Vol. 125, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1984 Pages 307-314

# USE OF FAST PROTEIN LIQUID CHROMATOGRAPHY IN THE PURIFICATION OF INHIBIN FROM BOVINE FOLLICULAR FLUID

Simon van Dijk, Frank H. de Jong and Henk J. van der Molen

Department of Biochemistry, Division of Chemical Endocrinology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

Received September 13, 1984

Inhibin from bovine follicular fluid was partly purified using affinity chromatography on immobilized Procion Red 3B, gel filtration on Sephadex G-25 and ion-exchange chromatography on the fast protein liquid chromatography system. Inhibin was subsequently characterized using preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroelution. Biological activity was associated with a protein with an apparent molecular weight of approximately 65 kD. © 1984 Academic Press, Inc.

The existence of inhibin as a testicular hormone has been postulated by Mottram and Cramer in 1923 (1), but there is still confusion about the exact molecular nature and physiological significance of inhibin. It is generally accepted, however, that inhibin is a protein or polypeptide which specifically suppresses follitropin (FSH)-release by the pituitary gland without affecting lutropin (LH)-release (2). The apparent lack of success in the purification of inhibin from various sources as reported by a number of authors (3-8) may be partly attributed to the a-typical behaviour of inhibin when classical purification techniques were used (5). Another difficulty in comparing results from different authors is caused by the various bioassays used with their large differences in sensitivity and specificity (9). The introduction of the fast protein liquid chromatography system (FPLC), using improved ion-exchange resins with a high resolution, offered new possibilities for separating proteins while maintaining their

0006-291X/84 $1.50

biological activity. Here we report the partial purification of inhibin from bovine follicular fluid (bFF) using FPLC and electroelution of biologically active proteins from sodium dodecyl sulfate polyacrylamide (SDS-PAA) gels.

## MATERIALS AND METHODS

bFF was obtained at a local slaughterhouse by aspiration of fluid from medium and large ovarian follicles. Inhibin-like activity was determined using the inhibition of FSH-release by cultured anterior pituitary cells obtained from male rats (10). Potencies were expressed relative to a standard bFF preparation which was given the arbitrary potency of 1 using a computer programme for parallel line statistics (11).

bFF, adjusted to pH 7.0, was applied to a column of immobilized Procion Red 3B (Matrex Gel Red A; Amicon, Lexington, Mass.) and proteins were eluted stepwise using 50 mM Tris, pH 7.0, containing 100 mM KCl; 50 mM Tris, pH 7.5, containing 350 mM KCl and 0.5 M urea; 50 mM Tris, pH 7.0, containing 1.2 M KCl and 0.5 M urea, essentially as described by Jansen et al. (5). The last fraction, which contains inhibin activity, was desalted on Sephadex G-25 (Pharmacia, Uppsala, Sweden) in 20 mM Tris, pH 7.9, containing 0.5 M urea. After centrifugation at 200,000 x g for 1 hour) or filtration over 0.22 µm filters, the protein samples were further fractionated on a prepacked Mono Q HR 5/5 anion-exchange column (Pharmacia, Sweden) using a salt gradient from 0-1 M NaCl in 20 mM Tris, pH 7.9, in the presence of 0.5 or 4 M urea. The inhibin containing fractions were pooled and rechromatographed on Mono Q in 20 mM ethanolamine, pH 9.2 with 0.5 M urea using a salt gradient from 0-1 M NaCl. Protein concentrations were estimated by UV-absorption at 280 nm assuming $OD^{1\%}_{280} = 10$. Protein samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12) using a 8-15% acrylamide gradient. Gels were stained either with Coomassie Brilliant Blue R 250 or with silver nitrate using the method described by Wray et al. (13).

Before preparative SDS-PAGE on 3 mm thick gels, protein samples were incubated in SDS-PAGE sample buffer (12) for 30 minutes at 37°C. Proteins were recovered from gel by electroelution (14).

## RESULTS

A bioactive fraction of bFF was obtained after chromatography on immobilized Procion Red 3B and Sephadex G-25 (relative specific activity (RSA) 10-18, recovery 50-80%, n=5). This fraction was chromatographed on the Mono Q column at pH 7.9. Protein elution pattern and distribution of bioactivity are shown in fig. 1. Two main protein peaks were obtained: the first while loading the column in the presence of 0.15 M NaCl to prevent protein denaturation, and the second which was eluted with the actual

