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Review

Chromatography of hydroxysteroid dehydrogenases

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Abstract

The structure–function study of hydroxysteroid dehydrogenases has stimulated the development of their chromatography, which in turn reveals more mechanisms of these enzymes. Due to the various membrane associations and mild hydrophobic nature of most of the enzymes studied up to now, hydrophobic interaction chromatography has played a crucial role in their purification, using media such as phenyl-Superose or Sepharose-PEG. At the same time, affinity chromatography, especially the dye-containing columns, proves very efficient for these dehydrogenases, as the latter utilizes adenylyl-containing cofactors. Elution by their specific ligand facilitates their purification. In this paper, the use of detergents in the purification of these enzymes is also reviewed. Hydroxysteroid dehydrogenase preparation is further improved by rapid purification which facilitates the elimination of protein microheterogeneity, caused in vitro by oxidation, reduction or partial proteolysis. This process was shown to increase the crystallizability of the enzymes [Lin et al., J. Cryst. Growth, 122 (1992) 242-245; Zhu et al., J. Mol. Biol., 234 (1993) 242-244]. The fast purification permitted a simpler procedure and better combination of various columns than conventional chromatography. This leads to even higher efficiency, yielding homogeneous and highly active preparations.

Keywords: Reviews; Hydroxysteroid dehydrogenases; Enzymes

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1. Introduction

Because of the importance of the hydroxysteroid dehydrogenases (HSDs) in endocrinology and oncology, their purification has been attempted by many laboratories to facilitate structure–function studies. For instance, the estrogenic form of 17β-HSD is responsible for the synthesis of the most potent estrogens which stimulate the proliferation of breast cancer cells. This enzyme thus becomes an important target in breast cancer therapy [1–4]. It has been purified partially or to apparent homogeneity since

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the 1950s by different laboratories [5–7], but relatively high specific activity has been achieved only in some reports [8,9]. Recent rapid purification methods have considerably increased the specific activity and homogeneity of the enzyme preparation [3,10].

3β-HSD catalyzes the essential steps in the biosynthesis of all classes of hormonal steroids, e.g., progesterone, glucocorticoids, mineral corticoids, androgens, and estrogens in gonadal and peripheral tissues [11–13]. The formation of these active steroids plays an important role in the growth of hormone-sensitive tumours. Considerable efforts have been made to purify 3β-HSD with membrane association, e.g. from human placental microsomes (Table 1), and the recent rapid purification resulted in a high specific activity of the enzyme preparation [Zhou et al., unpublished].

5α-Reductase (5α-R) catalyses the conversion of testosterone, 4-androstenedione, progesterone and other 4-ene-3-keto-steroids to the corresponding 5α-dihydro-3-keto-steroids. The great interest is its function. The best known conversion is from testosterone to the most potent androgen, dihydrotestosterone, which is responsible for the cell proliferation in prostate cancer. The existence of the enzyme was first discovered through the examination of patients with a genetic error in sexual differentiation that was ultimately shown to be caused by a deficiency of 5α-R activity. While knowledge of this deficiency is important for diagnostic purposes, the great interest is its function in stimulating the growth of prostate cancer. Thus it is a target for the development of drugs for the treatment of prostate tumors and benign prostatic hyperplasia.

3α-HSD and 3β-HSD have been purified (or partially purified) from human liver cytosol [14,15], porcine testicle cytosol [16], human prostate [17,18], human 3β-HSD/isomerase from placental microsomes ([11], Zhou et al., unpublished); 11β-HSD has been purified from human placental [19] and mouse liver microsomes [20]; 17α-HSD has been purified from intestinal *Eubacterium sp.*; 17β-HSD has been purified from human placenta [3], mouse prostate and seminal vesicles [21]; and 20α-HSD has been purified from a type of protozoan (*Tetrahymena*

