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MAGNETIC DNA AFFINITY PURIFICATION OF ECDYSTEROID RECEPTOR

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Summary—A new method for rapid purification to near homogeneity of the ecdysteroid receptor (EcdR) from *Drosophila melanogaster* nuclear extract is presented. In the first step of the purification procedure the EcdR molecules were radiolabelled with [^{3}H]ponasterone A and the [^{3}H]ponasterone A–EcdR complexes were chromatographed under very mild conditions on Fractogel EMD TMAE(s) ion-exchanger. A 23-fold purified receptor was obtained which can be stored in liquid N_2 without loss of activity. The second step involved the use of a magnetic DNA affinity technique where the double stranded hsp 27 oligonucleotide containing EcdR binding sequence was biotin 5′-end labelled and bound to monodisperse superparamagnetic particles coated with streptavidin (Dynabeads M-280 Streptavidin) giving magnetic DNA affinity beads. The chromatographed EcdR–ponasterone A complexes were bound to the magnetic DNA affinity beads and by magnetic separation, wash and elution, a 29,000-fold enriched EcdR preparation was obtained within 1.5 h. This procedure can be applied for other EcdR sources with minor modifications.

INTRODUCTION

20-Hydroxyecdysone (20-OH-ecdysone) is a steroid hormone which is found in many invertebrates [1]. It is involved in the regulation of many developmental processes, such as insect moulting and puparation. As with other steroid hormones [2], specific receptors for 20-OH-ecdysone (EcdRs) have been identified [3]. However, in comparison to other steroid hormone receptors only fragmentary information concerning amino acid sequence of the EcdR is available. Recently, the DNA sequence of the *Drosophila melanogaster* EcdR was published [4] and the predicted amino acid sequence yielded 90,000 Da receptor which is contradictory to biochemical data where the EcdR has been shown to have a M_w of 120,000 Da [5, 6]. This discrepancy indicates the necessity to purify the EcdR and determine its amino acid sequence in *Drosophila* and other invertebrates. To aid in the purification of EcdR molecules from different sources the availability of a general method to purify to near homogeneity the EcdR is of great importance.

Based on our recent experiments on characterization of the specificity of EcdR–DNA complexes [6] we have developed an EcdR purification procedure using magnetic solid phase technology [7]. The method is extremely rapid and after some modifications can be used with different EcdR sources.

EXPERIMENTAL

Preparation of nuclear extracts and labelling with [^{3}H]ponasterone A(PonA)

Nuclear extracts were prepared from embryos of the Oregon R stock of *D. melanogaster* according to Heiermann and Pongs [8] with the exception that the final dialysis was done with buffer containing 50 mM KCl.

[^{3}H]PonA labelling of the nuclear extract, removal of the [^{3}H]PonA excess and the determination of the [^{3}H]PonA–EcdR concentration were done as described previously [9].

Oligonucleotides: synthesis and 5′-biotin labelling

Double stranded (ds) oligonucleotides were obtained by annealing of appropriate strands

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Abbreviations: ds, double stranded; EcdR, ecdysteroid receptor; hsp27, gene for heat-shock protein 27; PonA, ponasterone A; ss, single stranded.

synthesized with a Gene Assembler (Pharmacia) and purified as recommended by the manufacturer. The concentration of the oligonucleotides was quantified spectrophotometrically [6].

Complementary DNA strands were annealed in TE buffer containing 100 mM KCl at 75°C for 10 min followed by cooling to room temperature over a period of 2 h.

