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THE USE OF AN ENZACRYL AA DERIVATIVE FOR AFFINITY CHROMATOGRAPHY OF SEX HORMONE BINDING GLOBULIN

R. HAMPL, O. TÁBORSKÝ, J. MUSIL and L. STÁRKA

Research Institute of Endocrinology, Prague, and Medical Faculty of Hygiene, Chair of Clinical Chemistry and Biochemistry, Charles University, Prague (Czechoslovakia)

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SUMMARY

A polymer of the polyacrylamide type with side-chains bearing free amino groups on the benzene ring (Enzacryl AA) with covalently attached 3-hemisuccinate of 5α-androstane-3α,17β-diol was used for the affinity chromatography of sex hormone binding globulin.

After washing with buffer of increasing ionic strength, the release of protein was achieved by treatment with a buffered solution of testosterone. An approximately 63-fold enrichment of the required protein was achieved.

The purified protein was characterized by its binding properties and by isoelectric focusing.

INTRODUCTION

Studies on selective steroid-binding proteins such as transcortin or sex hormone binding globulin (SHBG) have shown that these high-affinity but low-capacity plasmatic carrier proteins can act as reversible regulators of hormone action[1].

SHBG has already been purified by classical means[1,2] (ammonium sulphate precipitation followed by repeated ion-exchange chromatography and molecular sieving) and its physico-chemical properties have been well established[1–5]; however, these techniques did not seem to be suitable for preparative-scale work. Affinity chromatography utilizing the unique selectivity of steroid–protein interactions appeared to be an ideal method for this purpose.

"Amino-Sepharose" coupled with cortisol 21-hemisuccinate has been used for the separation of transcortin from plasma[6], and excellent results have been obtained by affinity chromatography of soluble oestradiol receptors[7,8]. An attempt to separate SHBG by this method was also made as early as 1969, but the yields were very low (0.4%) owing to the failure to remove SHBG from the column without denaturation[9]. In each instance, cyanogen bromide-treated Sepharose 4B coupled with various steroid derivatives with or without a spacer was used.

As activated "amino-Sepharose" is now available commercially, the inconvenient work with cyanogen bromide can be avoided. However, when used for the

separation of steroid-binding proteins, these materials require extensive washing in order to remove the physically adsorbed affinant, which is limited by the resistance of the gel to organic solvents[8,10-12].

We searched for another matrix with good flow properties and resistant to organic solvents, which would moreover permit the direct attachment of suitable steroid derivatives. A cross-linked polymer of the polyacrylamide type containing sufficiently long side-chains (Enzacryl AA) seemed to fulfil these requirements. To the free amino group on the benzene ring of Enzacryl AA, a steroid derivative containing a carboxyl group, *e.g.*, hemisuccinate, could be attached. As a free 17β-hydroxy group is necessary for SHBG binding, a steroid derivative succinylated on a position other than 17β- had to be prepared[2,3].

MATERIALS AND METHODS

Steroids and chemicals

Radioactive steroids, *viz.*, [1,2-^{3}H]testosterone, specific activity 56 Ci/mmole, and [4-^{14}C]-3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone), specific activity 52 mCi/mmole, purchased from the Radiochemical Centre, Amersham, Great Britain, were purified by paper chromatography in the system cyclohexane–toluene–methanol–water (9:1:8:2). The radiochemical purity (higher than 97%) was checked by chromatography in the same system using a non-labelled standard.

Non-radioactive steroids were obtained from Koch-Light (Colnbrook, Great Britain). All chemicals were of analytical grade and solvents were distilled before use.

5α-Androstane-3α,17β-diol 3-hemisuccinate was synthesized from androsterone by treatment with succinic anhydride in dry pyridine and by subsequent reduction of the resulting crystals (m.p. 183°) with sodium borohydride. The product was crystallized from acetone–*n*-hexane. The crystals, m.p. 221°, chromatographically homogeneous in the system Silica gel HF_{254}/ethyl acetate–methanol (92:8) (R_F = 0.36) and more polar than androsterone hemisuccinate (R_F = 0.58), were characterized as 5α-androstane-3α,17β-diol 3-hemisuccinate. The final yield was 31%.

5-Androstene-3β,17β-diol 3-hemisuccinate was prepared by the same method from dehydroepiandrosterone. The yield was 34%.

[4-^{14}C]-5-androstene-3β,17β-diol 3-hemisuccinate was prepared by the same method from [^{14}C]dehydroepiandrosterone (10 μCi) diluted with non-labelled steroid (100 mg). A product with a specific activity of 0.088 μCi/mg was obtained.

