*Journal of Chromatography*, 582 (1992) 71–76
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6529

# Rapid purification yielding highly active 17β-hydroxysteroid dehydrogenase: application of hydrophobic interaction and affinity fast protein liquid chromatography☆

Fu Yang, Dao-Wei Zhu, Jing-Yu Wang and Sheng-Xiang Lin☆☆

*MRC Group in Molecular Endocrinology, CHUL Research Center and Laval University, Ste-Foy, Quebec G1V 4G2 (Canada)*

(First received May 12th, 1992; revised manuscript received July 12th, 1992)

ABSTRACT

Homogeneous human placental 17β-hydroxysteroid dehydrogenase was obtained by a procedure consisting of two fast protein liquid chromatographic (FPLC) steps using Phenyl-Sepharose hydrophobic interaction and Blue-Sepharose affinity columns. In the first chromatography, the enzyme eluted only when an additional decrease in ionic strength was inserted after the ammonium sulphate concentration had reached zero, thus enhancing the separation. In the affinity chromatography, separation of contaminating proteins occurred at different stages of loading and washing. The specific elution of the enzyme by the co-factor $NADP^+$ is very efficient in obtaining a homogeneous preparation in high yield. The rapidity of FPLC was further increased by a maximum simplification of the intermediate steps, and the whole procedure lasted only two days. This preparation has a yield of more than 50% and a high specific activity, catalysing the formation of 7.9 μmol of estrone from estradiol per minute at pH 9.2 and 23°C. It has an apparent molecular mass of 35 000. This provides an efficient candidate for the purification of other membrane-associated proteins.

## INTRODUCTION

The development of protein structure studies requires highly homogeneous and active proteins. Since the early 1980s, fast protein liquid chromatography (FPLC) has been demonstrated to be very efficient in preparing high-quality proteins [1–5]. We have demonstrated the importance of the rapid purification to enzyme activity [6,7], which has been shown to be extremely important for crystal growth and other physicochemical studies of proteins [4,5,7–10].

A further need in the application of FPLC is the maximum simplification of the preparative steps. 17β-Hydroxysteroid dehydrogenase (17β-HSD, EC 1.1.1.62) is responsible for the conversion of most active androgens and estrogens, thus being of critical importance both in basic research and in the therapy of breast and prostate cancers [11–16]. In this work, we used the hydrophobicity of 17β-HSD from human placenta, and purified it efficiently first using a Phenyl-Sepharose hydrophobic interaction column. Combined with an affinity column (Blue-Sepharose CL-6B), we were able to purify 17β-HSD within two days, yielding highly active enzyme. With this method,

*Correspondence to:* Dr. S.-X. Lin, Laboratory of Molecular Endocrinology, CHUL Research Center, 2705 Laurier Boulevard, Ste-Foy, Quebec G1V 4G2, Canada.

☆ This project is supported by the Medical Research Council of Canada.

☆☆ Holder of a Chercheur-Boursier award from Le Fonds de la Recherche en Santé du Québec.

0378-4347/92/$05.00 

the dialysis process is completely avoided and the sample volume for loading is markedly reduced. This protocol may also be used for other membrane-associated proteins.

## EXPERIMENTAL

### *Materials*

$NAD^+$, $NADP^+$, glycerol, phenylmethylsulphonyl fluoride (PMSF) and protein standards for gel permation chromatography were obtained from Sigma (St. Louis, MO, USA). 17β-Estradiol, 2-mercaptoethanol (β-SH) and dithiothreitol (DTT) were purchased from Aldrich (Milwaukee, WI, USA). Protein markers (low molecular mass) for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were supplied by Bio-Rad Labs. (Richmond, CA, USA). Phenyl-Sepharose and Blue-Sepharose CL-6B columns were packed in the laboratory; the empty columns were obtained from Pharmacia LKB (Montreal, Canada). All reagents were of the best grade available. Centricon-30 and Centri-prep-30 concentrators were purchased from Amicon (Beverly, MA, USA).

