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ESTROGEN SYNTHETASE (AROMATASE). AFFINITY PURIFICATION OF ANTIBODY AGAINST THE CYTOCHROME *P*450 COMPONENT

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Summary—To procure an affinity gel capable of purifying antibody against the cytochrome *P*450 component of estrogen synthetase ($P450_{ES}$), we coupled purified $P450_{ES}$ to agarose supports. Our purpose was to compare two differently-activated agarose gels (Affi-Gel 15 and Tresyl-activated Sepharose) with the same $P450_{ES}$ preparation to compare the efficiency of coupling and the yield of purified antibody. Using supplier-directed protocols to covalently attach $P450_{ES}$ to each of the supports, and identical procedures to bind and elute anti-$P450_{ES}$, we reached the following conclusions. Tresyl-activated Sepharose bound greater amounts of antigen than Affi-Gel 15 based on the amount of residual antigen after the coupling procedure and the amount of bound antigen detected by an ELISA-type method. However, both ligand-coupled supports yielded comparable amounts of purified anti-$P450_{ES}$ at a 48-fold purification relative to the starting IgG preparation.

INTRODUCTION

Estrogen synthetase (aromatase) is the cytochrome *P*450-dependent enzyme responsible for estrogen biosynthesis. This enzyme is reported to be localized in specific areas of several tissues with relatively high aromatase activity [1–3], and its activity can be modulated within these and other estrogen-secreting tissues by a variety of regulatory factors [4]. Studies of the tissue localization and the mechanism of regulation of this enzyme require immunologic reagents specific for, and interactive with, this enzyme. Difficulties in examining these questions by immunotechniques arise in tissues containing particularly low basal levels of aromatase, such as testis, endometrium, brain and skin [5–8]. The use of monospecific antibodies is crucial for definitive immunocytochemical studies and in other techniques where physical separation of the antigen of interest is either not possible, or impractical. Monoclonal antibodies prepared against the cytochrome *P*450 component of aromatase ($P450_{ES}$)‡ have been described [9, 10]. However, their dependence on a single epitope sometimes limits their utility, particularly in protocols involving protein denaturation [11]. For that reason, monospecific polyclonal antibodies are, in general, more versatile reagents.

Monospecific polyclonal antibodies can be obtained either by injection of homogeneous antigen, or by affinity chromatography of the antiserum using highly purified enzyme coupled to an agarose gel. Even with affinity chromatography using as ligand the same highly purified antigen preparation, cross-reacting antibodies can be removed from the antiserum to improve the overall immunospecificity. This was dramatically illustrated by Vickery and Kellis [12] for $P450_{ES}$ in a Western blot of detergent-solubilized human placental microsomal proteins. Also, even if homogeneous antigen is used, IgG specific for the antigen can be obtained by affinity chromatography.

Although there are several reports of affinity purification of antibodies against various forms of *P*450, particularly from mammalian hepatic tissues [13, 14], we were unable to locate any systematic study comparing the coupling process for a *P*450 to differently-activated agarose gels, or independent assessment of the coupling efficiency. The purpose of this report is to (a) compare the coupling of purified $P450_{ES}$ to Affi-Gel 15 or Tresyl-activated Sepharose, (b) describe an ELISA-type assay which rapidly confirms the presence of coupled antigen, and (c) compare purification of anti-$P450_{ES}$ using purified antigen bound to the two differently-activated agarose gels.

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‡"$P450_{ES}$" is used here, rather than the more frequent designation "P450arom", since "estrogen synthetase" describes the function of this particular enzyme with greater specificity than the term "aromatase" [22].
Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; $P450_{ES}$, cytochrome *P*450 component of estrogen synthetase (aromatase); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; tSF, alpha toluenesulfonyl fluoride.

EXPERIMENTAL

Materials

Octyl-Sepharose CL-4B and DEAE-Sepharose CL-6B were purchased from Pharmacia. Bio-Rad supplied Affi-Gel 15 and DEAE-Affi-Gel Blue. Cholic acid, NADPH, diethanolamine and Tresyl-activated Sepharose 6B came from Sigma. Kao-Atlas supplied Emulgen 913 and BCA came from Pierce. HA-Ultrogel was purchased from IBF Biotechniques and leupeptin and pepstatin came from Chemicon. Cooper Biomedical supplied the alkaline phosphatase conjugated affinity purified goat anti-rabbit IgG.