Preparation of Enzacryl–affinant

A 200-mg amount of 5α-androstane-3α,17β-diol 3-hemisuccinate and 200 mg of dicyclohexylcarbodiimide in 10 ml of dimethylformamide were stirred carefully at room temperature for 80 min, filtered, the filtrate was added to a suspension of 6 g of Enzacryl AA (Koch-Light) in 40 ml of dimethylformamide and the mixture was stirred gently for a further 3 h. The Enzacryl–affinant was then washed with dimethylformamide (5 × 100 ml), dioxane (3 × 100 ml), water (6 × 100 ml) and 0.015 *M* phosphate buffer (pH 7.2) with 1 m*M* EDTA.

After the affinity chromatography had been performed, the following sequence of solvents was used for regeneration of the Enzacryl–affinant: 6 *M* urea, water, dioxane, dimethylformamide, water and buffer.

Purification of SHBG

The serum obtained from pooled placental blood collected at full-term deliveries was first freed from endogenous steroids by treatment with charcoal[13]. As confirmed with trace amounts (0.1 μCi) of labelled testosterone, less than 0.014% of testosterone was retained after such a treatment. Steroid-free serum was then precipitated twice with equal volumes of saturated ammonium sulphate solution and the final precipitate was redissolved in 0.015 *M* phosphate buffer with 1 m*M* EDTA (one quarter of the serum volume). The globulins were then dialyzed with demineralized water on a Sephadex G-25 column (40 × 2.5 cm) and the 60-ml fraction following the void volume (70 ml) was taken for affinity chromatography.

The sample was suspended with Enzacryl–affinant (6 g) in phosphate buffer (10 ml) and, after gentle stirring for 1 h at 4°, was allowed to stand overnight at 4°. The mixture was then applied on a chromatographic column with a sinter (3 cm diameter) and, after the bed had settled, the proteins were eluted step by step with 0.015 *M* phosphate buffer containing 1 m*M* EDTA and increasing concentrations of potassium chloride (0, 0.2 and 1.0 *M*) at 4°. Concomitantly, an aliquot (10 ml) of a dialyzed globular fraction of serum was suspended with untreated Enzacryl AA (1 g) and then eluted with the same buffers as above as the "Enzacryl control". The flow-rate was 1 ml/min. The Enzacryl–affinant with bound protein was then transferred into a flask to which testosterone with a trace amount of radioactive testosterone in buffer was added (600 μg of testosterone and 0.6 μCi of [^{3}H]testosterone were dissolved in 60 μl of ethanol and 60 ml of 0.015 *M* phosphate buffer with 1 m*M* EDTA and 0.2 *M* potassium chloride). After stirring for 1 h at room temperature, the solution was allowed to stand overnight at 4° and then separated on a Büchner funnel. The steroid was removed by treatment with charcoal as described previously[13] and the solution was lyophylized. In an aliquot, the radioactivity before and after charcoal treatment was measured in order to check for the complete removal of the steroid.

Determination of binding capacity and binding affinity

Both the testosterone binding capacity (TeBC) and testosterone binding affinity (TeBA) were determined by Sephadex gel dialysis in a batchwise fashion according to Pearlman *et al.*[3]. In this method (Method I in ref. 3), a ten times larger amount of testosterone, *i.e.*, 10 ng, was used.

The method of Lowry *et al.*[14], with human serum albumin as a standard, was used for quantification of proteins.

Isoelectric focusing

After dialysis on Sephadex G-25 followed by lyophylization, an equivalent of 20 ml of purified SHBG (TeBC = 215 ng; protein content 3.3 mg) with 1 μCi of [^{3}H]testosterone in 1.5 ml of water was subjected to isoelectric focusing on a 5% polyacrylamide gel column (12 × 0.8 cm) containing 2% of Ampholine, pH 3–10 (LKB, Stockholm, Sweden). The initial voltage of 10 V/cm was gradually increased to 35 V/cm during 10 h; the current did not exceed 1 mA per column. The course of the pH gradient was determined according to Finalayson[15]. After staining with Coomassie Brilliant Blue R-250 (ICI, Dyestuffs Division, Manchester, Great Britain)[16], the pattern was cut into 3-mm segments, which were transferred into counting vials, the gel

was solubilized according to Caputo and Hosty[17] using NCS solubilizer (Amersham-Searle, Arlington Heights, Ill., U.S.A.) and the radioactivity was measured.

