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CYCLIC BIOSPECIFIC AFFINITY CHROMATOGRAPHIC METHOD FOR THE PURIFICATION OF THE SEX STEROID BINDING PROTEIN (SBP): APPLICATON TO THE PURIFICATION OF SBP FROM TOAD

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SUMMARY

A novel biospecific affinity chromatographic procedure was developed for the purification of the sex steroid binding protein from *Bufo arenarum*. A charcoal column connected in series to the affinity column allows the removal of any ligand non-covalently bound to the matrix or released during its storage, thus avoiding the need for exhaustive and prolonged washing procedures. In addition, it is not necessary to remove the endogenous ligand from the starting material and the binding to the affinity column can be monitored to determine the time required to achieve the maximum yield. The advantages are the charcoal adsorption of the ligand "washed" from the affinity column by the protein to be purified and the amplification provided by the cyclic use of the system. The procedure improves the yield from less than 1% (by conventional procedures) to more than 50%. With minor modifications this procedure can be useful for the purification of binding proteins and receptors.

INTRODUCTION

Sex steroids are bound to a specific protein (steroid binding protein, SBP) present in the plasma of many species, including humans [1–6]. The protein is

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highly specific for steroids having a hydroxy group in the 17β-position and a generally planar steroid structure [7–9]. In a previous study [10] we showed that reproductively active *Bufo arenarum* females possess an SBP with these characteristics. The protein binds dihydrotestosterone (DHT) with high capacity (4 μM), moderate affinity ($K_a = 0.042/\text{n}M$ for DHT at 25°C) and has physico-chemical properties similar to those of the human protein.

In order to obtain further information about the structure, biosynthesis and functions of SBP from *Bufo arenarum*, we have purified this protein using three steps, one of which included affinity chromatography. However, our early attempts to purify SBP revealed that conventional affinity chromatographic procedures, using 5α-androstane-3β,17β-diol 3β-hemisuccinate as a selective adsorbant, failed to yield significant amounts of the protein (yield $<1\%$).

In this paper we describe a modified biospecific affinity chromatographic (BAC) procedure, based in a cyclic system, that allowed us to improve the yield in the affinity step from less than 1% to more than 50%. This system will be particularly useful when difficulties arising from ligand leakage cannot be ignored.

EXPERIMENTAL

Reagents and chemicals

Radioinert steroids were obtained from Steraloids (Wilton, NH, U.S.A.). Labelled steroids, [1,2-^{3}H]-dihydrotestosterone (40 Ci/mmol) and [1,2-^{3}H]dehydroepiandrosterone (50 Ci/mmol), were purchased from New England Nuclear (Boston, MA, U.S.A.). Dextran 70 from Pharmacia (Uppsala, Sweden), Norit-A charcoal from Amend Drug Chemical (Irvington, NJ, U.S.A.) (washed with 0.1 *M* sodium hydroxide solution and 0.1 *M* hydrochloric acid, neutralized and dried) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 3,3′-diaminodipropylamine-Sepharose CL-4B (DADAS) from Pierce (Rockfort, IL, U.S.A.). Electrophoresis reagents were obtained from Pharmacia and Sigma (St. Louis, MO, U.S.A.). All other chemicals were obtained from Sigma. Protein determinations were carried out by the method of Lowry et al. [11].

Animals

Reproductively active *Bufo arenarum* females (average mass 150 g) were transported to our laboratories and kept for 1 week in individual cages under controlled humidity conditions. After decapitation, blood was allowed to clot for 6 h. Sera were separated by centrifugation at 800 *g* for 15 min and immediately processed.

Assay for 5α-dihydrotestosterone (DHT) binding activity

Binding activity determinations were carried out with the charcoal assay, as described previously [10].

Synthesis of 5α-androstane-3β,17β-diol 3β-hemisuccinate

The synthesis of androstanediol hemisuccinate was carried out as described by Iqbal and Johnson [12].

