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# ISOLATION OF INHIBIN FROM BOVINE FOLLICULAR FLUID

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Bovine follicular fluid was used as a source for the isolation of gonadal inhibin, the activity of which was monitored by the dose dependent suppression of the FSH content of cultured pituitary cells. The procedures presented result in over 3000-fold purification of the starting material and the purified inhibin has an apparent molecular weight of 56000. The purified inhibin can be dissociated under reducing conditions into two subunits with molecular weights of 44000 and 14000 daltons. 

Accumulating evidence indicates that a gonadal protein termed inhibin is involved in the control of FSH secretion(1,2). Ovarian follicular fluid is a rich source of inhibin activity and this material has been shown to be active in suppressing FSH *in vivo*(2). However ovarian inhibin has not been extensively purified to date. A procedure is presented here for the purification of inhibin from bFF. Using a combination of techniques (gel filtration, reversed phase HPLC and SDS-PAGE, a highly potent, biologically active fraction was isolated which was capable of suppressing FSH cell content in an *in vitro* bioassay.

## METHODS

Inhibin activity was determined using an *in vitro* bioassay based upon the dose-dependent suppression of FSH (but not luteinizing hormone) cell content in pituitary cell cultures utilizing a parallel line bioassay design (3). Evidence of cytotoxicity was assessed using a number of procedures(4,5). A

ABBREVIATIONS: FSH - follicle stimulating hormone; bFF - bovine follicular fluid; SDS-PAGE - sodium dodecyl sulphate-poly acrylamide gel electrophoresis.

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charcoal-treated bFF preparation was employed as standard which had been previously standardised against an ovine testicular lymph reference preparation with an assigned unitage of 1 unit/mg protein (3).

The amino acid sequence determination was performed using a gas/liquid phase model 470A sequencer (Applied Biosystems Inc.) and PTH-amino acids were identified by HPLC (6).

## RESULTS

Bovine follicular fluid, free of cystic fluid, was collected in the presence of aprotinin (10 U/ml) and phenylmethylsulphonyl fluoride (24 g/ml). It was fractionated on a column of Sephacryl S200 at pH 7.0 (Fig. 1a). Fractions with Kd <0.2 (MW >100000) were further chromatographed on a column of Sephadex G100 or G200 in 4M acetic acid. Inhibin activity was found in a fraction with values of Kd 0.6-0.8 (MW 20000-60000) which is largely

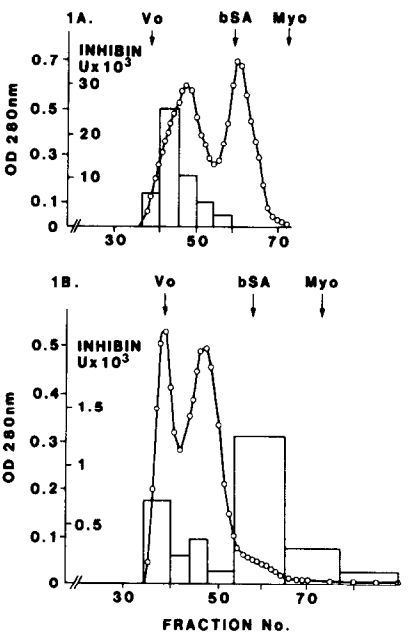


Fig. 1 a) Gel filtration of bovine follicular fluid on Sephacryl S200 (Pharmacia). bFF (10ml) was fractionated on a column of Sephacryl S200 (2.5 x 100 cm) in 0.05 ammonium acetate, pH7.0 at a flow rate of 40ml/hour at 4°C. b) Gel filtration of fractions 35-45 (Fig. 1A) on Sephadex G200. Following lyophilisation, fractions 35-45 (Fig. 1A) were extracted twice with 4M acetic acid (150 mg/7.5ml) for 1.5 h, centrifuged and the combined supernatants fractionated on a column of Sephadex G200 2.6 x 86.0 cm at a flow rate of 7ml/hr. Fractions 54-65 were pooled for further purification.
$V_o$ - void volume, bSA - bovine serum albumin (MW 67000), Myo-myoglobin (MW 18600)
Inhibin activity is presented in columns, protein in open circles.

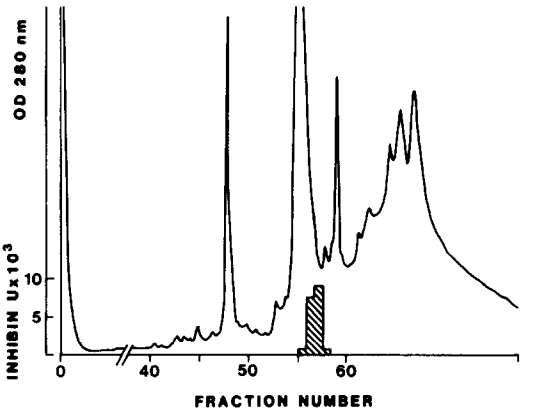


Fig. 2 Reversed phase HPLC of bFF. Fractions containing inhibin activity (tubes 54-65 Fig. 1B) were dissolved (8-10 mg/ml) in 4M acetic acid, centrifuged and the supernatant ( ∿ 100 µ l) applied to an Ultrapore RPSC column (Beckman, Berkley, CA.) and fractionated using a 30 min linear gradient of 0-50% acetonitrile in 0.1% trifluoracetic acid at 1 ml/min. Contents of individual fractions from up to ten experiments were pooled, acetonitrile removed under vacuum or nitrogen and lyophilised prior to assay. Inhibin activity was located in the hatched area.