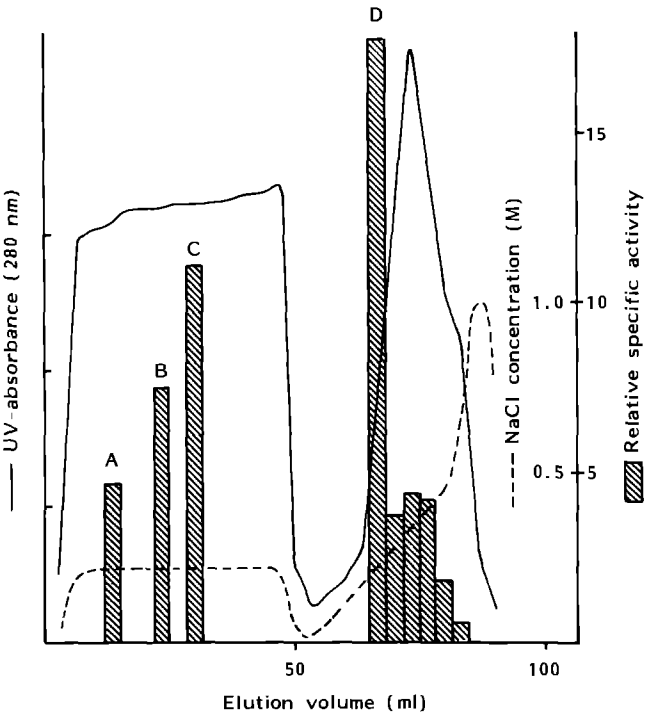



Figure 1
Protein-elution profile of an inhibin-containing fraction of bFF, which was obtained after chromatography on Matrex Gel Red A and Sephadex G-25, on Mono Q at pH 7.9 in the presence of 4 M urea.

salt gradient. Three fractions (fig. 1, A-C) from the first peak contained increasing amounts of bioactivity, whereas further bioactivity could be eluted in a sharp band at an NaCl-concentration between 0.18 and 0.25 M (fig. 1, D). When these fractions were analyzed on SDS-PAGE, the protein composition of fractions A-C appeared to be identical apart from an increase in the intensity of a 65 kD protein (fig. 2, arrow). In fraction D the 65 kD protein was more distinctly present, while the further protein composition of this fraction was different from the fractions A-C.

In subsequent experiments, where the desalted fraction from the immobilized Procion Red 3B column was brought onto the Mono Q column in the absence of 0.15 M NaCl, but in the presence of 0.5

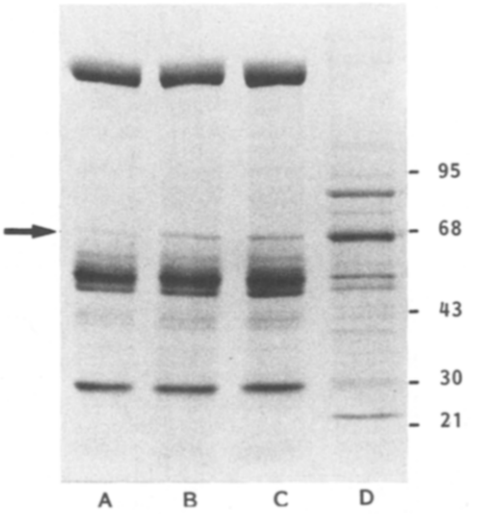
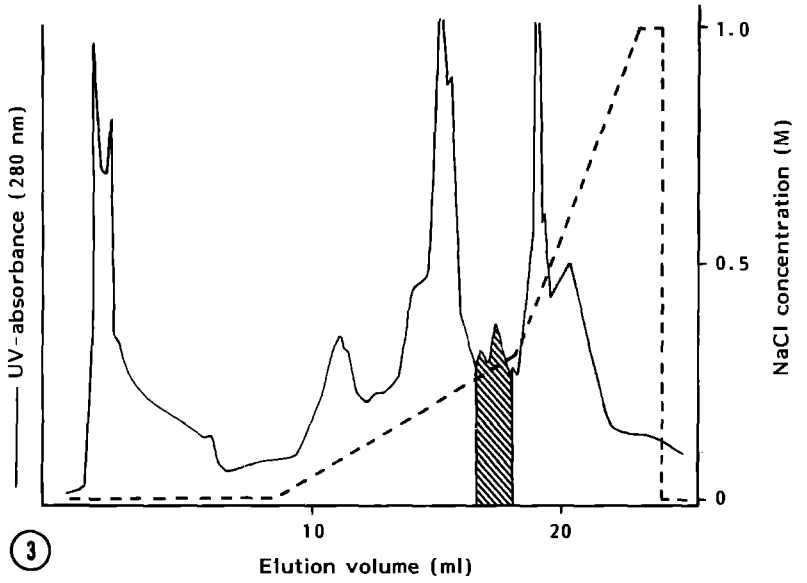



Figure 2
SDS-PAA-gel analysis of inhibin-containing fractions of bFF after Mono Q at pH 7.9 in the presence of 4 M urea (cf. Fig. 1); lanes A-C: fractions loaded in the presence of 0.15 M NaCl; lane D: fraction eluted with NaCl; position of molecular weight markers (in kD) as indicated. Arrow indicates protein, of which the relative amount is correlated with increase in bioactivity (see text).

