PARAMETERS INFLUENCING THE PURIFICATION OF CALF UTERUS ESTROGEN 'RECEPTOR' BY AFFINITY CHROMATOGRAPHY

Hélène TRUONG and Etienne-Emile BAULIEU

*Unité de Recherches sur le Métabolisme Moléculaire et la Physio-Pathologie des Stéroides de l'Institut National de la Santé et de la Recherche Médicale and Département de Biochimie, Faculté de Médecine Paris-Sud, 78 rue du Général Leclerc, 94270 Bicêtre, France**

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1. Introduction

The partial purification of the estradiol 'receptor' from calf uterine cytosol, using new 7α-estradiol derivatives for affinity chromatography, has been described in a preliminary report [1]. Early attempts were made to purify a '4 S' form of the 'receptor' from calcium treated cytosol [1–4]. However this form of the receptor was only obtained from fresh uteri, since all efforts to prepare it from frozen uteri resulted in very low yields [5]. Thus, it was difficult to undertake a program of large scale purification which a priori requires the storage of frozen uteri. This problem has been partially resolved by using a '4 S' form of the 'receptor', which can be obtained, from both fresh and frozen uteri, by trypsin treatment of cytosol [6].

This paper will present some systematic studies which were undertaken on the parameters affecting the purification of the 'receptor' by affinity chromatography.

2. Material and methods

2.1. *Biospecific adsorbents*

Two adsorbents were prepared by attaching the 7α-estradiol derivative (E_2-7α-$(CH_2)_{10}$-COO^-) to Sepharose as previously described [1]. The calculated concentrations of derivative bound to the Sepharose were 1.37 mg/ml and 0.03 mg/ml (subsequently referred to as adsorbent, A and B, respectively).

* Postal address: Lab Hormones, F-94270 Bicêtre.

2.2. *Biological material*

Cytosol was prepared, from calf uteri which had been stored in liquid nitrogen, as previously described [1] except that the buffer was 50 mM Tris–HCl pH 7.4.

The '4 S' form of the 'receptor' ('4 S'-Trypsin) was obtained by treating the cytosol with trypsin (100 μg/ml) for 12 hr at 0°C. The efficiency of the trypsin treatment was routinely assayed by Sephadex G-200 filtration. In general, 70% of the binding activity was associated with the '4 S' 'receptor' and the remaining

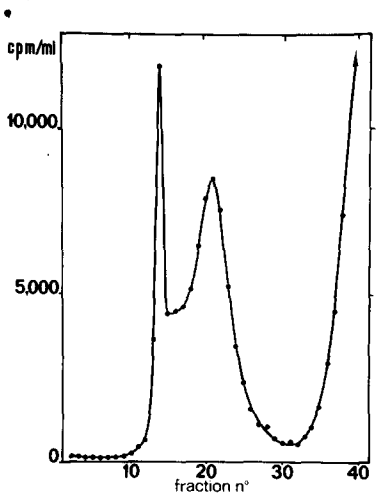

Fig. 1. Sephadex G-200 filtration of trypsin treated cytosol. Trypsin treated cytosol (3 ml) was incubated with [^{3}H]estradiol (20 nM), and filtered through a 200 ml Tris buffer equilibrated Sephadex G-200 column and 5 ml fractions were collected.

30% with large aggregates formed from 'receptor' tightly associated with cytosol proteins (fig. 1).

2.3. *Measurement of 'receptor'*

The concentration of estradiol 'receptor' complex was measured by the charcoal or the hydroxylapatite techniques (for details see [1]). Radioactive estradiol (55 Ci/mmole) was obtained from the CEA, Saclay, France.

2.4. *Binding of 'receptor' to the adsorbent and its specific elution*

The adsorbent was degassed, packed in a 1 ml column and equilibrated with the buffer (see [2–7]) used for the experiment. The cytosol was charged on the column at 0°C, at a controlled flow rate, and batchwise elution by [^{3}H]estradiol of 'receptor' from the adsorbent was performed at 30°C.

2.5. *Radioactivity counting*

The samples (500 μl) were counted in 10 ml of Bray's solution with a tritium efficiency of 16%.

2.6. *Protein*

The proteins were measured according to the technique of Lowry et al. [7].

2.7. *Buffers*

Tris: Tris 50 mM, EDTA 1.5 mM, pH 7.4; Tris–KCl: Tris 50 mM, EDTA 1.5 mM, KCl 0.3 M, pH 7.4.