Table 1
Some representative hydroxysteroid dehydrogenases

HSD type	Source	Detergent (for solubilization)	Detergent (for full enzyme activity)	Reference
3α-HSD	Human hyperplastic prostate	Na-cholate	Na-cholate	[17]
3β-HSD	Human hyperplastic prostate	CHAPS	CHAPS	[17]
3β-HSD	Human placental microsomes	Na-cholate Emulgen 913[a]		[11]
		Na-cholate Emulgen 913	β-OG decylmaltoside	Zhou et al., unpublished
		Na-cholate	NP-40	[33]
11β-HSD	Mouse liver microsomes	Emulgen 913[a]		[20]
17β-HSD	Human placental microsomes	Triton X-100		[3]
17α-HSD 17β-HSD	Equine placental microsomes	Na-cholate		[34]
5α-Reductase	Human prostate cytosol	Triton X-100 Digitonin MEGA 9 Tween 80 Tween 20 Lubrol PX	Lubrol PX	[35]

[a] Emulgen 913 is a non-ionic detergent from Kao Atlas, Tokyo.

pyriformis) [22], and rabbit ovary [23]. In this paper, we review the most commonly used chromatographic methods for the hydroxysteroid dehydrogenases and propose the employment of the fast purification technique. The purification of HSDs is discussed in the light of their structure–function relationship.

2. Hydrophobic interaction chromatography

The hydrophobic nature of the proteins, from mild to strong, has been observed in most of the hydroxysteroid dehydrogenases [24] which interact with the hydrophobic steroids. This hydrophobicity gives rise to many difficulties in the purification of these enzymes. However, by making appropriate use of this property, i.e. applying hydrophobic interaction chromatography, often excellent purification results are obtained. For example, the human estrogenic 17β-HSD is abundant in the soluble sub-cellular fraction of placenta, but with a low aqueous solubility of 2 to 3 mg/ml. The phenyl-Superose hydrophobic interaction chromatography played an important role in the purification of the labile 17β-HSD, a preparation of highly active enzyme with a crystal diffraction of 2.2 Å [27,28]. The crystallization of this critical enzyme in steroid metabolism has been attempted since the 1970s in other international laboratories, but not even primary diffraction data has ever been reported before our study, in spite of a report on its crystallization in 1976 [29]. In our purification, 17β-HSD eluted at the end of the reversed gradient on phenyl-Superose, yielding highly active and homogeneous preparations (Fig. 1). This also demonstrates that the 17β-HSD protein is more hydrophobic than the other proteins in the cytosol homogenate, and thus this chromatographic method can be used as the first column to separate components in the homogenate [28]. In some cases, when the ratio of the column volume to the sample volume is too large, 17β-HSD may be difficult to elute, due to its strong interaction with the media. Though the problem can often be solved by further decreasing the ionic strength of the elution buffer by its dilution, a correct saturation of the column in loading is highly recommended.

It was also reported recently that the separation of

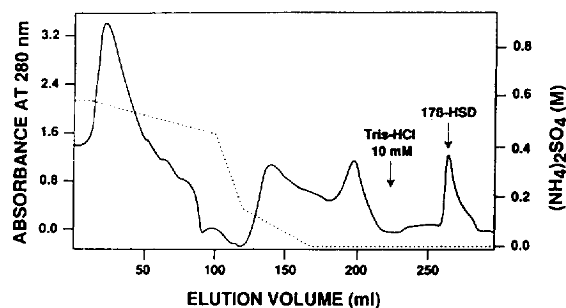

Fig. 1. Hydrophobic interaction chromatography. A 17β-HSD sample after ammonium sulphate fractionation was dissolved in the presence of 0.6 *M* $(NH_4)_2SO_4$ and loaded directly on a phenyl-Sepharose column (65×26 mm I.D.). The sample was only eluted when the ammonium sulphate concentration reached zero and with a further decrease in Tris–HCl concentration yielding fractions containing up to 20% of 17-HSD protein [28].