The synthesis of the 5′-biotin-labelled single stranded (ss) hsp 27_a oligonucleotide (5′GATCCAGACAAGGGTTCAATGCACTTGTCCAATGAA3′) containing a binding sequence for EcdR [6, 10] was carried out as follows. In the first step 6-(*N*-trifluoro-acetylo-amino)-hexyl-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (TFA-Aminolinker Amidite; Autoprimer Synthesis Kit, Pharmacia) was attached to the newly synthesized oligonucleotide (0.2 μmol). Since DNA was synthesized in a Gene Assembler, the TFA-Aminolinker Amidite was placed at a spare valve position on the synthesizer and was added to the 5′-end of the ss hsp 27_a oligonucleotide in the final cycle of the synthesis. After synthesis all protecting groups, including the TFA moiety on the linker, were removed by incubation with concentrated ammonia for 5 h at 55°C. After evaporation of ammonia the 5′-aminolinkered oligonucleotide was dissolved in 1 ml 10 mM Hepes/KOH pH 7.7 and chromatographed over a NAP 10 column (Pharmacia) equilibrated with the same buffer. Then the 200 μl sample containing approx. 30 nmol of the 5′-aminolinkered ss hsp 27_a oligonucleotide was mixed with 100 μl DMF solution of 2 mg biotin-*N*-hydroxysuccinimid at 20°C, overnight. The incubation mixture was subsequently chromatographed over a NAP 10 column equilibrated with TE buffer. Finally the 5′-biotinylated oligonucleotide was purified by HPLC chromatography. The elution of the biotinylated oligonucleotide on the Lichrosorb RP 18 column (Merck) was carried out with solution A (100 mM triethylammonium acetate buffer, pH 7.0, containing 5% acetonitryle) and solution B (100 mM triethylammonium acetate buffer, pH 7.0 containing 30% acetonitryle). The elution conditions were a linear gradient of 25 to 50% B in 70 min with a flow rate of 0.5 ml/min. The purified 5′-end biotin-labelled ss hsp 27_a oligonucleotide was annealed with complementary ss oligonucleotide as described above. The resulting ds hsp 27 oligonucleotide was subsequently used for affinity chromatography of the [^{3}H]PonA–EcdR (see below).

EcdR purification procedure

Unless otherwise stated all purification steps were carried out at 0–4°C and all buffers were prepared at 20°C.

Ion-exchange chromatography

Ion-exchange chromatography of the nuclear extract was carried out in a Pharmacia FPLC apparatus equipped with a HR 10 column. 2 ml of [^{3}H]PonA-labelled nuclear extract containing approx 8 pmol of [^{3}H]PonA–EcdR complexes were, after removal of the unincorporated [^{3}H]PonA, applied on the column containing 1.7 ml of Fractogel EMD TMAE(s) (Merck). The column was equilibrated with 20 mM Hepes/KOH, 50 mM KCl, 2 mM dithiothreitol, 10% (v/v) glycerol, pH 7.6 containing 1 mM $MgCl_2$. After application of the sample the column was washed with 4.5 ml of the same buffer without $MgCl_2$ followed by step-elution with 20 mM Hepes/KOH, 110 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 2 mM KH_2PO_4, 10% (v/v) glycerol, pH 7.6. The flow rate was 0.5 ml/min and 0.3 ml fractions were collected.

The fractions containing EcdR activity were pooled and concentrated 4-fold using the Centricon-30 concentrator (Amicon), frozen and stored in liquid N_2.

Affinity chromatography

Dynabeads M-280 Streptavidin (Dynal AS) were supplied as a suspension containing $6–7 \times 10^8$ beads/ml (10 mg/ml) in a phosphate buffered saline solution containing 0.1% bovine serum albumin and 0.02% NaN_3. Prior to the coupling of the ds biotinylated hsp 27 oligonucleotide (see above) 1 mg Dynabeads M-280 Streptavidin were washed with 1 ml 20 mM Hepes/KOH, 110 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 2 mM KH_2PO_4, 10% (v/v) glycerol, pH 7.6, containing 0.1% ovalbumin. The washing of the Dynabeads was performed with the aid of the Magnetic Particle Concentrator (Dynal AS) as recommended by the manufacturer and repeated six times. The prewashed Dynabeads M-280 Streptavidin were mixed with 52 pmol of 5′-end biotinylated ds hsp 27 oligonucleotide and incubated for 30 min at 0–4°C. In order to remove unbound DNA, the Dynabeads M-280 Streptavidin were washed three times with the same buffer using the Magnetic Particle Concentrator.

The concentrated material from three ion-exchange chromatographies was thawed, pooled

and incubated with 1 mg of Dynabeads M-280 Streptavidin at 0–4°C. After 30 min incubation the magnetic separation was performed and the beads were washed four times with 0.5 ml buffer 20 mM Hepes/KOH, 110 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 2 mM KH_2PO_4, 10 (v/v) glycerol, pH 7.6. Finally the Dynabeads M-280 Streptavidin were eluted with 50 μl of 20 mM Hepes/KOH, 10% (v/v) glycerol, pH 7.6, containing 400 mM KCl. After magnetic separation the sample containing [^{3}H]-PonA–EcdR was dialysed against 20 mM Hepes/KOH, 110 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 2 mM KH_2PO_4, 10% (v/v) glycerol, pH 7.6, using a Millipor VSW PO 1300 microfilter. The final material was stored in liquid N_2.