3. Results

3.1. *Factors affecting the binding of the 'receptor' to the adsorbent*

3.1.1. Concentration of the ligand bound covalently to the adsorbent

Saturation curves for 'receptor' binding to adsorbents A and B were compared. At identical flow rates, an increase in the concentration of the steroid linked to the resin enhanced binding of 'receptor' to the adsorbent (fig. 2).

3.1.2. Charge flow rate

For absorbent B, the charge profile of the 'receptor' was compared at different flow rates (fig. 3). There was not much difference between flow rates of

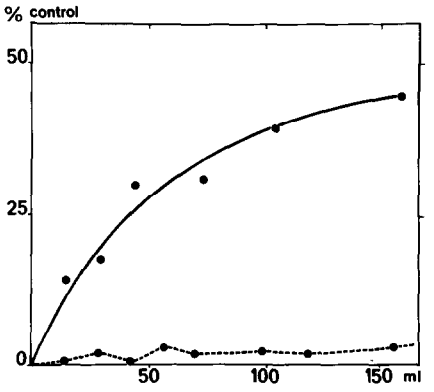

Fig. 2. Binding of 'receptor' to adsorbents A and B. Two columns (1 ml) containing (●---●) adsorbent A or (●——●) adsorbent B were charged with trypsin treated cytosol. The flow rate was 27 ml/hr. Effluent fractions, as well as a control cytosol, were incubated with 20 nM [^{3}H]estradiol. Binding activities in the cytosol and in the effluent fractions were measured by the charcoal technique. The binding activity in the effluent is expressed in per cent of the control value, which was: 174 000 cpm/ml.

33 ml/hr and 4 ml/hr. On the contrary with a flow rate of 159 ml/hr the pattern was significantly different. Furthermore, at 33 ml/hr and 159 ml/hr the adsorbent is rapidly saturated by the agregates present in the preparation (table 1).

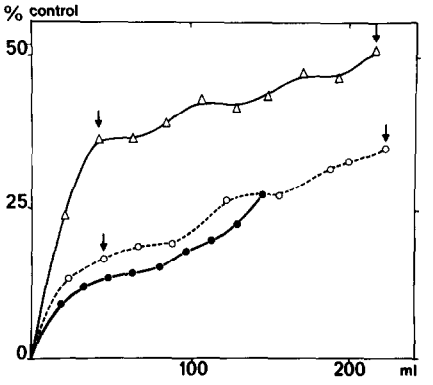

Fig. 3. Binding to adsorbent B of 'receptor' at different flow rates. Three columns (1 ml) were charged by trypsin treated cytosol (148 300 cpm/ml) at different flow rates: (●——●) 4 ml/hr, (○---○) 33 ml/hr, (△——△) 159 ml/hr. The results are expressed as in fig. 2. Arrows indicate the position of the fractions used for the experiment reported in table 2.

Table 1
Comparative binding of the aggregate and '4 S' form of the 'receptor' to adsorbent B

		Aggregates (%)	'4 S' (%)
Control		100	100
Column I	a	27	33
	b	77	43
Column II	a	25	37
	b	85	53

Aliquotes (3 ml) of the second (a) and the last (b) fractions of the column effluents (depicted in fig. 3), charged at 33 ml/hr (I) and 159 ml/hr (II), were incubated with 20 nM [^{3}H]-estradiol and filtered through Sephadex G-200 columns under the same conditions as reported in fig. 1. Simultaneously a control cytosol was subjected to the same incubation and filtration as described for the effluent fractions. Calculations from these results lead to a slight overestimation of the receptor present in the effluent (compare to fig. 3), because the background corresponding to 'non-specific' binding of the hormone to non 'receptor' proteins was not substracted from the values of gel filtration experiment.