### *17β-HSD assay*

The enzyme was assayed by measuring $NAD^+$ reduction monitored by the increase in absorption at 340 nm with a Beckman DU-70 spectrophotometer. The reaction mixture contained 25 μ*M* estradiol, 0.5 m*M* $NAD^-$ in 50 m*M* sodium hydrogencarbonate buffer (pH 9.2). An enzyme unit (U) is defined as the amount of enzyme protein that catalyses the reduction of 1 μmol of $NAD^+$ in 1 min under the above conditions. The absorbance change per minute is divided by 6.22 to yield the reduction of $NAD^+$ in m*M*/min, which is equivalent to the number of enzyme units per ml (m*M*/min = μmol/ml/min = U/ml). A blank value using the same reaction mixture but containing no estradiol was subtracted during assays throughout the purification. The reaction is as follows:

$$\text{estradiol} + NAD^+ \xrightleftharpoons{17\beta\text{-HSD}} \text{estrone} + NADH + H^+$$

The resulting homogeneous 17β-HSD activity was checked with a direct assay of the formation of [$^{14}$C]estrone from [$^{14}$C]estradiol under similar conditions. At different time intervals, aliquots were taken and the reaction was stopped with cold dichloromethane. Estradiol and estrone were extracted and separated by thin-layer chromatography, then cut and counted in a scintillation liquid, as described by Wahawsan and Gorell [17].

### *SDS-PAGE*

SDS-PAGE was carried out in the Laemmli discontinuous buffer system [18] using a Bio-Rad Mini-Protean II or Pharmacia PhastSystem. The gels were 12% polyacrylamide–N′,N′-bismethyleneacrylamide (37:1) and the samples contained 0.5% SDS and 10% glycerol. When doing immunoblotting, duplicate gels were run: one underwent Coomassie Brilliant Blue staining and the other was electroblotted with nitrocellulose. The blots were washed and treated with polyclonal anti-17β-HSD serum from rabbit and $^{125}$I-labelled anti-rabbit immunoglobulin G from goat, similarly to the method of St. John [19].

### *Protein concentration measurements*

We used the optical method of Warburg and Christian [20] and the measurements were made with a Beckman DU-70 spectrophotometer. For determination of homogeneous 17β-HSD, a microcuvette requiring 50 μl of sample was used.

### *Purification steps*

Unless mentioned otherwise, buffer A, which contained 40 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 0.2 m*M* DTT and 20% glycerol, was used as the principle buffer of low ionic strength after placenta fractionation. A Pharmacia FPLC system was used, consisting of two P3500 pumps, a UV-M monitor and an LCC-500 controller.

### *Placental homogenization and cell extract*

The placentae were refrigerated on ice immediately after delivery and treated within 45 min. The umbilical cord, blood clots and membranes were removed and the placentae were washed

with 0.9% sodium chloride Irrigation USP (neutralized). About 260 g of wet tissue were obtained per placenta, which was then cut into pieces and mixed with 520 ml of buffer containing 50 m*M* Tris–HCl (pH 7.2), 7 m*M* β-SH, 0.25 *M* sucrose, 5 m*M* EDTA and 1 m*M* PMSF. The tissue was then ground in a liquefier–blender (Osterizer) three times for 10 s at 1-min intervals. All the above treatments were carried out in a cold room (4°C). The homogenate was centrifuged for 30 min at 800 *g* in a Sorvall RC-3 centrifuge. The resulting supernatant was decanted and recentrifuged at 10 000 *g* in a Sorvall RC-5 centrifuge. The above supernatant was finally recentrifuged at 100 000 *g* for 60 min in a Beckman L5-65 ultracentrifuge. The centrifugations were also carried out at 4°C.