Determination of displacement of [^{3}H]testosterone from purified SHBG by various steroids

The relative displacement effect of various steroids was assessed according to Heyns *et al.*[3]. Briefly, the procedure was as follows. Increasing amounts (2–100 ng) of various steroids were added with stirring to the system containing a constant amount of purified protein (1 ml; TeBC = 10.73 ng), [^{3}H]testosterone (50 000 dpm) and Sephadex G-10 (100 mg) in a total volume 2 ml. After equilibrium had been achieved, the radioactivity in the supernatant was measured. The amount of each steroid producing the same displacement as 2 ng of testosterone was determined.

Radioactivity measurements

The radioactivities of both ^{3}H and ^{14}C were measured on a Betaszint BF 5000 liquid scintillation spectrometer (Berthold, Frieseke, Wildbad, G.F.R.) with a computing programme for the calculation of disintegrations per minute using the external standard channels ratio method. The scintillation fluid consisted of 4 g of PPO and 50 mg POPOP in 1 l of toluene. When aqueous samples (below 0.3 ml) were measured, a scintillation fluid containing 25% (v/v) of ethylene glycol monomethyl ether was used.

RESULTS

Determination of the coupling of affinant to Enzacryl

As 5-androstene-3β,17β-diol 3-hemisuccinate did not differ from 5α-androstane-3α,17β-diol 3-hemisuccinate in its binding affinity to SHBG (see below), [^{14}C]-5-androstene-3β,17β-diol 3-hemisuccinate was used for the determination of coupling to Enzacryl. A 15-mg amount of labelled hemisuccinate with a specific activity of 0.088 μCi/mg was coupled with 100 mg of Enzacryl as described above. The Enzacryl–affinant complex was then washed thoroughly with dimethylformamide, dioxane and water until no radioactivity could be detected in the effluent. The remaining Enzacryl with covalently bound steroid was then subjected to hydrolysis with 10% hydrochloric acid in methanol and the hydrolysate was extracted with dichloromethane. After evaporation of the solvent, the radioactivity in the dry residue was measured. The value of 34,000 dpm found corresponded to 195 μg, *i.e.*, 0.5 μmole of steroid bound to 100 mg of polymer.

In order to exclude the participation of physical adsorption, the same amount of labelled steroid was added to a suspension of Enzacryl AA in dimethylformamide without the addition of a condensation reagent (dicyclohexylcarbodiimide) and washed successively as above. The solid was hydrolyzed and the radioactivity in the hydrolyzate was measured; none was found.

Purification of SHBG

In a typical procedure, 40 ml of placental serum was worked up in one step. After removal of endogenous steroid with charcoal, the serum was precipitated twice with ammonium sulphate and the precipitate of globular proteins was dialyzed on

Sephadex G-25. The dialyzate was then applied in a batchwise fashion on Enzacryl–affinant and washed successively with 0.015 *M* phosphate buffer (pH 7.2) containing 1 m*M* EDTA and increasing concentrations of potassium chloride (0, 0.2 and 1.0 *M*). Simultaneously, an aliquot of the dialyzed protein mixture was applied on the column with untreated Enzacryl AA and worked up as above as an "Enzacryl control", in order to exclude non-specific adsorption. The bound protein was released by treatment with a concentrated testosterone solution containing trace amounts of labelled hormone. The steroid was removed again with charcoal. The completeness of the steroid removal was checked by measurement of tracer radioactivity; practically none could be detected after charcoal treatment.

In all fractions, including those of the "Enzacryl control", both the total protein concentration and the testosterone binding capacity were determined. The results obtained after individual purification steps are summarized in Table I. In the "Enzacryl control" pattern, almost complete recovery of TeBC was achieved within the first two fractions. As demonstrated in Table I, a product with a TeBC of 66 ng of bound testosterone per milligram of total protein was obtained, which corresponds to approximately 63-fold enrichment of the required protein.

