AFFINITY CHROMATOGRAPHY: LARGE-SCALE PURIFICATION OF THE SOLUBLE OESTRADIOL-17-β DEHYDROGENASE OF HUMAN PLACENTA

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1. Introduction

Several methods have been described [1–3] for the isolation of the soluble human placental oestradiol-17-β dehydrogenase (EC 1.1.1.62) but a larger scale purification was needed to perform structural studies of this protein. This enzyme binds its substrate (oestradiol-17-β or oestrone) in the absence of cofactor [4, 5] and we recently reported [6] that its active site is able to bind oestradiol-17-β with bulky substitution at carbon 3 of the steroid nucleus; thus we tried successfully a purification of this protein by affinity chromatography with an oestrone-3 linked Sepharose column. As an immediate application of this large-scale purification by affinity chromatography, we report also in this paper the results of the amino acid analysis of this dehydrogenase.

2. Experimental

2.1. *Preparation of oestrone–Sepharose*

Three oestrone–Sepharose derivatives were synthetized using cyanogen bromide activation of Sepharose 4 B [7]: oestrone hemisuccinate–ethylene diamine–Sepharose; oestrone–aminocaproate–Sepharose and oestrone–amino undecanoate–Sepharose. The first phase was synthetized according to the method of Cuatrecasas [8]. The other phases were obtained by esterifying oestrone with the Sepharose coupled amino acid: this reaction was carried out in dimethylformamide containing *N,N'*-dicyclohexyl carbodiimide.

The oestrone concentrations in the packed oestrone–Sepharose columns were in the same range (5×10^{-6}–2×10^{-4} M) as determined by hydrolysis, extraction and gas liquid chromatography on a 1% SE_{30} column.

2.2. *Affinity chromatography*

A preliminary and rough purification was carried out by single DEAE-cellulose chromatography and ammonium sulphate precipitation; the affinity was performed on either of the oestrone derivatives:

2.2.1. Sepharose containing high concentration of bonded oestrone ($\simeq 10^{-4}$ M)

The enzyme was applied to a 1 × 5 cm column in 0.3 M phosphate buffer, 0.001 M EDTA, 20% glycerol (medium A) and washed with a hundred column volumes of the same medium. The oestradiol-17-β dehydrogenase was eluted by medium A with 10^{-4} M of oestrone hemisuccinate.

2.2.2. Sepharose containing low concentration of bonded oestrone ($\simeq 10^{-6}$ M)

The enzyme solution was applied and retained on a 1 × 10 cm column of oestrone–Sepharose equilibrated with 0.3 M phosphate buffer, 0.001 M EDTA, 20% glycerol, 1 M ammonium sulphate (medium B). The column was washed with 600 ml of medium B and the enzyme was eluted by lowering the ammonium sulphate concentration.

2.3. *Analytical methods*

Analytical polyacrylamide gel electrophoresis, determinations of enzymatic activity and protein concentration were performed as previously described

[1, 2]. Specific activity is expressed in I.U. (μM of oestradiol oxidized or NAD reduced/min at 25°/mg of protein).

2.4. *Amino acid analysis*

The protein (0.7–1 mg) was hydrolysed in vacuo at 110° for 24, 48 and 72 hr with 3 N paratoluene sulfonic acid [9] or 6 N HCl. The hydrolysates were analysed using a Beckman 120 B and (or) a Technicon amino acid analyser. The tryptophan content was determined by both procedures of Liu [9] and Spies [10]. The half cystine content was determined by the method of Hirs [11].

3. Results

The chromatographic behaviour of oestradiol-17-β dehydrogenase on two oestrone Sepharose columns is illustrated in fig. 1 (Sepharose column containing high concentration of bonded oestrone) and fig. 2 (Sepharose column containing low concentration of bonded oestrone):

i) fig. 1 shows an example of the elution pattern obtained with an enzyme solution of specific activity 0.02 I.U./mg; medium B with 2×10^{-4} M oestrone hemisuccinate eluted 30 mg of oestradiol-17-β dehydrogenase with a specific activity of 1 I.U./mg; this specific activity was raised to 2–2.5 I.U./mg (pure enzyme) by a single DEAE-cellulose chromatography. The homogeneity of the enzyme was confirmed by analytical polyacrylamide gel electrophoresis.