The supernatant after the ultracentrifugation was submitted to ammonium sulphate fractionation. During the fractionation, the sample was always kept neutral with ammonia solution. The enzyme fractions precipitating between 30 and 50% saturation were collected and dissolved in buffer A made 0.85 *M* in ammonium sulphate. The sample volume at this stage was about 250 ml per placenta.

## RESULTS

### *Hydrophobic interaction chromatography*

The sample obtained as described above was loaded directly on a Phenyl-Sepharose column (65 mm × 26 mm I.D.) and washed with buffer A made 0.6 *M* in ammonium sulphate. A large amount of contaminating proteins was removed during the loading. Then a reverse gradient of 0.6–0 *M* ammonium sulphate in buffer A (Fig. 1) was generated by the LCC-500 controller, which decreased slowly at the beginning to separate well many contaminating proteins. The decrease in ammonium sulphate concentration was fastest between 0.45 and 0.15 *M* during the gradient where few proteins were eluted. 17β-HSD activity was eluted only after the ammonium sulphate concentration had reached zero and when the column was further washed with a buffer similar to buffer A but containing less Tris–HCl (10 m*M*), yielding fractions containing up to 20% of 17β-HSD protein, as revealed by gel scanning using an Amersham PAS system and GL-1000 software (Fig. 3, lanes 1 and 2). Only the fractions with four times or more 17β-HSD specific activity than that in the applied sample, *i.e.*, fractions between 35 and 52 ml elution volume after 10 m*M* Tris buffer was applied, were collected. In this way, a 24-fold purification of the enzyme could be obtained with this column. In this step, a flow-rate of 10 ml/min was easily achieved with the joint functioning of two P3500 pumps. Here, 0.5 m*M* PMSF was added to the buffers.

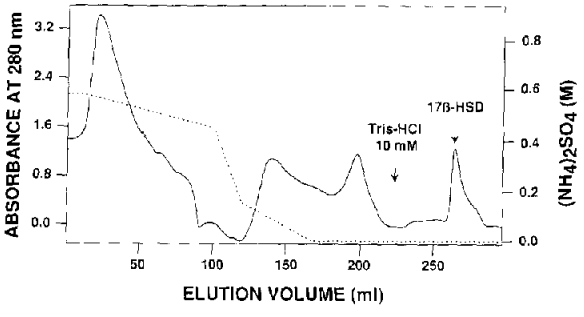


Fig. 1. Hydrophobic interaction chromatography. A 17β-HSD sample after ammonium sulphate fractionation was dissolved in the presence of 0.6 *M* $(NH_4)_2SO_4$ and loaded directly on a Phenyl-Sepharose column (65 mm × 26 mm I.D.). The sample was only eluted when the ammonium sulphate concentration reached zero and with a further decrease in Tris–HCl concentration yielding fractions containing up to 20% of 17β-HSD protein.

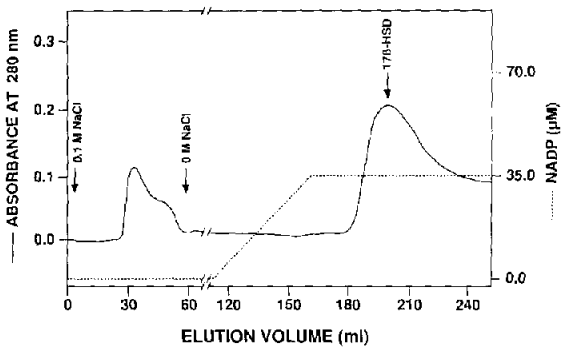


Fig. 2. Affinity chromatography. The Phenyl-Sepharose fractions of 17β-HSD were loaded directly on a Blue-Sepharose CL-6B column (100 mm × 16 mm I.D.). The column was first washed with buffer A of low NaCl content (0.1 *M*) and re-equilibrated with the same buffer ("0 *M* NaCl"), then eluted with $NADP^+$. Homogeneous 17β-HSD fractions eluted at 35 μ*M* $NADP^+$. The fraction size is 2 ml each.