TABLE I

SCHEME FOR PURIFICATION OF SEX HORMONE BINDING GLOBULIN

Treatment	*Volume (ml)*	*Total protein content (mg)*	*Testosterone binding capacity*	
			Total TeBC (ng)	*Specific TeBC (ng/mg protein)*
Initial serum	40	4068	4280	1.05
Ammonium sulphate precipitation (globular fraction)	10	1530	4210	2.75
Dialysis on Sephadex G-25	68	1000	4148	4.15
Fractionation on Enzacryl–affinant:				
fraction 1 (0.015 *M* phosphate buffer)	88	455	1804	3.96
fraction 2 (0.015 *M* phosphate buffer)	100	52	133	2.56
fraction 3 (0.015 *M* phosphate buffer with 0.2 *M* KCl)	100	195	386	1.98
fraction 4 (0.015 *M* phosphate buffer with 1.0 *M* KCl)	100	133	380	2.86
fraction 5 (0.015 *M* phosphate buffer with 1.0 *M* KCl)	100	83	35	0.42
fraction 6 (0.015 *M* phosphate buffer with 1.0 *M* KCl)	100	5	0	—
Elution with testosterone solution and removal of steroid with charcoal	80	13	858	66.0

Characterization of purified SHBG

The binding affinity expressed as an intrinsic association constant at 25° with testosterone as a tracer was determined in both a globular fraction of placental serum and purified material. The respective values (mean of five determinations, ± S.D.) were $3.4 \pm 0.43 \cdot 10^8$ and $1.92 \pm 0.28 \cdot 10^8$ l/mole. In both instances, complete loss of binding affinity after heating to 60° occurred.

The relative displacing effect of various steroids as a percentage (the testosterone effect being taken as 100%) is shown in Table II.

Isoelectric focusing of purified protein showed only two bands: the first in the area between p*I* 5.2–5.5 and the second at the cathodic end area, corresponding to immunoglobulins. The radioactivity distribution showed two peaks, the first, probably the unbound testosterone, containing more than 70% of the total radioactivity was located as a diffuse band in the anodic area, and the second peak was associated with the band of p*I* between 5.2 and 5.5.

TABLE II

RELATIVE DISPLACEMENT OF [³H]TESTOSTERONE BY VARIOUS STEROIDS

Testosterone effect is taken as 100%.

Steroid	*Relative displacement (%)*
Testosterone	100
5α-Androstane-3α,17β-diol	106
5-Androstene-3β,17β-diol	98
5α-Androstane-3α,17β-diol 3-hemisuccinate	2.6
5-Androstene-3β,17β-diol 3-hemisuccinate	2.5
17β-Oestradiol	58
4-Androstene-3,17-dione	1.8
Cortisol	<0.1

DISCUSSION

Using Enzacryl AA with covalently attached 3-hemisuccinate of 5α-androstane-3α,17β-diol, approximately 63-fold enrichment of SHBG was achieved in one step. Under the conditions used, approximately 84% recovery of the initial testosterone binding capacity was obtained and in the highly purified form about 20% of the binding capacity was found (Table I).

In order to establish a reliable technique for the release of SHBG from its binding to Enzacryl–affinant, various procedures involving changes of pH and ionic strength as well as elution with buffered solutions of various 17β-hydroxysteroids were tried. The most effective method appeared to be elution with a concentrated aqueous testosterone solution. A disadvantage of this procedure was the neccessity for intensive treatment with charcoal in order to remove the steroid moiety, in which a certain loss of binding capacity may occur.

The isoelectric focusing and the displacement experiments and the binding constants found confirmed the identity of SHBG in the purified fraction. The p*I* value of the band associated with radioactivity was in accordance with that reported previously for SHBG[5]. However, this method also revealed that some by-products with the mobility of immunoglobulins accompanied SHBG and could not be separated completely by the described method.

In addition to its convenient flow properties, another advantage of Enzacryl AA is its ability to give direct attachment of known amounts of steroid ligands. A ligand concentration of 30 μmole of 5-androstene-3β,17β-diol 3-hemisuccinate per 6 g of Enzacryl AA was obtained.

Although the displacing ability of androstanediol 3-hemisuccinate is not very high (2.5% relative to testosterone, see Table II), this derivative seems to be a reliable affinant, because, on the other hand, the binding protein can be released without great difficulty. In general, very strong binding of protein to the affinant is undesirable, as was found by experience with the affinity chromatography of oestradiol receptors on Sepharose derivatives[7,8].

Previously, Enzacryls had been used only for immobilization of biopolymers, especially enzymes. In the present study, Enzacryl AA was used for the affinity chromatography of selective steroid–carrier protein.

In comparison with other polymers used for affinity chromatography, Enzacryl seems to have several advantages, as follows. The synthesis of Enzacryl–affinant does not require previous activation of the carrier and the steroid is bound covalently in good yields. The resistance of Enzacryl AA to organic solvents permits the effective removal of physically adsorbed ligands without the use of large volumes of solvents. An enrichment of protein could be achieved by a relatively simple and rapid procedure.

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