M urea, all bioactivity was eluted in a single peak at a salt concentration between 0.1 and 0.2 M NaCl. RSA for these fractions varied between 25 and 45, with an overall recovery between 5 and 20% (n=5).

Rechromatography of these latter bioactive fractions on Mono Q at pH 9.2 resulted in the elution of inhibin-like activity at a NaCl-concentration between 0.26 and 0.32 M (fig. 3). The bioactive fraction contained a single 65 kD protein (fig. 4, lane B). Recovery and RSA of this fraction were 3% and 20, respectively.

Finally, when the proteins in the bioactive fraction from the Mono Q run at pH 7.9 were electrophoretically separated on a preparative SDS-PAA-gel, bioactivity (RSA>35) was again found to be associated with a protein with an apparent molecular



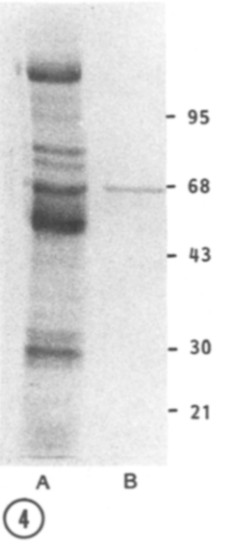




Figure 3
Protein-elution profile of an inhibin-containing fraction of bFF on Mono Q at pH 9.2 in the presence of 0.5 M urea after Matrex Gel Red A, Sephadex G-25 and Mono Q at pH 7.9.
Hatched area: bioactivity.

Figure 4
SDS-PAA-gel analysis of inhibin-containing fractions of bFF after Mono Q at pH 7.9 (lane A) and pH 9.2 (lane B); position of molecular weight markers (in kD) as indicated.

weight of 65 kD (fig. 5, lane D) after electroelution of the various gel slices.

## DISCUSSION

Studies on the purification of inhibin from different sources using conventional methods for protein purification have resulted in claims that inhibin-like activity is associated with polypeptides of molecular weight ranging from less than 1,500 D (15) to more than 100,000 D (4). Our experiments indicate that ion-exchange chromatography using FPLC can be used in the purification of a protein with an apparent molecular weight of 65 kD, which contains inhibin-like activity. The main advantage of FPLC in the purification of inhibin, when compared to e.g. HPLC as reported earlier (7, 16-18), is that biological activity is

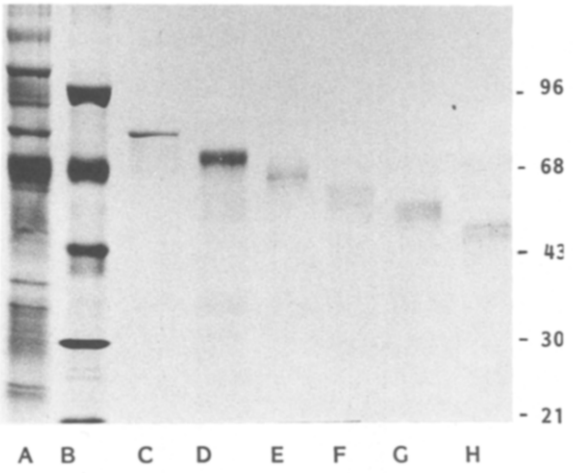



Figure 5
SDS-PAA-gel analysis of proteins electroeluted from preparative Gel Red A, Sephadex G-25 and Mono Q at pH 7.9 (lane A); lane B: marker proteins; lane D-H: electroeluted proteins; lane D contains bioactivity.

not lost during chromatography. Dialysis and lyophilization techniques, as employed after reversed phase-HPLC, may result in a large loss of bioactivity. Specific activities relative to bFF were between 25 and 45 after Mono Q at pH 7.9, with recoveries of activity of about 20%. The relatively low recovery during this step might be caused by denaturation and subsequent loss of protein during storage of desalted fractions, filtration or centrifugation.

The increase of bioactivity (fig. 1), associated with the increased presence of a 65 kD protein in the eluted fractions, (fig. 2) supports the apparent molecular weight of 65 kD for inhibin. After chromatography at pH 9.2 the RSA was increased 20-fold with a recovery of only 3%. This might reflect a pH-dependent inactivation of the biologically active molecule, apart from the factors mentioned above. Moreover, although only a single protein band appears on SDS-PAA gel (fig. 4, lane B), the chromatographic elution pattern suggests the presence of at

least two proteins in this fraction with the same apparent molecular weight, one of which could be inactive.