3α-HSD, 3β-HSD and isomerase from *Pseudomonas testosteroni* crude extract could be performed with Sepharose-PEG chromatography [30]. Their elution profile with decreasing potassium phosphate concentration indicated the increasing hydrophobicity from 3α-HSD to isomerase (Fig. 2). The author suggested a pre-study on mild hydrophobic interaction chromatography in actual fractionations of complex mixtures, and excellent purification ratio and recovery can be obtained with optimization of the chromatographic conditions.

Other examples of hydrophobic interaction chromatography in HSD purification are: a phenyl-Sepharose CL-6B column was used for the purification of 3α-HSD and 3β-HSD from human prostate cytosol [18]; 11β-HSD from mouse liver microsomes was purified with the use of octyl-Sepharose CL-4B chromatography [20]; a phenyl-Superose column was utilized in the purification of 17α-HSD from intestinal *Eubacterium sp.* [31].

3. Some results on membrane association and detergents used for HSDs

Most HSDs studied have membrane association or hydrophobic properties. A recent systematic study on their hydrophobicity showed the mild hydrophobic nature of a large number of HSDs [Campbell et al., unpublished results], in agreement with former re-

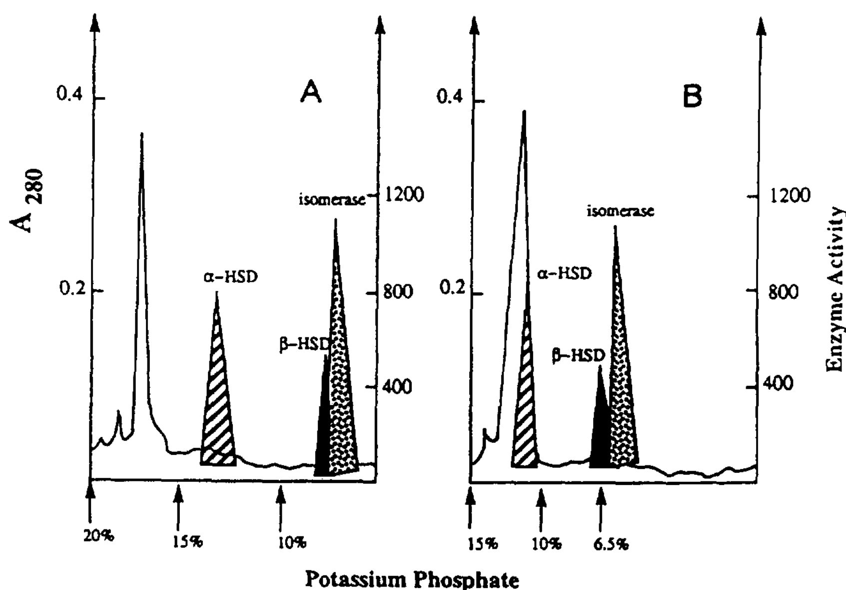

Fig. 2. Sepharose-PEG stationary phase. Elution profile of *Pseudomonas testosteroni* crude extract, starting with (A) 20% or (B) 15% potassium phosphate in the mobile phase. (From Ref. [32], with permission).

ports [30]. Different levels of hydrophobicity have been demonstrated for various members of the HSD group, for instance, the 3β-HSD showed a much higher hydrophobicity and membrane association than the 3α-HSD, both having been obtained from human hyperplastic prostate and human placenta [17]; the 3β-HSD from human placenta is localized mainly in microsomes and mitochondria, and is much more hydrophobic and intimately membrane-associated than the estrogenic 17β-HSD activity which is localized in both cytosol and microsomes [3]. A recent report on the 3-dimensional structure of estrogenic 17β-HSD afforded more direct evidence of the membrane association (mostly peripheric) of this enzyme form. In fact, the two helix–turn–helix motifs found near the C-terminal of the enzyme, which are new to the short chain dehydrogenase family, may be responsible for its observed membrane association [24].

Because of their hydrophobic nature and membrane-association, detergents have been often used for solubilizing HSDs in their active form. In Table 1, examples of some representative hydroxysteroid dehydrogenases are given from the recent literature.

