $[\alpha]_D = -118.5$ (c = 1 1% AcOH). Peptide was greater than 98% pure by analytical HPLC.

Peptide Antibodies were raised in rabbits using IN(1-6)-Gly-Tyr BDB coupled to human α-globulin as previously described for hGRF and rGRF (16). The antiserum from each bleeding was characterized with respect to titer and affinity for H-Ser-Thr-Ala-Pro-Leu-Pro-Gly-^{125}I-Tyr-OH. The antibody used for immunoneutralization bound 21% of this ligand at 1:3000 final dilution.

RESULTS AND DISCUSSION

Two similar schemes of purification were followed in order to obtain an IN preparation for biochemical and biological characterization. The first used propanol precipitation prior to preparative HPLC and is described in Table 1. The first three steps of the second scheme involved ammonium sulfate precipitation, preparative HPLC equivalent to step 1 of Table 1 and a preparative gel permeation chromatography step as described above; all other steps were similar to those reported in Table 1 (steps 3-9). When the supernatant of the ammonium sulfate precipitation step was used as starting material recovery of biological activity was ca 13% which partitioned in two zones: A) a fraction which was not retarded on preparative RP-HPLC under the usual conditions tested in our laboratories including Vydac C_{18} columns and 0.1% TFA or TEAP buffers (12) and acetonitrile, and which upon further purification by gel permeation FPLC under dissociating conditions exhibited IN activity associated with zones of >50K daltons; B) fractions which were retarded on RP-HPLC which included both FSH releasing (Vale and Rivier, in preparation) as well as FSH release inhibiting (IN) zones. Subsequent purification and analysis of the retarded IN fractions suggested at least 3

TABLE 1. PURIFICATION SCHEME I

	SAMPLE ml/ml-eq	COLUMN a) (cm)	SYSTEM A b)	SYSTEM B	FLOW (ml/min)	GRADIENT	RET.VOL. c) (ml)	PERCENT RECOVERY
1	121/1000 d)	5x30	TEAP 5.0	TEAP 5.0,60% PrOH	50	10%-45'-60%B	1600-1950	7.12
2	1000/350	5x30	TEAP 6.5	TEAP 6.5,60% PrOH	50	20%-45'-50%B	1030-1180	8.39
3	100/81	1x30	0.1% TFA	0.1% TFA,60% CH_3CN	2.5	35%-40'-95%B	60.8-63.9	4.7
4	10/1410	0.46x25	0.1% TFA	0.1% TFA,60% CH_3CN	0.7	10%(25')-20'-85%B	18.9-20.2	2.7
5	0.5/1400	1x60 e)			0.4		27.4-28.6 e)	
6	2.5/1350	0.46x25	0.1% TFA	0.1% TFA,60% CH_3CN	0.7	10%(25')-20'-85%B	16.28-16.98	2.7
7	2.5/1300	0.46x25	0.1% HFBA	0.1% HFBA,80% CH_3CN	0.7	30%(10')-20'-65%B	18.83-19.47	0.27
8	0.5/560	1x60 e)			0.4		25.6-26.4 e)	
9	5/560	0.46x25	0.1% TFA	0.1% TFA,80% CH_3CN	0.7	30%(5')-20'-65%B	14.28-15.54	0.33

a) RP-HPLC suports were end-capped Vydac C_4 silicas. Particle size was 5 µm except for steps 1 and 2, which used 20 µm silica. The column temperature was thermostated at 60°C for the first two steps, the remainder of the chromatography was done at room temperature.

b) TEAP 5.0 buffer (pH 5.0) was made as described in ref. 12. B was 60% n-propanol or CH_3CN diluted in buffer.

c) Retention volume of the active zone was measured in ml from the time of start of the gradient (does not include loading volume). DMS was added to all fractions (collected in polypropylene tubes) as they were eluted from the column (final concentration, 0.2-0.5%). Fractions were stored at -20°C. Aliquots for assays (0.1-1% of total fraction volume; never less than 5 µl) were measured with micropipettes and plastic tips and were transferred into polypropylene tubes containing bovine serum albumin (10 µl;10 mg/ml) and dried in a Savant rotary evaporator. Losses prior to assays and inconsistent results could be minimized in that manner.

d) When 1000 ml-eq were loaded (step 1), 3 similar runs were performed to process the total of 3 l-eq.

e) Gel permeation on Pharmacia FPLC system used two 10 µm Superose 12-B columns in tandem (V_t=50ml V_o=17ml), 0.22 µm filtered and degassed 6M Gdn·HCl, 0.1M NH_4OAc pH 4.75, 0.05% DMS in 18 mΩ deionized H_2O. Small aliquots for assay were removed and transferred into glass tubes containing 0.5 ml 10 mM Hepes + 0.1% BSA pH 7.5. Squares of dialysis tubing with MW cut off ca 1000 were secured over the tops with rubber bands. The tubes were inverted and dialyzed against 10 mM Hepes to remove salts which would interfere in the cell culture assays. Fractions were dried in a savant rotary evaporator and resuspended in cell culture assay medium.

forms of IN including 2 of similar size (17K Daltons on gel permeation FPLC or 32K Daltons based upon SDS-PAGE under reducing conditions) which could be separated by analytical RP-HPLC and a slightly larger form (35K on FPLC gel permeation).

We first concentrated on the purification of the smaller, less abundant IN forms which were retarded on preparative RP-HPLC. From Purification Scheme I we isolated 7 µg IN (single sharp peak on RP-HPLC) and obtained information on the size (Fig. 1), amino acid composition (not reported), biopotency (Fig. 2) and partial sequence. From Purification Scheme II we obtained 19 µg purified IN which was reduced, carboxymethylated, further purified by HPLC and used to confirm, and expand sequence data.