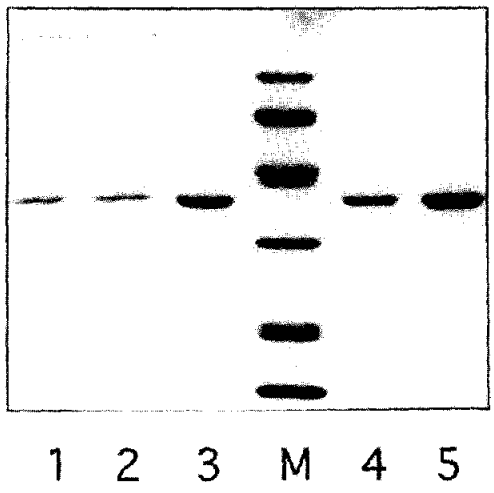


Fig. 3. SDS-PAGE of different 17β-HSD fractions. Lanes 1–2 = fractions eluted from Phenyl-Sepharose column. Lane M = protein markers; from top to bottom: phosphorylase *b* ($M_r$ 974 000), serum albumin ($M_r$ 662 000), ovalbumin ($M_r$ 45 000); bovine carbonic anhydrase ($M_r$ 31 000), main soybean trypsin inhibitor ($M_r$ 215 000) and lysozyme ($M_r$ 144 000). Lanes 3–5 = 17β-HSD fractions eluted from the Blue-Sepharose affinity chromatography.

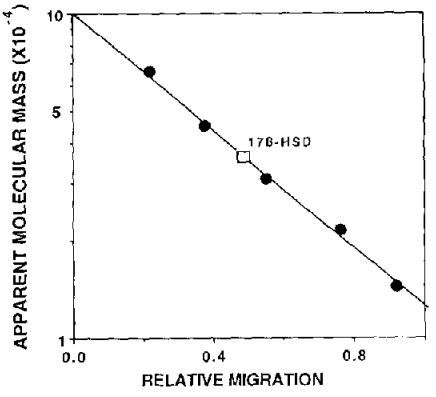


Fig. 4. Apparent molecular mass evaluation of 17β-HSD from SDS-PAGE. Protein markers (●) are of $M_r$ 66 000, 45 000, 31 000, 215 000 and 144 000 from top to bottom.

*Affinity chromatography*

The above-collected fractions (only about 27 ml) were applied directly to a Blue-Sepharose CL-6B column (100 mm × 16 mm I.D.) at a flow-rate of 3–4 ml/min. About 45–50% of the proteins passed directly through the column and possessed no 17β-HSD activity. The column was then washed with 0.1 *M* sodium chloride in buffer A, separating further about 10% of interfering proteins (over the total applied protein) and then re-equilibrated with buffer A. Stepwise washing of $NADP^+$ was carried out at a flow-rate of 2–3 ml ml/min and the enzyme activity eluted at 35 μ*M* $NADP^+$ in the same buffer (Fig. 2) where most fractions were homogeneous, as verified by SDS-PAGE (Fig. 3). A further increase in $NADP^+$ did not elute more 17β-HSD activity. The yield was about 80% in this step. PMSF (0.2 m*M*) was included in all buffers during the affinity chromatography.

*Concentration, specific activity and storage*

The homogeneous fractions resulting from the affinity column were collected, then concentrated and equilibrated in a buffer containing 40 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 0.4 m*M* DTT and 50% glycerol with Centricon (or Centri-

TABLE I

17β-HSD (PLACENTA) PURIFICATION BY TWO CHROMATOGRAPHIC STEPS

| Step | Total units | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Cell extract | 50 | 12238 | 0.0040 | 100 | 1 |
| 50% $(NH_4)_2SO_4$ | 40.6 | 971 | 0.0418 | 81 | 10.5 |
| Phenyl-Sepharose | 34.5 | 34.2 | 1.01 | 69 | 253 |
| Blue-Sepharose | 26.9 | 3.4 | 7.9 | 54 | 1980 |

prep) concentrators using centrifugation at 4500 *g*. This preparation has a high specific activity which can catalyse the oxidation of nearly 8 nmol of estradiol per minute and per milligram of enzyme protein [21–24]. When stored at −20°C in the above buffer in the presence of 50% glycerol, the activity can be maintained for many months.