Strong detergents are usually needed for proteins with more intimate membrane association. For example, in the presence of sodium cholate, 3α-HSD activity was solubilized from the membranes while 3β-HSD remained mostly bound to the membrane in human hyperplastic prostate.

Here CHAPS is required for 3β-HSD solubilization as compared to sodium cholate for 3α-HSD [17]. Some HSDs, even from soluble sub-cellular fractions, need the help of detergents for their effective purification by increasing their solubilities, because of their hydrophobic nature, such as human placental 17β-HSD [26]. Of course, in the sample loading of this enzyme form on the first column, only 0.5% of mild Triton X-100 was used.

As the mild sugar detergents, especially those with shorter hydrophobic chains, are favourable for maintaining the enzyme activity, they are used more often now for the purification and storage of HSDs. β-D-Octylglucoside (β-OG) and similar sugar detergents are often considered, e.g., it is used in the purification and storage of human placental 3β-HSD [Zhou et al., unpublished] and in the stockage and crystallization of human estrogenic 17β-HSD [25,26]. β-OG is nonionic and favorable for protein crystallization. The inclusion of a low detergent concentration of 0.06% β-OG in 17β-HSD sample increased the solubility of the dehydrogenase from

2.5 mg/ml to more than 40 mg/ml (which enabled the first crystallization of human steroid-converting enzymes at diffraction quality) [25,26]. In protein preparation for crystallization, all ionic detergents should be removed in the last chromatographic run; such considerations should be incorporated in the purification strategy [Zhou et al., unpublished].

4. Affinity chromatography

As all hydroxysteroid dehydrogenases use adenylyl-containing cofactors such as NAD, NADP, NADH or NADPH, dye affinity chromatography (e.g., Blue-Sepharose columns) is often used. For example, Blue-Sepharose chromatography was used in the preparation of 3α-HSD from human liver [14], $3\alpha/\beta$-HSD from human prostate cytosol [18] and human estrogenic 17β-HSD from placental cytosol [3]; a Matrex gel blue A column was used for the purification of $3\alpha/\beta$-HSD from porcine testicle cytosol; AMP agarose chromatography achieved a 1000-fold purification for human placental 11β-HSD; a Red-Sepharose column played an important role in the homogenization of 11β-HSD from mouse liver microsomes; Blue Agarose chromatography was used in 17α-HSD purification from intestinal *Eubacterium sp.*

Of course, the correct choice of the ligand in the elution is important for the efficiency of the chromatography. For example, the specific elution of the estrogenic 17β-HSD by the co-factor NADP was very efficient in obtaining a homogeneous preparation with a high yield [10]. A comparison of the NADP and NAD elution was striking, in spite of the fact that the enzyme could utilize both cofactors in the catalytic reaction (unpublished results). A 30 μM concentration of NADP can fully elute the enzyme protein under our experimental conditions while a 4 mM concentration of NAD often results in incomplete elution with a much lower yield.

5. Fast purification

The advantage of fast purification is its greater efficiency. It allows simplification of the intermediate steps and very rapid procedures. The latter can yield homogeneous and highly active enzyme preparations, as has been shown by many researchers [3,25]. The high specific activity obtained by fast purification may be due to the elimination of protein microheterogeneity, caused in vitro by oxidation–reduction or partial proteolysis [22].

This is especially important for the purification of hydroxysteroid dehydrogenases, as they are often hydrophobic and labile ([3], Zhou et al., unpublished, [29]). In the study reported in Ref. [29], we purified the overproduced human 3β-HSD from placental microsomes using fast purification. This purification yielded a specific activity of 500 nmol/min/mg which was much higher than the results reported in Ref. [11].