Methods

Purification of $P450_{ES}$. Two different preparations of $P450_{ES}$ were used in this study: a partially purified preparation was used to obtain the antisera, and a highly purified preparation was used for coupling to the agarose gels.

Procurement of the partially purified $P450_{ES}$ was described [15]. This preparation contained a single major band at 53 K and several minor bands, as seen by silver staining after SDS–PAGE. The highly purified $P450_{ES}$ preparation was prepared as described [16]. All buffers contained 20% glycerol, 1 mM EDTA, 0.1 mM DTT, 2 μM androstenedione, adjusted to pH 7.4, and all purification steps were carried out at 4°C. Briefly, soluble placental microsomal proteins in buffer A (40 mM potassium phosphate, 1% Na cholate, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 68 μg/ml tSF) were applied to an octyl-Sepharose column (13 × 1.2 cm) and, after washing, the bound fraction containing $P450$ was eluted with a 0–1% Emulgen 913 gradient in buffer A at about 0.15% Emulgen 913. The $P450$ preparation was dialyzed against buffer B (10 mM potassium phosphate, 0.05% Emulgen 913) before application to the DEAE–Sepharose column (18 × 1.2 cm) equilibrated in buffer B. Part of the $P450_{ES}$ eluted during the isocratic wash and the remainder eluted during the 10–500 mM potassium phosphate buffer gradient in buffer B at 42 mM phosphate. The latter preparation was dialyzed against buffer C (10 mM potassium phosphate, 0.1% Emulgen 913) before application to the HA-Ultrogel column (14 × 1.2 cm) equilibrated in buffer C. After washing, the $P450_{ES}$ was eluted during a 10–500 mM potassium phosphate buffer gradient in buffer C at 42–46 mM phosphate. The $P450_{ES}$ preparation was concentrated 31-fold and adjusted to 100 mM potassium phosphate, pH 7.4. The $P450$ specific content was 2.8 nmol/mg protein. This preparation was at least 95% pure, based on silver-stained SDS–PAGE, with a single major band at 55 K* and a single minor band at about 90 K (see Fig. 1, lane 1). Aromatase activity (V_{max} of 39.8 nmol/min/mg protein and K_m of 43.7 nM) was obtained when $P450_{ES}$ was mixed with phosphatidylcholine (10 μg), saturating amounts of purified placental microsomal NADPH-cytochrome $P450$ reductase (27.4 nM), and [1B-^{3}H,4-^{14}C] androstenedione (6–129 nM).

Antibody procurement. A female rabbit (3 kg) was injected into two popliteal lymph nodes with 19 μg protein (partially purified $P450_{ES}$ preparation) in complete Freund's adjuvant [17]. The rabbit was boosted i.m. at six weeks with 30 μg protein and at sixteen weeks with 15 μg protein. The IgG preparation of serum containing potent aromatase-inhibition ability [15], PHPM4 (No. 304), was obtained using the DEAE Affi-Gel Blue protocol (Bio-Rad bulletin 1062).

Antigen coupling to agarose. Affi-Gel 15 (300 μl of gel; binding capacity approx. 10 mg BSA) was washed by centrifugation in cold deionized water before incubating with purified $P450_{ES}$ (188 μg protein) in 300 μl of 67 mM phosphate buffer, pH 7.4, for 4 h at 4°C. The bound ligand was separated by centrifugation, and the supernatant was used to determine the relative amounts of residual ligand. The antigen-coupled gel was incubated with 300 μl of 1 M diethanolamine at 4°C to block any remaining reactive groups, and then washed several times with 100 mM potassium phosphate buffer, pH 7.4. Tresyl-activated Sepharose (75 mg; equivalent to 300 μl of hydrated gel; binding capacity approx. 2.4 mg chymotrypsin) was hydrated in 1 mM HCl, and washed several times by centrifugation in 100 mM phosphate buffer, pH 8, containing 0.5 M NaCl. The Tresyl-activated Sepharose gel was then incubated with purified $P450_{ES}$ as described for Affi-Gel 15, except the coupling reaction was carried out at 21°C. The residual ligand was separated by centrifugation, and the antigen-coupled gel was blocked with diethanolamine and washed as described above.

