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Note

High-performance affinity chromatography of human progesterone receptor

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Obtaining a pure protein molecule can be a difficult task, yet it is often the only way in which new structural or regulatory information may be obtained. Affinity chromatography has proven to be a powerful tool in obtaining highly purified proteins in a single step with minimal elution volume while retaining the biological activity of the molecule[1].

High-performance liquid chromatography (HPLC) is a means of separating biological components rapidly and reproducibly due to the sophisticated technology and the availability of different stationary phases for specific separations. This technique has gained wide acceptance in many fields of study by providing data of high resolution while retaining small sample volumes. A thorough characterization and/or purification of a particular protein may now be carried out by HPLC alone.

One particular HPLC separation mode that has only recently found extensive application has been high-performance affinity chromatography (HPAC). We present here the first use of a commercially-available epoxide-reactive column matrix to purify high affinity (K_d = 0.1 n*M*) steroid-binding progesterone receptors by HPAC. This method takes advantage of the availability of the 21-hydroxy group contained in the synthetic progestin steroid 16-α-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione. The final product resulted in a functional affinity matrix which may be utilized for the affinity separation of progestin receptors with the retention of biological activity.

EXPERIMENTAL

Unlabeled and tritiated 16-α-ethyl-21-hydroxy-19-nor[6,7-^{3}H]-pregn-4-ene-3,20-dione (Organon 2058, 52 Ci/mmol) [2,4,6,7-^{3}H]estradiol and [^{35}S]methionine (1280 Ci/mmol) were purchased from Amersham. The Ultraffinity-EP column was obtained from Beckman. All other chemicals were analytical-grade or better.

HPAC separations were performed using an LKB GTi 2150 HPLC system attached to a Model 2152 controller. All solvents were filtered trough 0.45-μm HA Millipore filters prior to use.

Incubation with saturating amounts of tritiated steroid plus or minus a 200-fold excess of unlabeled steroid to assess non-specific binding was performed at 4°C as previously described[2]. Specific receptor binding is defined as the total minus non-specific binding after adsorption of excess, non-protein bound steroid with dextran-coated charcoal and expressed as fmol/mg protein.

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Human breast cancer cells or human uterine tissue were the two sources of tissue used for progesterone receptor purification. T47D human breast cancer cells[3] were cultured at 37°C in Earle's minimal essential medium (MEM) with 10% fetal calf serum and containing 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. Incorporation of [^{35}S]methionine into proteins was as described in ref. 4.

Human uteri obtained at surgery from hysterectomies were used as alternative starting material and these were stored under liquid nitrogen until use. All purification procedures were performed at 4°C unless otherwise indicated. A standard cytosol fraction in 10 m*M* Tris, 2 m*M* EDTA, and 1 m*M* dithiothreitol, pH 7.6 (buffer A) was prepared by homogenization and ultracentrifugation (100 000 *g*) as previously described[2]. This was either used directly or more commonly, receptors from uterine tissue were prefractionated and concentrated from DEAE-Sephacel (0.1 ml bed volume per mg protein) in a batchwise fashion. After pouring into a column and washing thoroughly with buffer A containing 80 m*M* sodium chloride, receptors were collected in a solution of buffer A containing 0.5 *M* sodium chloride. The yield of receptor at this stage was 70% and progesterone receptor concentrations were five-fold enriched. Cytosol fractions from the cultured cells were used directly.

A 50 × 4.6 mm I.D. Ultraffinity-EP column (0.5 ml volume) was used for all separations. The epoxide-activated silica is supplied ready to use. The affinity matrix was prepared by recycling 100 nmol Organon 2058 (7 μl of a 5 mg/ml acetonitrile solution) in 10 ml acetonitrile–water (50:50) at a flow-rate of 0.2 ml/min for 24 h at 25°C according to the manufacturer's instructions. Any remaining reactive groups were blocked overnight (25°C) with 0.5 *M* β-mercaptoethanol in 0.1 *M* potassium phosphate, pH 6.5. Following extensive washing with buffer A the column was ready for use.

Protein solutions were filtered through a 0.45-μm membrane filter before pumping onto the affinity column. The mixture was pumped through the solvent inlet line at 0.05 ml/min.

The column was then washed at 1 ml/min with buffer A for 2 h. Elution was performed by injecting 1.0 ml of a solution containing 10% N,N-dimethylformamide, 0.5 *M* sodium thiocyanate, 20 μ*M* [^{3}H]Organon 2058 (diluted to 1 Ci/mmol) in buffer A, stopping the flow, and warming the column to 25°C for 1.5 h.

The column temperature was then decreased to 4°C when the flow was started and 0.5 ml fractions of eluate were collected. The column was subsequently cleaned with ten column volumes of 0.2 *M* glycine, 10% N,N-dimethylformamide, 0.5 *M* sodium thiocyanate, pH 3.5, followed by washing with distilled water overnight. The column was re-equilibrated for 1 h with buffer A just prior to re-use.

Denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemmli[5] and protein content determined by the Bradford dye-binding assay[6].

RESULTS

The binding of the progesterone receptor to the column matrix was rapid: only one pass of sample through the column was required to bind 90–100% of receptor (Table I). Under the same conditions, the estradiol-17β receptor, possessing little

TABLE I

HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY OF HUMAN UTERINE PROGESTERONE RECEPTOR

Experiments 1 and 2 refer to data generated from two separate attempts at receptor purification.

Sample	*Experiment*	*Volume (ml)*	*Progesterone receptor**	*Recovery (%)*	*Estrogen receptor**	*Recovery (%)*
DEAE-Sephacel	1	75	15763	(100)**	10852	(100)
eluted fraction	2	100	33170	(100)	4463	(100)
Affinity column	1	75	953	8	7784	91
flow through	2	100	0	0	5044	113
Affinity column	1	0.5	6678	43	–	–
eluted fraction	2	0.5	16702	50	–	–

* Determined by association with saturating concentrations of radioactive ligand according to ref. 2 and expressed as total number of femtomol of receptor protein.

** 100% Recovery is taken here to indicate the first step in the affinity purification procedure.

affinity for progestins, was unable to compete with progesterone receptor for the matrix. Once progesterone receptor was bound, exhaustive washing was continued and elution of receptor could only be accomplished in the presence of free steroid. Recoveries were consistently in the range of 40 to 50% as long as the chaotropes thiocyanate and N,N-dimethylformamide were included in the elution buffer and to less than 10% in their absence. The 116000-dalton polypeptide thus observed after sodium dodecyl sulfate gel electrophoresis from affinity column eluates (Fig. 1) agrees with reported values[7] for the mass of the intact progesterone receptor and was not present if unlabeled Organon 2058 was incubated with the sample prior to HPAC. The

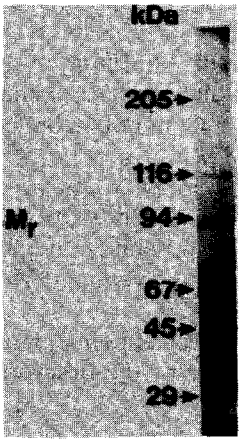

Fig. 1. Autoradiography of affinity purified ^{35}S-labeled progesterone receptor from T47D human breast cancer cells. [^{35}S]methionine (200 μCi/ml) was incorporated into total cellular proteins over 6 h in culture as described in ref. 4. Affinity-eluted proteins were separated by gel electrophoresis using a 7.5 to 15% polyacrylamide gradient. The gel was fixed and impregnated with fluor, dried down, and exposed to an X-ray film at −70°C. Lane 1 shows the elution pattern of proteins obtained from the HPAC column. Progesterone receptor migrates as a 116000-dalton polypeptide. kDa = kilodalton.

degree of purification obtained through one affinity step from the crude protein mixture used as starting material was satisfactory although at least a dozen other bands were also present in the sample shown in Fig. 1. Although the absolute capacity of the column was not measured, in one experiment 234 pmol of progestin receptor as determined by ligand binding was purified from human uterus with 45% recovery (data not shown).

DISCUSSION

The recent availability of this epoxide reactive silica has allowed the efficient coupling of small molecules or proteins to the solid matrix under very mild conditions[8]. The only requirement is that the molecule contains a free sulphydryl, primary or possibly secondary amino, or hydroxyl group. In cases where affinity interactions are high, *i.e.* $K_d \leq 1.0$ n*M*, reduced ligand concentrations as used here (100-fold less concentration than manufacturer's recommendation) for column derivatization have proved to be beneficial since high ligand concentrations on the support dictate that high concentrations of eluting ligand must be used to elute the binding protein. In the present application, a desirably small elution volume was possible with the affinity column. Greater utility in a preparative sense might be obtained if the 100 × 10 mm I.D. column is employed although the larger derivatizable column and amount of radioactive steroid needed for elution may prove considerably more expensive.

As presented here, attachment via the 21-hydroxyl of Organon 2058 to the solid silica support maintained the activity of the steroid for successful binding of the progesterone receptor. Although an affinity constant was not measured, this retention of steroid binding ability by the receptor protein and the harsh conditions required for elution indicate that biological activity was maintained although the receptor was probably recovered in the smaller dissociated form instead of the native, hetero-oligomeric form[9]. There also did not appear to be any proteolytic degradation of the receptor protein. The potential affinity interaction of the column with other steroid receptors, however, remains a possibility although the gluco-corticoid receptor's affinity for progestational agents is 10–100 fold lower than for gluco-corticoids[10]. Interaction of the androgen receptor with progesterone is similarly inefficient[11]. Inclusion of the appropriate unlabeled steroid to samples just before affinity purification could also be used to block any potentially interfering binding sites.

In conclusion, affinity isolations of progesterone receptor on both large and small samples have been performed with ease and completed in 3–4 h. This HPAC method has found particular application in this laboratory in the analysis of post-translational modifications (phosporylation) related to receptor function[12].

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