Coupling of androstanediol hemisuccinate to diaminodipropylamine-Sepharose (DADAS)

A 20-ml volume of DADAS gel (approximately 20 μmol/ml of settled gel) were equilibrated with 70% dioxane. Androstanediol hemisuccinate (1 mmol) was dissolved in 10 ml of 70% dioxane and added to 20 ml of the DADAS. The pH was adjusted to 5 and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.5 g) was added to the mixture in two equal portions, 6 h apart. After the first 6 h, the mixture was poured into a column and the solution was recycled for 12 h at 25 °C. The substituted gel was then washed successively at room temperature on a Buchner funnel with 2 l of 70% dioxane, 2 l of 80% methanol, 2 l of 50% methanol and 2 l of water. The affinity adsorbent was diluted five-fold with unsubstituted Sepharose CL-4B. The mixture was poured on to a column (2.5 cm I.D.) and washed with buffer A (10 m*M* Tris–HCl, 50 m*M* $CaCl_2$, 150 m*M* NaCl, pH 7.4, at 25 °C).

Charcoal–Sepharose column

Activated charcoal (1 g) was added to a Sepharose CL-4B slurry (30 ml), the mixture was shaken for 10 min, decanted and the charcoal remaining in the supernatant was discarded. The procedure was repeated until no charcoal was detected in the supernatant. A solution of 10% Dextran 70 (50 mg) was then added to the slurry, shaken for 10 min and washed. The mixture was poured on to the column and washed with buffer A. The affinity column and the charcoal column were connected in series.

Ammonium sulphate fractionation of serum

Ammonium sulphate (saturated solution at 4 °C) was added gradually to a concentration of 57% saturation at 4 °C. The mixture was stirred for 6 h, protein precipitate was removed by centrifugation at 10 000 *g* for 30 min and the pellet was dissolved in half the original volume of buffer A. This solution was stirred overnight, centrifuged at 10 000 *g* for 30 min and the pellet discarded.

Biospecific affinity chromatography

The affinity column was cooled to 0–4 °C and the charcoal column was maintained at 25 °C in order to facilitate the steroid–protein dissociation. The SBP solution, partially purified by ammonium sulphate fractionation, was recycled through the system until a constant amount of SBP was detected in the recycled solution. The charcoal column was removed and the affinity column washed at 0–4 °C with buffer A containing 10% glycerol until the absorbance at 280 nm was constant. The affinity column was then eluted with buffer A containing 10% glycerol and 0.07 m*M* DHT (DHT buffer). The column was brought to room temperature by removing it from the cooling system and was eluted with an additional amount of DHT buffer until the absorbance at 280 nm remained constant. The active fractions were pooled, concentrated to 3–5 ml by sequential pressure ultrafiltration in TCF-10 and 8 MC cells (Amicon, Oosterhout, The Netherlands) using PM 10 membranes (Amicon) and dialysed against phosphate buffer (10 m*M*, DHT 0.07 m*M*, pH 7.4).

Preparative polyacrylamide gel electrophoresis

Preparative gel electrophoresis was performed with a Canalco Pre Disc apparatus according to the method described by Mickelson et al. [13]. The active fractions were pooled, concentrated to 3–5 ml, dialysed against DHT buffer and stored at 4°C.

Analytical polyacrylamide gel electrophoresis

Analytical disc gel electrophoresis was performed at 4°C using 7% T (2% C) gels according to the methods described by Davis [14] {T refers to the total percentage monomer concentration [acrylamide+bis(acrylamide)] and C refers to the concentration of bis(acrylamide) expressed as a percentage of T}. Samples (50 μl) containing 50–100 μg of protein were applied to the gels, which were stained with Coomassie Blue. SBP was located by running parallel steady-state polyacrylamide gels as described by Ritzen et al. [15], except that only one concentration of the steroid was used. Stacking and resolving gels were polymerized in the presence of 1 n*M* [^{3}H]DHT. The samples were incubated with 1 n*M* [^{3}H]DHT at 25°C for 15 min and then applied to the steady-state gels. After electrophoresis, the gels were sliced transversely with a gel slicer into 1 mm sections for counting.