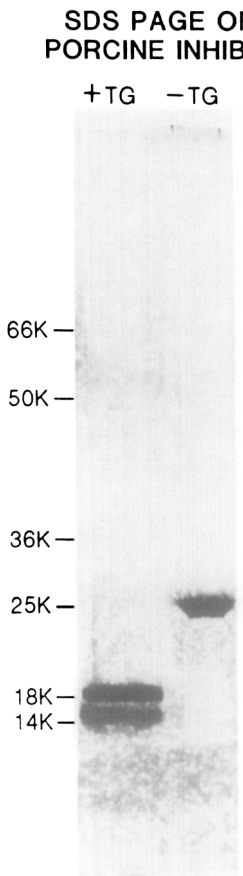

Fig. 1. Analytical SDS-PAGE of purified IN. The Gel (15% acrylamide) was silver stained. Column 1: 1 µg reduced sample (with 5% TG)
Column 2: 1 µg non-reduced sample

SDS-PAGE illustrated in Fig. 1 suggested that the isolated material was indeed highly purified (column 2 unreduced conditions) and that it consisted of 2 chains (column 1, reduced conditions). Estimated molecular weights were 14K and 18K for the two chains. Preliminary amino acid composition of the fraction from Purification Scheme I indicated that this molecule was particularly rich in Cys residues suggesting the possibility of internal as well as interchain disulfide bridging. IN is also proline rich which may favor a secondary structure with several turns. When also considering the large number of Cys residues one may suggest a globular stucture which would explain the low apparent MW on gel permeation and its MW of 25 K when non-reduced as compared to 32K, the sum of the MW of the two denatured chains on SDS-PAGE (Fig. 1). Partial sequence data on both chains was obtained from this preparation. The carboxymethylated RP-HPLC purified A chain from Purification Scheme II was recognized and analyzed with an Applied Bio System gas phase sequencer. The sequence of the first six residues could be ascertained and found to be H-Ser-Thr-Ala-Pro-Leu-Pro- confirming and extending the sequence information obtained with the first fraction. This sequence is in agreement with the four residues of the 18K A chain disclosed by Miyamoto in his oral presention at the 67th Endocrine Society Meeting (17) yet is not related to the N-terminal 3 amino acids of the 44K bovine IN chain reported by Robertson et al. (5). Residues one to three of the B Chain (H-Gly-Leu-Glu-) could be deduced by subtraction of the A chain residues from the sequence obtained using underivatized IN (from step 9, Table 1). Residues Leu^2-Glu^3 of the B chain are also consistent with Robertson et al.'s report (5) on bovine IN. Thus, it can be postulated that Robertson et al. (5), Miyamoto et al. (17) and we have characterized inhibin-like molecules sharing a closely related B chain while Robertson et al. may have isolated an IN with an N-terminally extended A chain.

Antibodies raised against a synthetic peptide related to IN A chain significantly immunoneutralized a given maximally active dose of the purified fraction from Scheme II in our <u>in vitro</u> dispersed rat pituitary cell culture

TABLE 2

IMMUNONEUTRALIZATION OF INHIBIN BY ANTI [IN(1-6)-Gly-Tyr]-BDB IN α GLOB

Treatment	ng FSH/ml ± SEM	Treatment	ng FSH/ml ± SEM
Control	63.4 ± 3.0	IN	23.4 ± 1.4*
NRS	55.9 ± 1.4	IN + NRS	23.1 ± 1.0*
Ab_1	65.5 ± 2.0	IN + Ab_1	43.7 ± 1.3*

IN: active fraction was an aliquot of the Scheme II batch tested at 100 eq/ml

Ab_1: Rb anti IN(1-6)-GlyTyr-BDB-h α Glob. #104

*: $p < .01$

assay (see Table 2). Normal rabbit serum or antibody by themselves had no effect on FSH secretion, nor did normal rabbit serum inhibit the IN response.

The results of these immunoneutralization experiments when taken together with the high potency (EC_{50} = 0.3 ng/ml or 10pM, Fig. 2) of our most purified IN preparations strongly suggests that the observed biological activity is associated with the partially characterized protein rather than with an as yet not recognized minor impurity.

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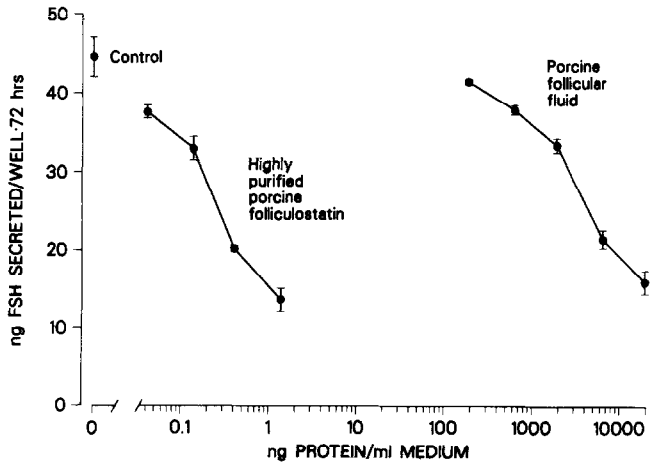

Fig. 2. Inhibition of basal FSH secretion in vitro by crude pFF and purified IN. Each point represents the mean ± SEM of three replicates.

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