*SDS-PAGE and immunoblotting*

Our preparation showed a unique band on SDS-PAGE (Fig. 3, lanes 3–5). This band hybridized with polyclonal anti-17β-HSD serum from rabbit in immunoblotting (data not shown). The plot of the relative migration *versus* the logarithm of the molecular mass of protein standard determined the apparent molecular mass of 17β-HSD as 35 000 (Fig. 4).

## DISCUSSION

Placental 17β-HSD is a hydrophobic and relative unstable enzyme, and therefore has been relatively difficult and long to prepare to high homogeneity by conventional chromatography [21–24]. Using FPLC, we have substantially simplified the chromatographic steps to the use of two columns. The conventional dialysis, often a time-consuming and destabilizing step for enzyme activity, is completely avoided and the sample volume is minimized by making use of Phenyl-Sepharose in FPLC. On this column, the enzyme is well separated because of its high hydrophobicity. When the enzyme is eluted at nearly the lowest ionic strength possible, it is ready to be loaded on a second column, avoiding any dialysis or dilution. In this way, the sample volumes are substantially reduced.

Because of the rapidity of FPLC and the simplicity of the procedure, we can obtain large amounts of 17β-HSD with high specific activity. This is probably due to the elimination of protein microheterogeneity from *in vivo* modifications, *e.g.*, oxidation–reduction effects or partial proteolysis [25,26] using the fast procedure.

Such a procedure may also be applied to many other hydrophobic and membrane-associated proteins. The laboratory-packed FPLC columns are fairly inexpensive and can provide quantitative and fast preparations because of their high flow-rates and large diameters. The column size mentioned is suitable for purification using two placentae, but larger-scale purification can be easily achieved by increasing the column size. For example, a Phenyl-Sepharose column of 130 mm × 26 mm I.D. combined with a Blue-Sepharose column of 100 mm × 26 mm I.D. afforded good separations for three or four placentae, although the elution profile was slightly changed, *i.e.*, 17β-HSD eluted from the first column slightly before the end of the reverse gradient, earlier than in the work described here. After the Phenyl-Sepharose chromatography more than a 200-fold purification was achieved.

Hydrophobic interaction chromatography is suitable for many membrane-associated proteins, *e.g.*, human placental 3β-hydroxysteroid dehydrogenase [27], while the second step can be alternated with another high-resolution FPLC column, such as ion-exchange (anionic or cationic, *e.g.*, Pharmacia Q or S series), affinity columns other than Blue-Sepharose or gel permeation. It is important to note that the present procedure yields very reproducible results. Application of the above procedure to several other hydrophobic proteins is in progress to develop a more general purification method for membrane-associated proteins whose biological importance has been increasingly recognized in recent years.

## ACKNOWLEDGEMENTS

The authors thank Dr. A. Rosler for his careful reading of the manuscript and Elaine Leclerc for its preparation.

## REFERENCES

1 F. Markey, *FEBS Lett.*, 167 (1984) 155.
2 M. Tam, A. Taylor and L. Giri, presented at the *4th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Baltimore, MD, 1984.*
3 S. X. Lin, J. P. Shi, X. D. Cheng and Y. L. Wang, *Biochemistry*, 27 (1988) 6343.
4 J. D. Orbell, L. W. Guddat, K. J. Machin and N. W. Isaacs, *Anal. Biochem.*, 170 (1988) 393.