Detection of agarose-bound antigen. The association of antigen with agarose was verified using an ELISA-type assay. Aliquots (30 μl) of coupled and control (freshly prepared gels) agarose preparations were incubated with rocking at 21°C for 1 h each with 5% BSA (in TBS buffer: 20 mM Tris–Cl, 500 mM NaCl, pH 7.4), then with anti-$P450_{ES}$ IgG (PHPM4 (No. 303); 52 μg protein) in 5% BSA. The gel samples were washed three times by centrifugation in 1% BSA before incubating at 21°C with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2000 dilution) for 1 h followed by extensive washing in TBS. The alkaline phosphatase color reaction was developed using *p*-nitrophenyl phosphate as substrate [18] and measured by $A_{410\,nm}$.

Antibody purification. An aliquot (300 μl) of $P450_{ES}$-coupled gel (Affi-Gel 15 or Tresyl-activated Sepharose) was incubated with PHPM4 (No. 304) IgG (2.3 mg protein) in 67 mM phosphate buffer,

*In our hands, different preparations of $P450_{ES}$ separated by SDS–PAGE at different times run with or slightly ahead of the 55 K marker protein indicating some ambiguity in the molecular weight between about 53 and 55 K.

pH 7.4, for 1 h at 21°C. Unbound anti-$P450_{ES}$ was separated by centrifugation, and the gel pellet was washed three times with 67 mM phosphate buffer, pH 7.4. Bound antibody was eluted by incubating the washed gel with 0.2 M glycine, pH 2.7, for 1 min, and quickly centrifuged before neutralizing the supernatant with 1 M Tris–Cl, pH 8.

Miscellaneous procedures. The procedure used for SDS–PAGE, silver staining and Western blotting were described previously [15]. Protein was determined using the BCA micro assay [19]. Aromatase specific inhibitory activity is defined as the fractional inhibition (in per cent of control) of placental microsomal aromatase activity per milligram of antibody protein. A similar measure of antibody specific inhibitory activity has been described [20].

RESULTS AND DISCUSSION

Preparation and detection of agarose-coupled $P450_{ES}$

An affinity column for $P450_{ES}$ was prepared by covalently attaching the highly purified $P450_{ES}$ to either Affi-Gel 15 or Tresyl-activated Sepharose, as described in Methods. The relative coupling efficiency between antigen and solid support was assessed in two ways: (a) by comparing the amounts of unbound antigen protein remaining after the coupling procedure, and (b) comparing the amounts of antigen protein coupled to the gel. To determine the residual antigen protein, the amount of protein in equivalent aliquots of the supernatant at the end of the coupling procedure were compared with the amount in the starting material by SDS–PAGE and aromatase reconstitution. Most (>90%) of the $P450_{ES}$ added to the coupling reaction was transferred to the agarose gel, as shown by the small amount of $P450_{ES}$ detectable by SDS–PAGE in the supernatant (Fig. 1) after the coupling reaction relative to the initial amount. The apparent loss in soluble $P450_{ES}$ protein is supported by the loss of aromatase reconstitution activity using the supernatant as the source of $P450_{ES}$ (aromatase activity before coupling was 7662 pmol/min, and in equivalent aliquots after coupling: Affi-Gel 15–120 pmol/min; Tresyl-activated Sepharose—161 pmol/min). Less $P450_{ES}$ was detected by SDS–PAGE in the supernatant from the Tresyl-activated Sepharose, suggesting a greater degree of coupling between ligand and this particular activated support.

$P450_{ES}$ bound to the agarose gels was detected using an ELISA-type assay. Control and antigen-coupled agarose gels were blocked with BSA, incubated with anti-$P450_{ES}$ IgG, and washed thoroughly before incubating with alkaline phosphatase-linked second antibody. The $A_{410\,nm}$ after conversion of the alkaline phosphatase substrate to *p*-nitrophenol for $P450_{ES}$ bound to agarose were: Affi-Gel 15: antigen coupled—1.347, control—0.284; Tresyl-activated Sepharose: antigen coupled—2.70, control—0.48. These results show that the color intensity difference between coupled and control gels is greater for the Tresyl-activated Sepharose than Affi-Gel 15, again suggesting that more ligand is bound to the Tresyl-activated Sepharose gel.