Sodium dodecyl sulphate (SDS) gel electrophoresis

Analytical gel electrophoresis in the presence of SDS was performed according to the method described by Weber and Osborn [16], in 10% T (2.63% C) slab gels. Protein samples were denatured in 1% SDS, with or without 1% 2-mercaptoethanol, at 100°C for 5 min. Samples containing 0.5 μg of protein were applied to the gels. The gels were stained with silver according to the method described by Sammons et al. [17].

Determination of radioactivity

Aqueous samples were placed in scintillation vials containing 4 ml of scintillant (4 g/l of Omnifluor in toluene). The radioactivity was extracted from the aqueous phase in 12 h.

RESULTS

Synthesis of 5α-androstan-3β,17β-diol 3β-hemisuccinate and its coupling with Sepharose

The yield of androstanediol hemisuccinate was approximately 90% and the concentration of steroid covalently bound to the Sepharose was approximately 1–2 μmol/ml of packed gel.

Purification of SBP

In the first step, SBP was partially purified by ammonium sulphate precipitation. The purification was about 1.4-fold with a 76% yield (Table I). The next step in the purification was the affinity chromatography. Preliminary experiments showed that less than 1% of SBP was adsorbed to the affinity column.

TABLE I

PURIFICATION SCHEME FOR SBP FROM *BUFO ARENARUM*

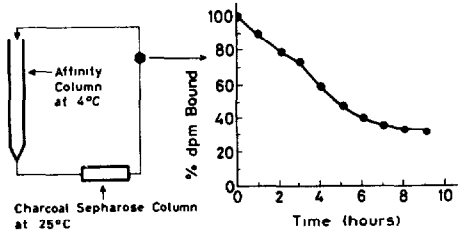
Protein was measured by the method of Lowry et al. [11].

Step	Total protein (mg)	Specific activity*	Yield in each step (%)	Total yield (%)
Serum, 120 ml	6720	7.6	100	100
Ammonium sulphate precipitation	3700	10.4	76	76
Affinity chromatography	29.4	715.6	55	41
Preparative PAGE	3.2	5656	86	36

*Specific activity is expressed as nanograms of DHT bound per mg of protein.

This low yield can be explained by the presence of free ligand that competed with the steroid attached to the matrix for SBP binding sites. The presence of the free ligand was detected by assessing the binding capacity of the eluted protein before and after charcoal treatment (data not shown). Therefore, a cyclic system was designed to remove the unbound steroid and improve the efficiency of the column.

The method is illustrated in Fig. 1 (left). During passage through the first column (the affinity column), SBP removed any steroid that could have been non-covalently attached to the Sepharose. During passage through the second column, the steroids were adsorbed by charcoal and the eluted proteins were applied again on the affinity column. By repeating this process several times, almost complete "washing" of the column was achieved, thus allowing maximal SBP binding. Prior charcoal treatment of the sample is not required using this method. The charcoal adsorption to the Sepharose matrix is necessary to obtain



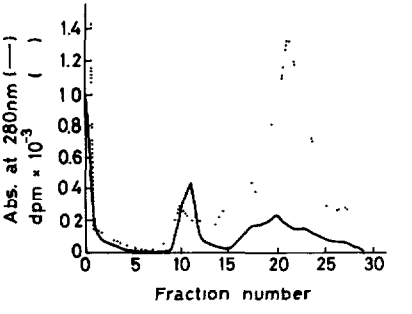


Fig. 1. Illustration of the cyclic BAC step. The sample containing SBP was recycled through the system until a constant amount of SBP was detected in the recycled solution. The charcoal column was then removed and the affinity column was eluted as shown in Fig. 2. The amount of DHT bound is expressed as a percentage of the initial DHT bound.