5 A. E. Franke, D. E. Danley, F. S. Kaczmarek, S. J. Hawrylick, R. D. Gerard, S. E. Lee and F. Geoghegan, *Biochim. Biophys. Acta*, 1037 (1990) 16.
6 S. X. Lin, F. Yang, J. Z. Jin, A. Brisson, J. H. Liu and J. Lapointe, presented at *FASEB Meeting, Atlanta, GA, April 1991*.
7 S. X. Lin, A. Brisson, J. H. Liu, P. H. Roy and J. Lapointe, *Protein Expression Purif.*, 3 (1992) 41.
8 R. Giegé, B. Lorber, V. Mikol, D. Moras, M. Ruff, A. Thobald and J. C. Thierry, *Bull. Inst. Pasteur Paris, 86 (1988) 9*.
9 M. Ruff, A. Mitschler, J. Cavarelli, R. Giegé, V. Mikol, J. C. Thierry, B. Lorber and D. Moras, *J. Mol. Biol.*, 201 (1988) 235.
10 M. Ruff, S. Kirshnaswamy, M. Boeglin, A. Poterszman, A. Mitschler, A. Podjarny, B. Rees, J. C. Thierry and D. Moras, *Science*, 252 (1991) 1982.
11 E. E. Beaulieu, *C. R. Acad Sci.*, 251 (1960) 1421.
12 F. Labrie, A. Dupont, A. Bélanger, L. Cusan, Y. Lacourcière, G. Monfette, J. G. Laberge, J. Emond, A. T. A. Fazekas, J. P. Raynaud and J. P. Husson, *J. Clin. Invest. Med.*, 5 (1982) 267.
13 F. Labrie, A. Bélanger and A. Dupont, in V. T. De Vita, S. Hellman and S. A. Rosenberg (Editors), *Important Advances in Oncology*, J. B. Lippincott, Philadelphia, PA, 1985, p. 193.
14 J. M. Hall, M. K. Lee, B. Newman, J. E. Morton, L. A. Anderson, B. Hucy and M. C. King, *Science*, 250 (1990) 15841.
15 M. J. Reed, *J. Endocrinol.*, 129 (1991) 163.
16 M. J. Reed, A. Singh, M. W. Ghilchick, N. G. Coldham and A. Purohit, *J. Steroid Biochem. Mol. Biol.*, 39 (1991) 791.
17 R. Wahawsan and T. A. Gorell, *Steroids*, 36 (1980) 115.
18 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
19 T. P. St. John, in F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, T. G. Seidman, T. A. Smith and K. Struhl (Editors), *Current Protocols in Molecular Biology*, Wiley, New York, 1987, 6.7.1–6.7.4.
20 O. Warburg and W. Christian, *Biochem. J.*, 310 (1942) 384.
21 B. Descomps, J. C. Nicolas and A. Crastes de Paulet, *Bull. Soc. Chim. Biol.*, 50 (1968) 1681.
22 J. Jarabak, *Methods Enzymol.*, 15 (1969) 746.
23 S. Karavola, M. L. Baedecker and L. L. Engel, *J. Biol. Chem.*, 245 (1970) 4948.
24 G. L. Murdock, C. C. Chin and J. C. Warren, *Biochemistry*, 25 (1986) 641.
25 R. Giegé, in D. Moras, J. Drenth, B. Strandberg, D. Suck and K. Wilson (Editors), *Crystallography in Molecular Biology (NATO ASI Series A*, No. 126), Plenum Press, New York, 1987, p. 15.
26 R. Giegé, A. C. Dock, D. Kern, B. Lorbere, J. C. Thierry and D. Moras, *J. Crystall Growth*, 76 (1986) 554.
27 V. Luu-The, M. Takahashi and F. Labrie, in L. Castagnetta, S. d'Aquino, F. Labrie and H. Bradlow (Editors), *Steroid Formation, Degradation and Action in Peripheral Tissues*, Academy of Sciences, New York, 1989, pp. 386–388.