Polyacrylamide gel electrophoresis(PAGE)

One-dimensional SDS–PAGE was run in a discontinuous system [3% (w/v) stacking gel, 9% (w/v) separating gel] according to Laemmli [11]. Following electrophoresis proteins were stained with GELCODE Silver-Stain kit (Pierce) according to the manufacturer's instructions.

Gel retardation assay

One hundred and seventy picomoles of ds hsp 27 oligonucleotide was labelled using T4 polinucleotide kinase and [γ-^{32}P]ATP (NEN). The labelled oligonucleotide was separated from unincorporated nucleotide using NAP 10 column (Pharmacia) and stored at −20°C. The binding reactions were carried out in 20 μl at 4°C for 30 min in reaction mixtures containing approx. 0.09 pmol of the [^{3}H]PonA–EcdR complexes and 0.02 pmol [^{32}P]hsp 27 ds oligonucleotide in 20 mM Hepes/KOH, 110 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 2 mM KH_2PO_4, 10% (v/v) glycerol, pH 7.6. In some experiments incubation was carried out in the presence of 100-fold molar excess of specific competitor, i.e. of the non-labelled ds hsp 27 oligonucleotide or non-specific ds competitor: 5′GATCCCGAC-CGCCGGGCGCGGTGGCGCGT3′ (only one strand shown). Protein–DNA complexes were separated from protein-free DNA by non-denaturing 5.6% PAGE [12]. Gels were run at constant voltage of 120 V, dried and exposed at −70°C.

Radioactivity measurement

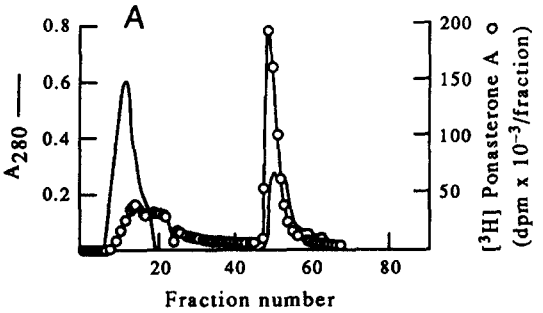
Radioactivity was measured as described previously [6].

RESULTS

Until now purification to homogeneity and the structural analysis of the EcdR have been impeded by low abundance and the fact that [^{3}H]PonA–EcdR complexes are very unstable [13]. This prompted us to develop a purification procedure which is fast and does not require high salt concentrations.

As a first step [^{3}H]PonA–EcdR is chromatographed on an ion-exchanger column. In view of the fact that elevated salt concentrations lead to dissociation of the [^{3}H]PonA–EcdR complexes followed by the irreversible denaturation of the receptor, the conditions of the chromatography were optimized as follows. Firstly, the Fractogel EMD TMAE(s) was used as a chromatographic material (Fig. 1A). According to the manufacturer the special feature of the Fractogel EMD TMAE(s) is that its charged groups are not directly bound to the matrix, but to the polymeric spacers. This implies that due to the mobile polymeric arms the matrix binds proteins without changing their native structure. Secondly, due to the optimization of the pH of the running buffer (data not shown) the salt concentration could be as low as 50 mM during the application of the nuclear extract and 110 mM KCl during the elution of the [^{3}H]-PonA–EcdR from the column. The material obtained from the column containing the highest concentration of the [^{3}H]PonA–EcdR was routinely stored in liquid N_2 until a sufficient amount was collected for further purification. Under these conditions we have not observed a significant reduction in the amount of [^{3}H]PonA–EcdR complexes in contrast to the initial experiments in which the partially purified [^{3}H]PonA–EcdR was stored at −25°C (data not shown). According to our previous observations [6] the material obtained from Fractogel EMD TMAE(s) columns does not contain significant amounts of other DNA binding proteins, therefore magnetic DNA affinity chromatography was used as a second purification step. The principles and general strategy for the use of magnetic DNA affinity particles in the purification of sequence specific DNA binding proteins were described by Gabrielsen *et al.* [7].