ii) The elution pattern shown in fig. 2 is representative of the results obtained with an enzyme solution of 160 mg of protein (specific activity of 0.2 I.U./mg) applied to the column in medium B containing 1 M ammonium sulphate. After washing the column with 600 ml of the same buffer, lowering the ammonium sulphate concentration to 0.6 M resulted in the elution of 13 mg of pure oestradiol-17-β dehydrogenase (specific activity 2–2.5 I.U./mg). However, the polyacrylamide gel electrophoresis showed the existence of some weakly active polymers accounting for less than 10%. The aggregation of the oestradiol-17-β dehydrogenase into inactive [12] or weakly active polymers [13] has been previously reported. An 8 mg protein fraction

Table 1

Amino acid	Molecular weight of residue (M.W.)	Molar* percentage	Number of residues per 68,000 g	Nearest integral number (ri)
Lys	128.17	4.8	29.8	30
His	137.14	2.3	14.2	14
Arg	156.18	5.5	35.4	34–36
Asp	115.10	8.8	54.6	54–56
Thr	101.10	5.3	33.4	32–34
Ser	87.07	6.5	40.2	40
Glu	129.11	11.2	69.4	68–70
Pro	97.11	6.5	40.2	40
Gly	57.05	7.8	48.2	48
Ala	71.07	8.7	54	54
$\frac{1}{2}$ Cystine**	102.13	1.9	11.8	12
Val	99.13	8.4	52	52
Met	131.19	1.9	11.8	12
Ile	113.15	3.3	20.4	20
Leu	113.15	10.7	66.4	66
Tyr	163.17	2.5	15.6	16
Phe	147.17	3.8	23.6	24
Trp	186.2			4
Total number of residues				624 ± 4
Molecular weight (ri × M.W.)				68,250 ± 500

* Mean value of 5 analyses (3 time of hydrolysis for each analysis).

** Determined after performic oxidation [11].

*** Determined by two independent methods: Liu [9], Spies [10].

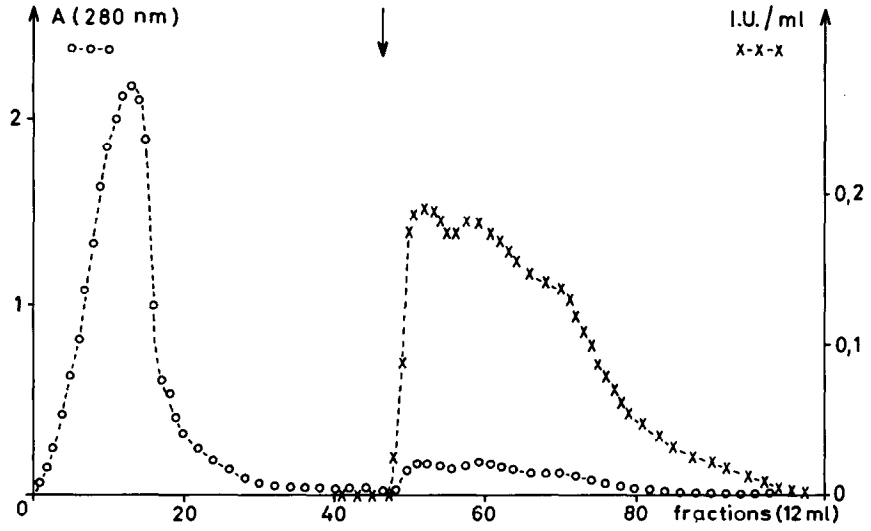

Fig. 1. Affinity chromatography of oestradiol-17-β dehydrogenase on a Sepharose column containing high concentration of bonded oestrone. Crude enzyme solution (protein 1.5 g, specific activity 0.02 I.U.; in 150 ml 0.3 M phosphate buffer pH 7.2, 0.001 M EDTA, 20% glycerol) was applied to a 1 × 5 cm column. After a 100 column volume washing with the same buffer, elution was obtained by adding 0.2 mM oestrone hemisuccinate to the buffer (arrow). Fractions 48–75 contained 30 mg of oestradiol-17-β dehydrogenase (specific activity 1 I.U./mg).