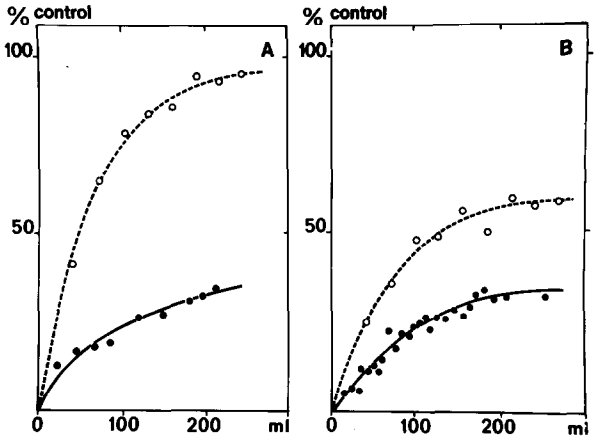

Fig. 4. Binding of different forms of the 'receptor' to adsorbent B. Columns (1 ml) were charged at a flow rate of 33 ml/hr, by (o----o) native cytosol or (•——•) trypsin treated cytosol. A) Low ionic strength 'receptor' (control native cytosol: 73 360 cpm/ml and control trypsin treated cytosol: 174 260 cpm/ml). B) High ionic strength 'receptor' (control native cytosol: 59 590 cpm/ml and control trypsin treated cytosol: 137 280 cpm/ml). Results are expressed as in fig. 2.

3.1.3. Molecular form of the 'receptor'

Comparative binding to adsorbent B of 1) native low salt 'receptor' '8 S' (Tris buffer), 2) native high salt 'receptor' '5 S' (Tris–KCl buffer) and 3) the '4 S' trypsin 'receptor', in both low and high salt medium, is presented in fig. 4.

Binding of the trypsin treated form of the 'receptor' is independent of the ionic strength. On the contrary, the low salt 'receptor' aggregates and rapidly saturates the adsorbent. The high salt 'receptor', which has less aggregates is more readily bound, but the binding remains lower than that of the '4 S' trypsin form.

3.2. *Parameters affecting elution*

The elution of 'receptor' bound to the adsorbent is obtained by displacing the 'receptor' with an excess of [^{3}H]estradiol.

Three factors may affect the elution recovery: the duration of exchange, the concentration of steroid bound to the adsorbent and the concentration of [^{3}H]estradiol used for the elution.

3.2.1. Duration of exchange

To determine the optimal time of incubation with [^{3}H]estradiol for the specific elution of 'receptor' from the adsorbent, cytosol was first incubated, until equilibrium, with 850 nM of the free derivative (E_2-7α-$(CH_2)_{10}$-COO^-). Then [^{3}H]estradiol, 20 nM, was added and the samples incubated for various periods of time at 30°C. After cooling at 0°C, binding activity was measured by the charcoal technique. The exchange equilibrium was reached after one hour (fig. 5).

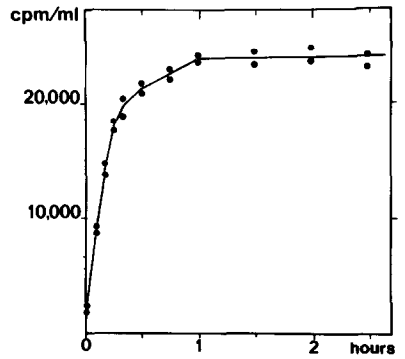

Fig. 5. Kinetic of exchange of the E_2-7α-$(CH_2)_{10}$-COO^- derivative against [^{3}H]estradiol. For details see text section 3.2.1.

Table 2
Elution of the 'receptor' from adsorbents A and B with different concentrations of estradiol

	'Receptor' bound to adsorbent (10^6 cpm)	[^{3}H]estradiol used for elution (μM)	Eluted 'receptor' (10^6 cpm)	Elution recovery (%)
Adsorbent A	40.8	20[1]	2.8	6.8
	55.1	80*[2]	14.2	25.7
Adsorbent B	24.4	20[3]	16.7	68.4
	10.8	30[4]	10.2	94.4

One ml columns were charged with trypsin treated cytosol. The 'receptor' bound to the adsorbent was estimated from the difference between the hormone 'receptor' binding activities of the control cytosol and the effluent. The specific radioactivities of [^{3}H]estradiol were: [1] 5 Ci/mmole, [2] 2.5 Ci/mmole, [3] 5 Ci/mmole, [4] 3.3 Ci/mmole. The binding activities of the eluted 'receptor' for the steroid were measured by the hydroxylapatite technique and corrected to a standard specific radioactivity of 55 Ci/mmole.

* In this experiment, the elution medium contained 10% dimethyl sulfoxide for solubilizing the steroid, without modifying the stability of the 'receptor'.

3.2.2. Effect of the concentration of [^{3}H]estradiol used for the specific elution with adsorbents A and B.

As shown in table 2, the concentration of tritiated steroid used for the elution is a limiting factor for adsorbent A. It is not possible to increase the concentration of [^{3}H]estradiol above 80 μM, which is the limit of solubility of the steroid in a medium which maintains the integrity and the binding activity of the receptor. With adsorbent B, 30 μM of [^{3}H]estradiol results in complete elution of the 'receptor'.