The ds hsp 27 oligonucleotide, which binds specifically to EcdR [6], was biotinylated at the 5′-end and was subsequently bound to the magnetic beads coated with streptavidin, resulting in a magnetic DNA affinity material with high



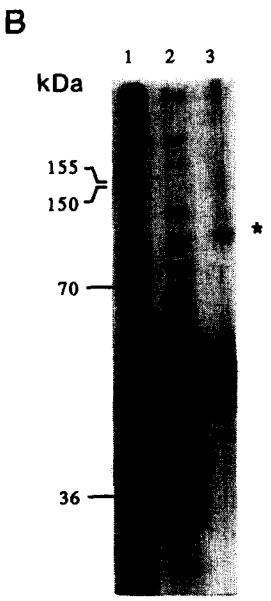


Fig. 1. A. Purification of the [^{3}H]PonA–EcdR, chromatography on the Fractogel EMD TMAE(s) column. Two millilitres of the *D. melanogaster* nuclear extract containing approx. 8.0 pmol [^{3}H]PonA–EcdR complexes were chromatographed over a PD 10 column in order to remove the excess of [^{3}H]PonA. The sample was subsequently applied to a HR 10 column which contained 1.7 ml of the Fractogel EMD TMAE(s) equilibrated with 20 mM Hepes/KOH, 50 mM KCl, 2 mM dithiothreitol, 10% (v/v) glycerol, pH 7.6 containing 1 mM $MgCl_2$. After washing with 4.5 ml of the same buffer without $MgCl_2$ the column was eluted with 20 mM Hepes/KOH, 110 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 2 mM KH_2PO_4, 10% (v/v) glycerol, pH 7.6. The flow rate was 0.5 ml/min and 0.3 ml fractions were collected. Fractions containing the highest [^{3}H]PonA–EcdR concentration were pooled, concentrated 4-fold, frozen and stored in liquid N_2 until sufficient amount of material was collected for affinity chromatography step. B. Purification of the [^{3}H]PonA–EcdR, electrophoretic analysis of the purification of the [^{3}H]PonA–EcdR. The efficiency of the purification and the protein composition during the purification procedure was analysed by SDS–PAGE [11]. The proteins were visualized using GELCODE Color Silver Stain kit (Pierce) and *E. coli* subunits of the RNA-polymerase were used as size markers indicated in kilodaltons (kDa). Lane 1, crude nuclear extract, 180 ng; lane 2, material after ion-exchange chromatography, 80 ng; and lane 3, 4 ng of the preparation obtained from affinity chromatography. The polypeptide corresponding to EcdR is indicated by the asterisk.

binding capacity. The EcdR containing protein preparation was bound to the beads, which are separated from other proteins remaining in the solution by placing a strong magnet against the wall of the tube. After a few washes with a buffer, EcdR was eluted by resuspending the beads in elution buffer, incubating and removing the beads by a magnetic separator.

One of the critical points in the purification using magnetic streptavidin beads is optimalization of the binding kinetics of the specific protein to the affinity beads [7]. The kinetics of the binding of the [^{3}H]PonA–EcdR complexes was monitored by measuring the amount of [^{3}H]PonA–EcdR [9] in the supernatant after magnetic removal of the beads. Similarly, as in the case of the τ-factor [7], the affinity beads rapidly removed the [^{3}H]PonA–EcdR from the solution; more than 50% after 2 min and about 70% after 5 min. Maximal binding was observed after 15–20 min (data not shown).

After the binding step the magnetic beads were washed in order to remove unbound proteins and the EcdR was eluted by resuspending the beads in a buffer containing 400 mM KCl for 20 min followed by removal of the beads by magnetic separation. In order to reduce high KCl concentration the final sample was dialysed using a microfilter (see Experimental).

The two-step purification scheme described above resulted in a 29,000-fold enrichment of the receptor (Table 1). Purification efficiency was analysed by SDS–PAGE (Fig. 1B). The silver stained gel shows a major 120,000 Da protein in addition to some other proteins of lower molecular weight. The low molecular weight protein contamination varied from one batch to another (data not shown). Our previous studies strongly suggest that the homogenous material of 120,000 Da is EcdR [5, 6].

When the purified EcdR was used in a mobility shift assay [12] with ds hsp 27 oligonucleotide, two DNA–protein complexes (indicated by b in Fig. 2) characterized by similar mobility were detected. EcdR–hsp 27 DNA complex formation could be prevented by molar excess of the specific competitor (Fig. 2, lane 5) but not by a competitor containing an unrelated sequence motif (Fig. 2, lane 6). We have previously shown that EcdR in the presence of ds hsp 27 oligonucleotide forms dimers whereas without specific DNA sequence it exists as a monomer [6]. This observation seems to be supported by results obtained in mobility shift experiments. Similarly as for glucocorticoid

Table 1. Purification of the [^{3}H]PonA–EcdR from the nuclear extract of *D. melanogaster*

Step	Total protein (mg)	Total activity (pmol [^{3}H]PonA bound)	Sp. act. (pmol [^{3}H]PonA bound/mg)	Purification (-fold)	Yield (%)
Nuclear extract	78.0	24.3	0.31	1	100
Fractogel EMD TMAE	0.72	5.0	6.94	22.4	20.6
Affinity chromatogr.	~0.0001	0.9*	9000	29,000	3.7

*This value is based on amount of the [^{3}H]PonA–EcdR complexes bound to the affinity beads. Values were not corrected for possible dissociation of [^{3}H]PonA–EcdR complexes in the 400 mM KCl elution buffer. The protein concentration was determined according to Bradford [19].

receptor [14] two DNA–protein complexes are formed which probably correspond to the monomer and dimer of EcdR bound to the ds hsp 27 oligonucleotide.