Fig. 2. Elution profile of SBP from the affinity column. Aliquots of 100 μl of each fraction were assayed for binding activity by the dextran-coated charcoal method. The column was washed with buffer A (plus 10% glycerol) at 4°C (fractions 0-7), then buffer A (plus 10% glycerol and 0.07 m*M* DHT) at 4°C (fractions 8-15) and finally at 25°C with the same buffer (fractions 15-30). The active fractions were pooled, concentrated and purified by preparative PAGE.

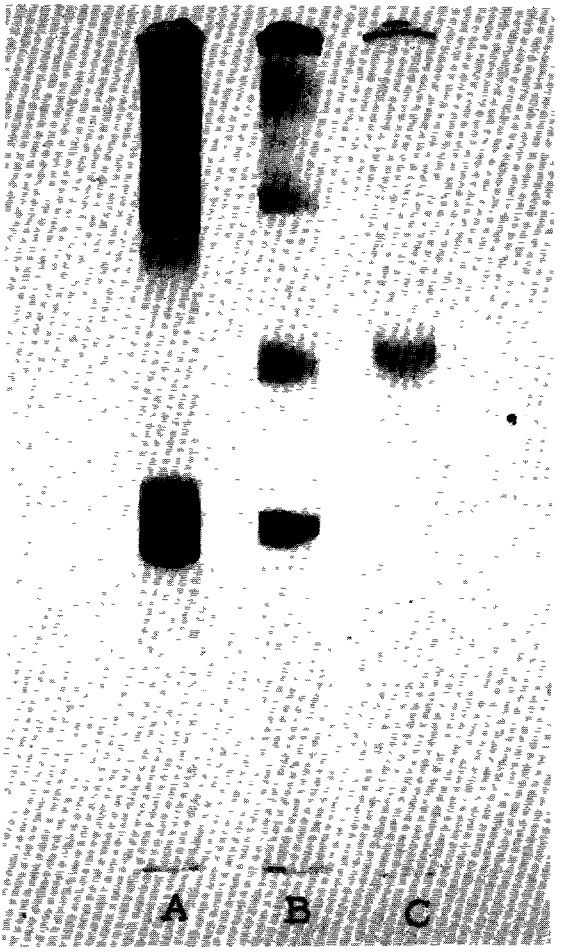
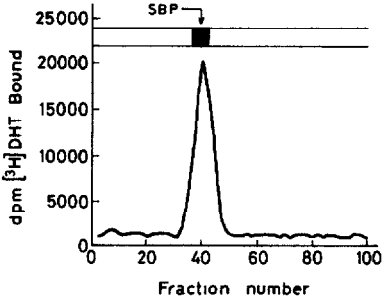


Fig. 3. Polyacrylamide gel electrophoresis: (A) serum; (B) DHT eluate from affinity column; (C) SBP from preparative PAGE.

high flow-rates. Fig. 1 (right) shows the binding activity of SBP detected in the recycled solution during the procedure. The maximal yield was achieved in about 9 h, by which time the system had reached equilibrium.

The mode of elution of SBP from androstanediol–Sepharose is shown in Fig. 2. Analytical polyacrylamide gel electrophoresis (PAGE) of the pooled active fractions revealed the presence of albumin and slowly migrating impurities as shown for gel B in Fig. 3. The active fractions were purified by preparative PAGE and the gel C in Fig. 3 represents the final state of purity of SBP. A single band was obtained after staining with Coomassie Blue. Single bands were also obtained in 4, 6, 8 and 10% T gels and by SDS-PAGE (not shown). Fig. 4 shows that the final preparation also gave a single band associated with [^{3}H]DHT on steady-state gel electrophoresis. The same R_F (0.4) was obtained when electrophoresis was performed on unfractionated serum. The molecular mass of the native SBP was reported to be 93 300 ± 3600 [10]. Here we report a molecular mass of 52 600