3.3. *Washing of the adsorbent*

Since some charged groups are introduced at the surface of the gel during the attachment of the derivative and the chain linking the steroid to the Sepharose is hydrophobic, the biospecific adsorbent may interact non specifically with protein [4,8,9]. Thus to obtain a good purification, before elution of the 'receptor', it is important to eliminate the maximum amount of proteins bound non specifically to the adsorbent.

To elute the 'receptor', the temperature is increased to 30°C and a large concentration of estradiol is added. The change of temperature decreases the ionic interactions, and consequently provokes a release of proteins retained by a mechanism of ionic exchange. The large concentration of steroid may act as a detergent, which could remove also the non specifically bound proteins.

The elution of the proteins non specifically bound to the adsorbent was studied during washing of the gel at 30°C with Tris buffer or at 0°C with Tris buffer containing various amounts of KCl or 17β-hydroxy-5α-androstan-3-one 10 μM (in order to mimic the

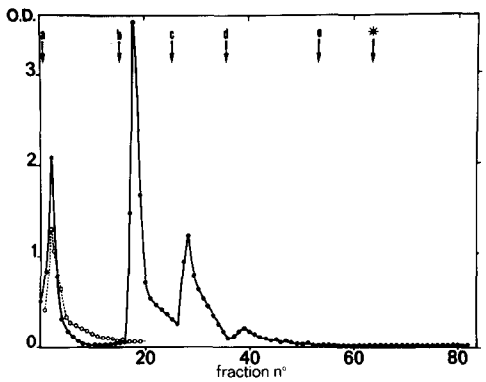

Fig. 6. Removal of non specifically bound proteins by washing before specific 'receptor' elution. Two columns (1 ml) of adsorbent B were charged with 250 ml of trypsin treated cytosol (protein 11 mg/ml). Under these conditions, the capacity for non specific binding of proteins was saturated. Charge and washing were performed at the same flow rate (33 ml/hr). (o---o) Tris buffer washing at 30°C, (•——•) stepwise washing by Tris buffer containing various amounts of KCl (a: 0.15 M, b: 0.25 M, c: 0.50 M, d: 0.75 M and e: 1 M) at 0°C. The starred arrow indicates a washing by Tris buffer without KCl at 30°C. Proteins in each fraction (2.5 ml) were estimated by the OD at 280 nm.

possible estradiol effect without binding to the 'receptor'). This steroid does not provoke the release of significant amounts of proteins. Results of the washings at 30°C by Tris buffer and at 0°C by Tris buffer containing various concentrations of KCl are presented in fig. 6. At 0°C, a Tris buffer 0.5 M KCl wash is sufficient for eliminating the majority of the proteins that could interfer with specific elution of 'receptor'.

4. Discussion

The 7α-estradiol Sepharose adsorbents used in these experiments have some advantages when compared to others described previously for estrogen 'receptor' purification [3,4,10,11]. The 7α-derivatives maintain high specificity and affinity for estrogen 'receptors' binding [12] and these adsorbents may be stored at 0°C for several months without any release of the covalently bound ligand.

However this affinity material is still not suitable for purifying the native low salt '8 S' 'receptor' which tends to aggregate and then rapidly saturates the adsorbent at very low levels, possibly by steric hindrance.

This hypothesis is supported by the observation that specific binding of the native 'receptor' to the adsorbent is significantly enhanced in high ionic strength media, in which the size of the aggregates is decreased. Trypsin treatment gives a high yield of the low molecular weight '4 S' 'receptor' which is not susceptible to aggregation and consequently, the best yield of 'receptor' binding to the adsorbent. When this '4 S' 'receptor' is present together with aggregates, the latter decreases the specific binding of the '4 S' 'receptor' to the adsorbent. Thus, the efficiency of the procedure for 'receptor' purification described in this study should be increased, if prior to affinity chromatography, conventional methods of protein separation are used to eliminate 'receptor' aggregates.

From these experiments, conditions were established which resulted in a one step partial purification of estradiol 'receptor' with a final specific activity of 5.5. X 10^7 dpm/mg protein. These preparations have specific activities approximately 5 times higher than those previously obtained [1]. This procedure gives an approximately 3% pure protein, calculated on the basis of a molecular weight of 60 000 and of one steroid binding site per molecule of 'receptor'.

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