separate from the bulk of protein (Fig. 1b). A similar reduction in the apparent molecular weight of ovine rete testes fluid inhibin under acid conditions has been reported (7). The inhibin-containing fraction was then lyophilised and fractionated by reversed phase HPLC (Fig. 2). The active fractions were then subjected to vertical SDS-PAGE using the Laemmli gel system (8) under non-reducing conditions. Protein bands were visualised using a light Coomassie Blue stain before sectioning of the gel. Inhibin activity, as determined by *in vitro* bioassay, was recovered from the appropriate gel slices by electrophoretic elution (9). Details of specific activities and recoveries at various stages of the purification procedure are presented in Table 1. The biological specific activity of the purified fraction was 212000 U/mg protein with a 3365 fold purification factor in approximately 1% yield (Table 1). The purified preparation gave an FSH inhibitory dose response in the *in vitro* bioassay which was parallel with the bFF standard (slope values (± SEM) 15.5 ± 0.23 and 17.6 ± 0.16 respectively where FSH levels are expressed as a percentage of controls). In addition the pituitary cell content of luteinizing hormone, thyroid stimulating hormone and prolactin remained unaffected as measured by radioimmunoassay.

TABLE 1 Purification of Inhibin from bovine follicular fluid[a]

| Preparation | Specific activity (U/mg)[b] | Total Units ($x10^3$) | % Recoveries at each stage | Purification factor |
|---|---|---|---|---|
| 1. Bovine follicular fluid | 63[c] (4) | 250 | 100 | 1 |
| 2. Gel filtration Sephacryl S200 (0.05 M ammonium acetate pH7.0) | 231[d] (7) | 223 | 89 | 3.7 |
| Sephadex G100 (4M acetic acid) | 944[e] (12) | 38.2 | 17 | 15 |
| 3. RP-HPLC | 10260[e] (12) | 15.1 | 40 | 163 |
| 4. Preparative SDS-PAGE | 212000[e] (2) | 2.1 | 14 | 3365 |

a. For 50 ml samples of bFF, column dimensions were scaled up (cf. Fig. 1): Sephacryl S-200, 5 x 100cm, flow rate 70ml/hr; Sephadex G100, 10 x 60cm, flow rate: 70ml/hr. Profiles similar to those in Fig. 1 were obtained.

b. Average of several determinations (number in brackets).

c. U/mg protein determined by Lowry (17).

d. U/mg dry weight.

e. U/mg protein determined by amino acid analysis.

Analytical SDS-PAGE of the final product under non-reducing conditions gave a single band with a molecular weight of 56000±1000 (mean ± SD, 5 preparations) (Fig. 3, Track: B). Other proteins of higher molecular weight eluted from the preparative gel had low to negligible (<3000U/mg) inhibin bioactivity (Fig. 3, Tracks C & D); proteins with lower molecular weight were inactive (Fig. 3, Track A).

Under reducing conditions (Fig. 3, Track E) two major bands with molecular weights of 44000 ± 3000 and 14000 ± 2000 (5 preparations) were observed in preparation of high inhibin specific activity. Following reduction and alkylation the two subunits were separated on SDS-PAGE and recovered from the gel by electroelution. The $NH_2$-terminal amino acid sequences of the 44000 and 14000 molecular weight forms were asn-ala-val and tyr-leu-glu respectively. These amino acids were also found on sequencing of the intact hormone. In some experiments, slicing of the preparative gels led

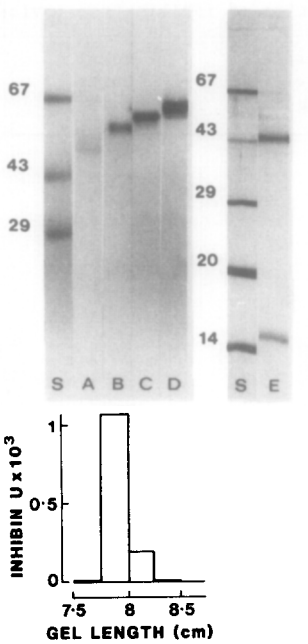


Fig. 3 Analytical SDS-polyacrylamide gel electrophoresis and inhibin bioactivity of fractions obtained by preparative SDS-PAGE. Inhibin-containing fractions (Fig. 2) were dissolved in 0.01 M phosphate-0.15 M NaCl (pH 7.0) and then made 1% in SDS (final protein concentration, 1 mg/ml). The samples were electrophoresed in 7.5% acrylamide slab gels (11). Following electrophoresis the gels were cut transversely into 0.25 cm slices in the region (50000-60000 MW) of inhibin migration, determined by preliminary experiments. Inhibin was recovered from gel slices by electrophoretic elution (11), and was localised predominantly to one slice (Track B); adjacent slices containing lower or higher molecular weight proteins showed low to undetectable inhibin activity (Tracks A, C, D). Analytical SDS-PAGE (10% gel) of a portion of these fractions are shown in the upper part of the figure. The inhibin containing band (Track B) showed a single protein band (MW 56000) which on reduction with mecaptoethanol (12.5% gel; Track E) dissociated into two components (MW 44000 and 14000).
Track S: molecular weight standards BSA, 67000; ovalbumin 43000; carbonic anhydrate, 29000; goose egg lysozyme 20300; chick egg lysozyme 14300.