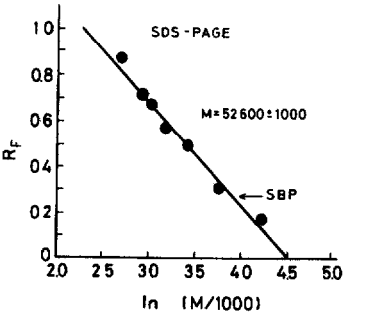


Fig. 4. Electrophoresis of purified SBP in 7% polyacrylamide gels containing 1 nM [^{3}H]DHT. Following electrophoresis, one gel was sliced into 1 mm segments for measurement of radioactivity. The mobility of SBP is plotted relative to that of the bromophenol blue (fraction 100). The parallel gel stained with Coomassie Blue (Fig. 3C) is illustrated at the top of the figure.

Fig. 5. Electrophoresis of purified SBP on 10% SDS polyacrylamide gel. The relative mobilities of the standards (albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 100; α-lactalbumin, 14 400; β-lactoglobulin, 18 400; trypsinogen, 24 000) are plotted against ln(molecular mass/1000). Each point represents an average of two determinations [mean ±S.D. (inter-assay, $n=2$)].

for the denatured protein (Fig. 5). This result suggests that the native protein would have two subunits. In the absence of mercaptoethanol, the same molecular mass (52 600) was obtained by SDS-PAGE, indicating that the subunits are not coupled through disulphide bonds.

Table I summarizes the scheme for the purification of *B. arenarum* SBP. The cyclic method allows the purification of at least 3 mg of homogeneous protein per preparation with total yield of 36%. A similar yield was obtained reproducibly in several experiments.

DISCUSSION

Early attempts to purify SBP revealed that conventional BAC procedures, using 5α-androstane-3β,17β-diol 3β-hemisuccinate as an adsorbent, failed to yield significant amounts of the protein. Several investigators have found that 3β-hemisuccinate derivatives are not efficient for the purification of human and rabbit SBP [3,5]. However, 5α-androstane-3β,17β-diol diacetate and similar compounds can be converted into the 17β-monoacetate by partial hydrolysis in weakly alkaline aqueous media [18]. Therefore, the low yield obtained with the 3β-derivatives probably arises from the high rate of hydrolysis of 3β-esters compared with that of 17β-esters. On the other hand, the presence, in the crude preparation, of some esterase activity more efficient for the hydrolysis of the 3β-position cannot be ruled out. Further work is required in order to clarify this point.

Regardless of the causes of the ligand release, the free steroid can compete effectively with the adsorbents avoiding SBP binding to the column. Taking into account that SBP from *B. arenarum* has a relatively low K_a (0.042/nM) and that the unavailability of the 17β-group on the steroid could produce less specific and

effective binding, we chose to synthesize a solid matrix with the steroid attached at the 3β-position instead of the 17β- or 17α-position, and to develop a method that avoids the problems associated with the ligand release.

The effectiveness of BAC for steroid binding proteins is not only related to the matrix stability. The coupled gel must be washed exhaustively to ensure complete removal of the material not covalently bound. Usually, complete removal of adsorbed material is very difficult and may require many days of continuous washing. In some instances, effective washing can be achieved only by passing through the column large amounts of crude preparation, with a consequent reduction in the final yield.

In order to circumvent these problems, we used a charcoal column connected in series to the affinity column, which allowed us to remove any steroid adsorbed on the matrix or released during the purification (including the endogenous steroid). The method is based on both the charcoal adsorption of the non-covalently-bound ligands from the affinity column by the protein to be purified and the amplification provided by the cyclic use of the system. The use of this technique improves the yield of SBP in this step from less than 1% by conventional BAC to 55–60%. After the purification on preparative gels, the protein was shown to be homogeneous by non-denaturant polyacrylamide gel electrophoresis as well as by SDS-PAGE.

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