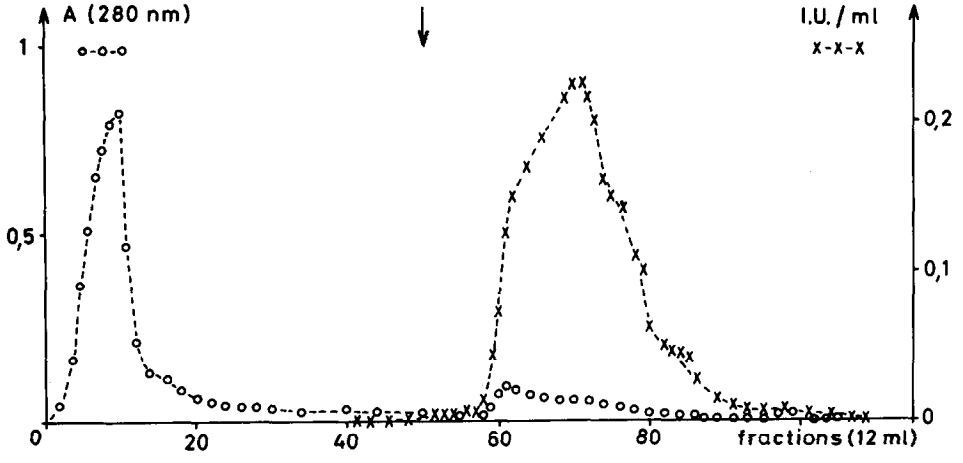

Fig. 2. Affinity chromatography of oestradiol-17-β dehydrogenase on a Sepharose column containing a low concentration of bonded oestrone and in the presence of ammonium sulphate. The enzyme solution (protein 160 mg, specific activity 0.2 I.U., in 105 ml of 0.3 M phosphate buffer pH 7.2, 0.001 M EDTA, 20% glycerol, 1 M ammonium sulphate) was applied to a 1 × 10 cm column. After a 600 ml washing with the same buffer, lowering the ammonium sulphate concentration of the buffer down to 0.6 M (arrow) eluted 8.5 mg (fractions 58–64) of enzyme and weakly active polymers (mean specific activity 0.5 I.U.) then 13 mg of pure enzyme (fractions 65–80; specific activity: 2.2–2.5 I.U./mg).

containing higher amounts of polymers or aggregates was eluted just in front of the main active fraction, leading to an assymetrical elution diagram.

The resulting amino acid analysis of the oestradiol-17-β dehydrogenase is given in table 1.

4. Discussion

We believe this to be the first reported purification of a hydroxysteroid dehydrogenase by affinity chromatography.

On high oestrone concentration Sepharose, the oestradiol-17-β dehydrogenase is retained by the column when applied in "normal" conditions (buffer 0.01 to 0.1 M), and can be eluted by addition to the buffer of $2–3 \times 10^{-4}$ M of oestrone hemisuccinate, a water soluble substrate: no elution could be obtained by adding oestrone or oestradiol to the buffer, because efficient competition for the enzyme between the Sepharose linked oestrone and the free steroids would require higher concentrations than the low solubility of the oestrogen allows.

On low oestrone concentration Sepharose, the oestradiol-17-β dehydrogenase is *not* retained when applied to the column in an usual buffer (0.01 to 0.1 M). Addition of 1 M ammonium sulphate to this buffer allows the retention of the enzyme; but in this case, the addition of 3×10^{-4} M of oestrone hemisuccinate to this 1 M ammonium sulphate buffer does not result in elution of the enzyme. This elution is easily obtained, even in the absence of substrate, by lowering the ammonium sulphate concentration. However, such a technique is in fact affinity chromatography as suggested by the following observations demonstrating the role of the oestrone molecule bonded to the Sepharose:

i) Whatever the ammonium sulphate concentration, the oestradiol-17-β dehydrogenase is not retained on columns packed with Sepharose, or ethylene diamine–Sepharose or aminocaproate–Sepharose.

ii) The ammonium sulphate concentration necessary to obtain the retention of the oestradiol-17-β dehydrogenase on an oestrone–Sepharose column is related to the steroid concentration of the solid phase: a column containing 10^{-4} M oestrone adsorbs the enzyme without ammonium sulphate (first method), but the *same phase* diluted in Sepharose 4B requires ammonium sulphate to retain the enzyme.

The chromatography of enzymes on DEAE-cellulose using high ammonium sulphate concentration recently reported [14] is quite different from the procedure described here since in our conditions the column is devoid of any ion-exchange property. Moreover, the ammonium sulphate does not increase the interactions between oestrone–Sepharose and the enzyme since the K_m is not altered by high concentrations of this salt. It is likely that ammonium sulphate, increasing the interactions between different molecules of enzyme gathered by several neighbouring oestrone molecules, lowers the mobility of the oestradiol-17-β dehydrogenase and facilitates its retention on the column.

The results of our amino acid analysis differ from Jarabak's [15]. We find a greater proportion of isoleucine, methionine, tyrosine and tryptophan, which are the less abundant amino acids in this protein. This could be related to differences in the analytical scale used: Jarabak determined the tryptophan content of the protein by spectrophotometric titration alone and performed microanalysis on 67 μg samples, whereas we could use one mg of enzyme for each analysis and two different and independent methods for tryptophan determinations: that of Spies [10] and the new method of Liu [9].

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