to contamination of the inhibin preparation by traces of the higher molecular weight proteins of negligible biological activity. These contaminants did not dissociate under reducing conditions. The presence of the 44000 and 14000 molecular weight forms on reduction was characteristic of all fractions containing measurable inhibin activity.

The data indicate that bovine follicular fluid inhibin is a protein with a molecular weight of 56000 composed of two disulphide-linked polypeptide chains of apparent molecular weight 44000 and 14000.

## DISCUSSION

The molecular weight of the purified preparation is in a similar range to that reported (60000-70000) for active inhibin fractions from bFF (10). The inhibin isolated from bFF differs from substances with inhibin-like action isolated from bovine and human seminal plasma. The molecular weight of the latter substances are lower being 18000 for bovine seminal plasma inhibin (11) and 5000 and 14000 for human seminal plasma inhibin (12-14). However there is some doubt that substances termed seminal plasma inhibin are of testicular origin since activity is detected in seminal plasma following vasectomy (15,16) and the sequence of one purified preparation was identical to that of a prostatic sperm coating antigen (18). Additionally, inhibin from gonadal sources is more acidic (bFF inhibin (10); ovine rete testis fluid inhibin (7)) than seminal plasma inhibin.

The present results provide for the first time, a scheme for the purification of inhibin from a gonadal source, namely bovine follicular fluid and will enable further studies of the physiology of this substance.

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## REFERENCES

1. Baker, H.W.G., Bremner, W.J., Burger, H.G., de Kretser, D.M., Dulmanis, A., Eddie, L.W., Hudson, B., Keogh, E.J., Lee, V.W.K. and Rennie, G.C. *Recent Prog. Horm. Res.* (1976) **32**, 429-476.
2. Franchimont, P., Verstraelen-Proyard, J., Hazee-Hagelstein, M.T., Renard, Ch., Demoulin, A., Bourguignon, J.P. and Huston, J. *Vitam. Horm.* (1979) **37**, 243-302.
3. Scott, R.S., Burger, H.G. and Quigg, H. *Endocrinology* (1980) **107**, 1536-1542.
4. Robertson, D.M. Au, C.L. and de Kretser, D.M. *Mol. Cell. Endocrinol.* (1982) **26**, 119-127.
5. Scott, R.S., Burger, H.G., Quigg, H., Dobos, M., Robertson, D.M. and de Kretser, D.M. *Mol. Cell. Endocrinol.* (1982) **27**, 307-316.
6. Zimmerman, C.L., Appella, E. and Pisano, J.J. *Anal. Biochem.* (1977) **77**, 569-573.
7. Baker, H.W.G., Eddie, L.W., Higginson, R.E., Hudson, B. and Niall, H.D. *Ann. N. York Acad. Sci.* (1982) **383**, 329-340.
8. Laemmli, U.K. *Nature* (1970) **227**, 680-685.
9. Hunkapiller, M.W., Lujan, E., Ostrander, F. and Hood, L.E. *Methods Enzymol.* (1983) **91**, 227-236.
10. Jansen, E.H.J.M., Steenbergen, J., de Jong, F.W. and Van der Molen, H.J. *Mol. Cell. Endocrinol.* (1981) **21**, 109-117.

11. Sairam, M.R. Ranganathan, M.R., Ramasharma, K. and Lamothe, P. *Mol.Cell Endocrinol.* (1981) **22**, 231-250.
12. Seidah, N.G., Ramasharma, K., Sairam, M.R. and Chretien, M. *Febs Lett.* (1984) **167**, 98-102.
13. Ramasharma, K., Sairam, M.R. Seidah, N.G., Chretien, M., Manjunath, P., Schiller, P.W., Yamashiro, D. and Li, C.H. *Science* (1984) **223**, 1199-1202.
14. Sheth, A.R., Arabatti, N., Carlquist, M. and Jornvall, H. *Febs Lett.* (1984) **165**, 11-15.
15. Peek, J.C. and Watkins, W.B. *J. Reprod. Fert.* (1979) **57**, 281-285.
16. Scott, R.S. and Burger, H.G. *Nature* (1980) **288**, 246-247.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. *J. Biol. Chem.* (1951) **193**, 265-275.
18. Johansson, J., Sheth, A., Cederlund, E. and Jornvall, H. *Febs. Lett.* (1984) **176**, 21-26.