Finally, electroelution of proteins from an SDS-PAA gel containing a purified inhibin-preparation showed that bioactivity was associated again with a protein (or proteins) with a molecular weight of 65 kD. The observation that the RSA is much lower than expected for a purified protein might be due to denaturation during SDS-PAA gel electrophoresis and electroelution. No estimation of protein content of the electroeluted fraction could be made due to UV-absorption of the glycerol-containing buffer; therefore no data on actual RSA and recovery can be given.

The molecular weight of inhibin as reported here agrees with earlier reports on bovine ovarian inhibin (5, 19), while for inhibin from ovine ovarian follicular fluid and rat testicular extracts apparent molecular weights of 80 kD (7) and 50-60 kD (20) have been reported. For inhibin from human seminal plasma molecular weights of 14,000 D (16) and 5,000 D (17,18) have been reported recently. No indication for "low molecular weight"-inhibin was obtained in the experiments described here.
In conclusion, the experiments presented in this report show that it is possible, using dye-ligand affinity and ion-exchange chromatography, to isolate a 65 kD protein from bovine follicular fluid possessing inhibin-like activity.

ACKNOWLEDGEMENT

This work was financially supported by the Dutch Foundation for Medical Research (FUNGO).

REFERENCES

1. Mottram, J.C. and Cramer, W. (1923) Quart. J. Exp. Physiol. 13, 209-222.

2. De Jong, F.H. (1979) Mol. Cell. Endocrinol. 13, 1-10.
3. Baker, H.W.G., Eddie, L.W., Higginson, R.E., Hudson, B. and Niall, H.D. (1982) Ann. N.Y. Acad. Sci. 383, 329-342.
4. Cahoreau, C., Blanc, M.R., Dacheux, J.L., Pisselet, C. and Courot, M. (1979) J. Reprod. Fertil. Suppl. 26, 97-116.
5. Jansen, E.H.J.M., Steenbergen, J., De Jong, F.H. and Van der Molen, H.J. (1981) Mol. Cell. Endocrinol. 21, 109-117.
6. Ramasharma, K. and Sairam, M.R. (1982) Ann. N.Y. Acad. Sci., 383, 307-328.
7. Dobos, M., Burger, H.G., Hearn, M.T.W. and Morgan, F.J. (1983) Mol. Cell. Endocrinol. 31, 187-198.
8. De Jong, F.H., Steenbergen, J., Van Dijk, S. and Van der Molen, H.J. (1983) in: Hormones and Cell Regulation (Dumont, J.E., Nunez, J. and Denton, R.M., eds.), vol. 7, pp. 203-216, Elsevier, Amsterdam.
9. Baker, H.W.G., Eddie, L.W., Higginson, R.E., Hudson, B., Keogh, E.J. and Niall, H.D. (1981) in: Intragonadal Regulation of Reproduction (Franchimont, P. and Channing, C.P., eds.), pp. 193-228, Academic Press, London.
10. De Jong, F.H., Smith, S.D. and Van der Molen, H.J. (1979) J. Endocrinol. 80, 91-102.
11. Finney, D.J. (1964) in: Statistical Method in Biological Assay; Griffin, London.
12. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
13. Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. 118, 197-203.
14. Stralfors, P. and Belfrage, P. (1983) Anal. Biochem. 128, 7-10.
15. Vijayalakshmi, S., Bandivdekar, A.H., Joshi, L.R., Moodbidri, S.B. and Sheth, A.R. (1980) Arch. Androl. 5, 179-188.
16. Sheth, A.R., Arabatti, N., Carlquist, M. and Jörnvall, H. (1984) FEBS Lett. 165, 11-15.
17. Seidah, N.G., Ramasharma, K., Sairam, M.R. and Chretien, M. (1984) FEBS Lett. 167, 98-102.
18. Ramasharma, K., Sairam, M.R., Seidah, N.G., Chretien, M., Manjunath, P., Schiller, P.W., Yamashiro, D. and Li, C.H. (1984) Science (Washington, D.C.) 223, 1199-1202.
19. De Jong, F.H., Jansen, E.H.J.M., Hermans, W.P. and Van der Molen, H.J. (1982) in: Non-steroidal Regulators in Reproductive Biology and Medicine (Fujii, T., and Channing, C.P., eds.), pp. 73-82, Pergamon Press, Oxford.
20. Au, C.L., Robertson, D.M. and De Kretser, D.M. (1983) Endocrinology 112, 239-244.