DISCUSSION

Since identification of the EcdR activity [15] various physico-chemical properties of this receptor have been characterized, using crude and partially purified preparations. Protocols for the purification of the receptor have been reported and the best enrichment factor obtained by means of classical chromatographic methods was 500- to 700-fold enrichment of the receptor from the K_c cells [13] or a 5000-fold enrichment from cytosolic extracts of pupal *D. melanogaster* could be obtained using calf-thymus DNA–cellulose chromatography [16]. The main problem of the purification procedures to date is the instability of the [^{3}H]PonA–EcdR complexes, which is especially pronounced at elevated salt concentrations and thus makes it difficult to monitor the EcdR binding activity [13]. This problem is not easily solved using classical chromatographic methods which are time consuming and frequently require elevated salt concentrations (e.g. 200 to 400 mM KCl). The introduction of DNA affinity chromatography using immobilized specific DNA fragments was a significant improvement that allowed the isolation of many sequence-specific DNA-binding transcription factors [17]. A further development of this affinity technique, was the magnetic DNA affinity purification methods, where column chromatography is substituted by a much more rapid and convenient magnetic solid phase separation [7]. Based on our recent experiments which defined the sequence of the EcdR response element [6], we were able to use a similar specific magnetic purification procedure based on specific interactions between EcdR and DNA. The receptor preparation obtained from ion-exchange chromatography was used as a starting material for the magnetic DNA affinity step. As indicated in the Results, the conditions of Fractogel END TMAE(s) chromatography were optimized for purification of [^{3}H]PonA–EcdR complexes. Firstly, a matrix was chosen which should not modify the native structure of the [^{3}H]PonA–EcdR complexes. Secondly, optimization of the pH of the chromatography buffer allowed us to carry out experiments under very mild salt conditions, i.e. 50–110 mM KCl. The EcdR preparation obtained after the ion-exchange step is stable, can be stored in liquid N_2 or be directly used for magnetic DNA purification which in comparison to classical DNA-affinity chromatography is a very simple and fast method. The binding of the [^{3}H]PonA–EcdR occurs in a few minutes, the magnetic separation takes seconds, and the elution step takes approx. 20 min. However, we have not carried out systematic experiments in order to optimize the elution time and it is possible that it can be speeded up. In our hands the preparation of EcdR of high purity was obtained in 1.5 h, including time

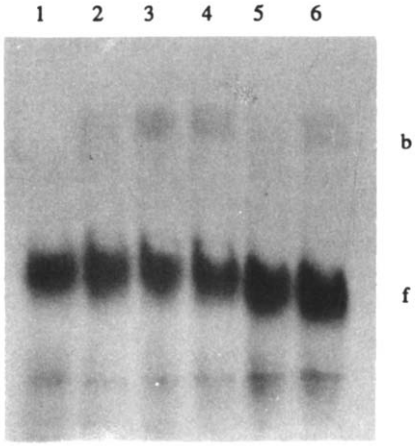


Fig. 2. Binding of the purified EcdR to the ds hsp 27 oligonucleotide. The purified EcdR was incubated with ^{32}P-labelled ds hsp 27 oligonucleotide as described under Experimental, either alone (lanes 1, 2, 3, 4) or in the presence of 100-fold molar excess of non-labelled ds hsp 27 oligonucleotide (lane 5) or non-specific competitor (lane 6). Samples were electrophoresed as described in Experimental. The EcdR amount used was 0.09 pmol (lanes 4, 5, 6) and 0.0, 0.02, 0.05 (lanes 1, 2, 3), respectively. b, f show the positions of bound (b) and free (f) DNA.

required for dialysis of the final preparation. In contrast, 2 days were required for the purification of EcdR from the *D. melanogaster* S3 cells [18]. We hope that our procedure will help in the future to solve the existing discrepancies between biochemically and molecular biologically characterized EcdR.

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