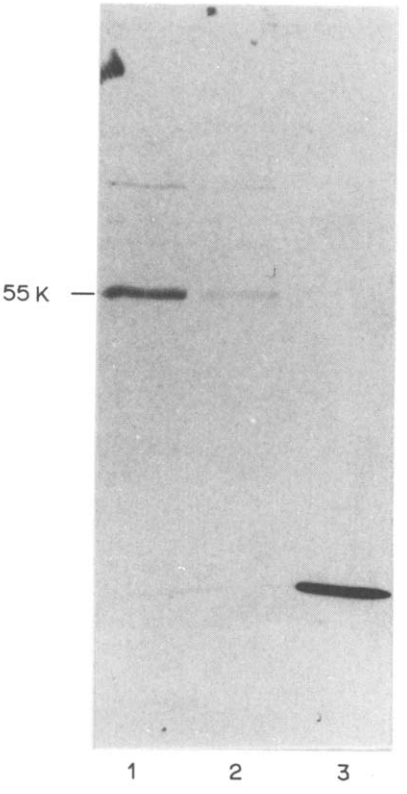

Fig. 1. Assessment of residual $P450_{ES}$ after coupling to the affinity gel matrix. Purified $P450_{ES}$ was subjected to SDS–PAGE before (lane 1: 1 μl, 0.6 μg protein) and from the supernatant after the covalently bound $P450_{ES}$-agarose was separated from the unbound $P450_{ES}$ by centrifugation (4 μl of supernatant; lane 2: Affi-Gel 15, lane 3: Tresyl-activated Sepharose). After electrophoresis, the proteins were visualized by silver staining. The position of a 55 K protein marker after co-electrophoresis on an adjacent lane is indicated on the left side of the figure.

Affinity purification of antibody against $P450_{ES}$

Anti-$P450_{ES}$ was obtained by injecting partially purified $P450_{ES}$ into rabbits and isolating the IgG fraction from the immune serum that contained aromatase-inhibiting activity. The IgG preparation recognized placental microsomal $P450_{ES}$ based on its ability to strongly inhibit aromatase activity and to recognize a 55 K protein on Western blots of solubilized placental microsomal proteins [15].

This IgG preparation was incubated with each $P450_{ES}$-coupled gel as described in Methods. The gels were then thoroughly washed, and the specifically bound antigen was eluted with glycine, pH 2.7. The relative amounts of unbound antibody remaining at the end of the incubation, and the relative amounts of glycine-eluted antibody were assessed by antibody-dependent aromatase inhibition assay and by Western blot of SDS-solubilized placental microsomal

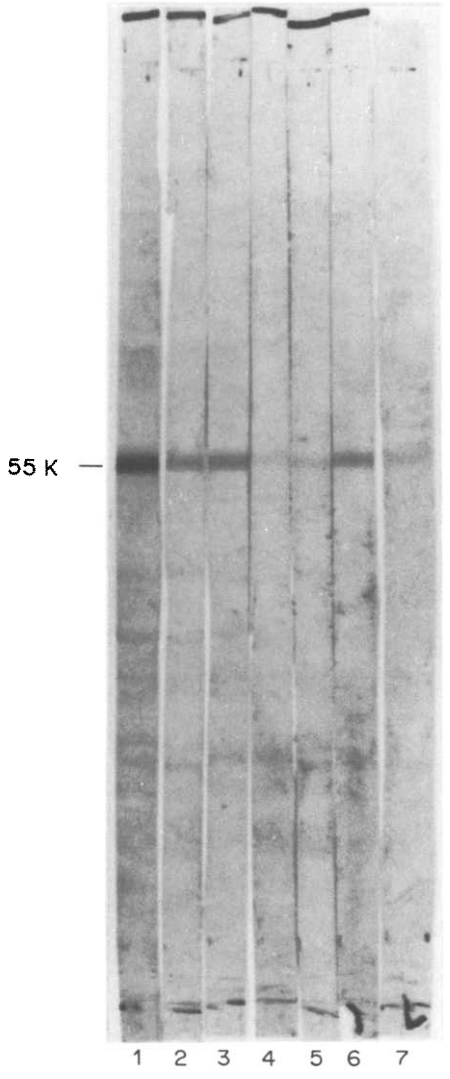

Fig. 2. Detection of affinity purified anti-$P450_{ES}$. The ability of anti-$P450_{ES}$ to immunostain $P450_{ES}$ in placental microsomes was determined using the starting IgG preparation (lane 1) and after various steps in the affinity purification protocol (lanes 2–4, Affi-Gel 15; lanes 5–7, Tresyl-activated Sepharose). Lanes 2 and 5 affinity gel flowthrough (unbound anti-$P450_{ES}$); lanes 3 and 6, first glycine elution; lanes 4 and 7, second glycine elution.