We used the fast flow properties of Sepharose to minimize the purification steps for some human hydroxysteroid dehydrogenases [27]. The samples were correctly diluted before every FPLC (fast protein liquid chromatography) and loaded directly onto the column to eliminate dialysis. For example, in 17β-HSD homogenization, the sample after ammonium sulfate fractionation was diluted to a conductivity close to that of a running buffer containing 0.09 M NaCl and applied directly onto a Q-Sepharose column 170×50 mm for 3 to 4 placentas (Fig. 3). Second, for a large sample volume, a low salt concentration was used during sample application, e.g. 0.09 M NaCl for 17β-HSD loading. In this way,

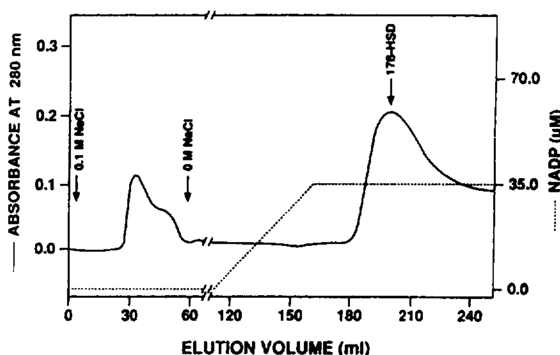

Fig. 3. Affinity chromatography. The phenyl-Sepharose fractions of 17β-HSD were loaded directly on a Blue-Sepharose CL-6B column (100×16 mm I.D.). The column was first washed with buffer A of low NaCl content (0.1 M) and re-equilibrated with the same buffer (0 M NaCl), then eluted with $NADP^+$. Homogeneous 17β-HSD fractions eluted at 35 mM $NADP^+$. The fraction size is 2 ml each [28].

we reduced the column back pressure by eliminating about 85% of the contaminating proteins during loading, thus maintaining a high flow-rate of 12 ml/min with the joint function of two Pharmacia 3500 pumps [3]. A cyclic program was used to allow large sample loading via a 10-ml Superloop. While this loop was being filled, simultaneous washing of the column reduced the back pressure. This chromatography, with large sample volumes of more than 1000 ml and fast gradient elution, can be accomplished within a few hours, as compared with several days for the conventional ion-exchange method used in the first chromatography.

With the above refinement, Q-Sepharose chromatography is now a very suitable replacement for the traditional DEAE media as a first column for many natural protein homogenizations. In the purification of human estrogenic 17β-HSD, this helped to shorten the whole procedure to ca. 3 days, as compared with several weeks using conventional chromatography [3].

The quality of our preparations is demonstrated in both the homogeneity and activity of the enzymes, made possible by the fast flow and high resolution of the column media, e.g., the different types of Sepharose, and by an appropriate combination of different chromatographic methods [3]. Protein separations take place even at different stages within one chromatography, e.g. during sample application and gradient elution of each column; this is also demonstrated in Blue-Sepharose affinity chromatography. For example, in the purification of 17β-HSD [3,10], the protein separation took place at several levels of sample application, low salt washing, and a specific elution with the cofactor. A high purification within one column was thus achieved.

Improvement of the homogeneity of 17β-HSD permitted us to obtain high-resolution crystals of this enzyme complexed with NADP [25]. Our success with crystallization is also attributable to the high activity of the preparation, as the fast purification may eliminate protein microheterogeneity. In the purification of 17β-HSD, we obtained enzyme specific activities that were ca. three-fold higher than those obtained using conventional chromatography [3].

After several years of practice in our group, the chromatographic methods based completely on FPLC have produced homogeneous and highly active protein preparations very rapidly and economically. This is because of the availability of fast flow and low pressure media, which made it possible to pack different columns from empty ones (e.g., XK series columns from Pharmacia Biotech.). It is easy to adjust the column length or change a column with a different width to maximize the resolution, e.g. to have a short one for absorption chromatography. These media also enable us to adapt different gel types to reach the best chromatographic combination. Of course, the overproduction of HSDs in various systems considerably facilitates their fast purification [3,13].

The above will be helpful in choosing suitable chromatographic conditions for the purification of new HSD species from different sources. These purifications are important for structure–function studies of the hydroxysteroid dehydrogenases. We believe further developments will be achieved for the purification of these enzymes as the feedback of their mechanism study, especially from the understanding of their interaction with membranes.

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