proteins. The Western blot analysis (Fig. 2) showed that not all of the anti-$P450_{ES}$ bound to the affinity gels, and that the amount of unabsorbed antibody (anti-$P450_{ES}$ in the flow-through fraction) was greater when using the $P450_{ES}$-Affi-Gel 15 gel. This result is also consistent with a greater degree of bound ligand on the Tresyl-activated Sepharose. The final two wash fractions contained no antibody detectable by Western blot (data not shown). The first glycine elution for both Affi-Gel 15 (Fig. 2, lane 3) and Tresyl-activated Sepharose (Fig. 2, lane 6) gave comparable levels of released antibody, while the second glycine elution yielded only trace amounts for each affinity support (Fig. 2, lanes 4 and 7, respectively). We conclude therefore that, although the Tresyl-activated Sepharose appears to bind the greater amount of ligand, both supports are equally effective in the actual purification of antibody.

The extent of antibody purification was determined by measuring the aromatase specific inhibitory activity in the starting anti-$P450_{ES}$ preparation and in each affinity-purified antibody preparations, using the standard anti-$P450_{ES}$-dependent aromatase inhibition assay [15]. In this manner, we determined that both affinity gels gave approximately equal amounts of anti-$P450_{ES}$, with a 48-fold purification of antibody (Table 1).

The emphasis of this work is on the methodology to procure purified polyclonal IgG specific for $P450_{ES}$. We argue in the Introduction that the highly specific monoclonal antibodies are, in general, less versatile than polyclonal antibodies—particularly for techniques that may modify by protein denaturation the epitope recognized by the monoclonal antibody. Our affinity-purified polyclonal antibody preparation appears to be comparable to the affinity-purified preparation described by Vickery and Kellis[12], and may be of better quality than the polyclonal preparations of Mendelson *et al.*[9] obtained without affinity purification of antisera generated by injecting the 55 K protein band (after recovery from SDS–PAGE) from a partially purified $P450_{ES}$ preparation. The polyclonal antisera against $P450_{ES}$ described by

Table 1. Immunoinhibition of placental microsomal aromatase at various steps of purification of anti-$P450_{ES}$[a]

Anti-$P450_{ES}$ fraction	Relative aromatase activity[b] (%)	Relative inhibition (%)	Anti-$P450_{ES}$ protein amount (μg)	%inhibition / μg protein
Control	100	0	0	—
Starting IgG preparation	38.9	61.1	116	0.525
Affinity column				
Flowthrough	93.8/84.6	6.2/15.4	95.4/104	0.065/0.15
Wash 1	80.7/84.0	19.3/16.0	24.1/26.6	0.8/0.6
Wash 2	88.7/89.7	11.3/10.3	2.5/4.7	4.52/2.2
Wash 3	86.0/96.5	14.0/3.5	ND/ND	—/—
Glycine elution 1	69.5/62.5	30.5/37.5	1.24/1.47	24.6/25.5
Glycine elution 2	86.2/86.0	13.8/14.0	0.46/0.615	30/22.8

[a]From the affinity column flowthrough down through the rest of the table, the data is presented as the results for Tresyl-activated Sepharose/Affi-Gel 15. [b]An aliquot (50 μl) of the fraction was used in the standard aromatase inhibition assay [15] from 1 ml total volume for the starting IgG preparation through wash 3, and 1.2 ml total volume for the glycine elutions 1 and 2. ND = not detectable.

Kitawaki *et al.*[21] was prepared using immunoaffinity purified $P450_{ES}$ as antigen. It is difficult to compare the immunospecific properties of their crude antisera with our purified IgG preparation due to the low resolution of their Western blot staining.

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