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Affinity chromatography of human estrogen receptor-α expressed in *Saccharomyces cerevisiae* Combination of heparin- and 17β-estradiol-affinity chromatography

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Abstract

Estrogen receptor-α is a member of the nuclear hormone receptor superfamily and is considered as a very important regulatory protein. Human estrogen receptor-α has been cloned into *Saccharomyces cerevisiae* as a fusion to ubiquitin and expression is controlled by a metallothionin promotor. Pilot scale quantities of receptor have been produced by a yeast strain transformed with expression plasmid YEpE13 [Graumann et al., J. Steroid Biochem. Mol. Biol. 57 (1996) 293] in a 14 l stirred tank reactor. The yeast extract contained 2–4 pmol of receptor protein per mg total protein. A purification scheme has been developed using heparin-affinity chromatography combined with affinity chromatography with immobilized 17β-estradiol 17-hemisuccinate. Heparin-affinity chromatography was very efficient to remove host cell protein. Accompanying proteins that stabilize unoccupied receptor have not been dissociated during elution. The receptor could be purified 5–10-fold in ligand-free state. In contrast to previous reports, we did not find a difference of the binding affinity of liganded and unliganded receptor for heparin immobilized onto Sepharose. The unoccupied receptor could be further purified 100-fold with ligand-affinity chromatography using 17β-estradiol 17-hemisuccinate–bovine serum albumin–Sepharose. The receptor could be kept in its native state, although saturated with 17β-estradiol. The purification sequence allows an efficient production of receptor. Further improvement of productivity can be only accomplished by increasing the expression level.

Keywords: *Saccharomyces cerevisiae*; Affinity chromatography; Proteins; Steroid hormone receptors

1. Introduction

Human estrogen receptor-α (ERα) plays a key role in the differentiation, functioning and growth of various reproductive tissues, in regulation of breast cancer and brain development [1–7]. It is a ligand-activated gene regulatory protein that is a member of the nuclear receptor superfamily [8]. In the nonactivated state, ERα is complexed with various chaperones and immunophilins such as heat shock protein 90, heat shock protein 70, FKBP52 or CyP-40 [9–12]. When the complex dissociates, the protein becomes unstable and looses some binding activity to the cognate hormone and estrogen response element and tends to aggregate. Upon binding of estrogen, the ERα undergoes a conformational change and traffics into the nucleus, where it binds its hormone responsive element and subsequently

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induces transactivation of the response gene. Recent advances in our understanding of the molecular mechanism of action of the receptor has supported an increased interest in discovery and development of new drugs which can modulate its function [13]. Therefore production and purification of liganded and unliganded receptor has become an important task in drug discovery and pharmaceutical biotechnology.

Normal mammalian cells are not programmed to express large quantities of steroid hormone receptors, although many human breast carcinomas exert elevated amounts of estrogen receptors. Breast cancer tissue contains up to 1000 fmol per mg extracted proteins. This corresponds to 0.07 μg receptor protein [14]. Breast cancer tissue is a very limited source for ERα. In addition, a lot of breast cancer tissues contain mutated and truncated receptor, which does not represent the structure and function of wild type receptor. An approach to study the role of hormone action is crystallization of the occupied receptor but the lack of a source of enough homogeneous, biologically active receptor makes this approach very difficult.

ERα has been overexpressed in various host cells, such as *Escherichia coli* [15], yeast [16], insect cells [17] and CHO cells [18]. Purification of ER from calf uteri has been studied for two decades by using various chromatographic methods [19,20]. 17β-Estradiol-affinity chromatography has been developed as selective tool for ERα purification. However, efficiency is low due to the ligand-bleeding and harsh elution conditions. 17β-Estradiol has been coupled via either the A-ring or D-ring [21]. A-ring substitution destroys the affinity to the receptor. D-ring substitution has been accomplished through hemisuccinate derivatization. The activated steroid has been further immobilized either via short spacers with various length or via macromolecular spacers such as bovine serum albumin (BSA) and poly(D-lysyl-DL-alanine) [19]. The macromolecular spacers gave a high purification yield, although ligand-bleeding and harsh elution conditions could not be completely avoided.

Heparin is a naturally occurring glycosaminoglycan which serves as efficient affinity ligand with ion-exchange function for a wide range of biomolecules including steroid receptors [19,22–24]. Affinity chromatography with heparin–Sepharose 6B was used as initial purification step for ERα to remove compounds, which have a destabilizing effect on estradiol–Sepharose and DNA–Sepharose. Therefore heparin-affinity chromatography is an excellent auxiliary step for receptor purification.

ERα has been expressed in *Saccharomyces cerevisiae* by fusion to ubiquitin and expression has been controlled by a metallothionin promoter. This intracellularly expressed receptor retained its biological activity [16] upon overexpression. Therefore this expression system has been considered as a favorable tool compared to tissue sources for production of starting material for our receptor purification studies. We describe here a strategy for the purification of ERα produced in yeast using a combination of heparin- and estradiol-affinity chromatography.

2. Experimental

2.1. hERα preparation

The preparation of recombinant yeast was carried out according to the protocol described previously [16]. Yeast cells transformed with the expression plasmid YEpE13 and YEpE28 have been inoculated into a 14 l stirred tank bioreactor (IMCS-2000 MBR, Wetzikon, Switzerland). The yeast cells were grown in synthetic medium [25] to midlog phase at 30°C and cell density was monitored photometrically at a wavelength of 600 nm. ERα expression was induced by addition of 100 μ*M* $CuSO_4$ for 6 h.

Cells were harvested by centrifugation and washed with distilled water. The cell pellet was resuspended in TMDG 0.1 buffer [20 m*M* Tris–HCl, pH 7.4, added with 10 m*M* Na_2MoO_4, 2 m*M* dithiothreitol (DTT), 10% glycerol, 0.1 *M* NaCl and protease inhibitors (0.1 m*M* L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and 1 m*M* phenylmethylsulphonyl fluoride (PMSF))] and disintegrated by glass beads in a vibration cell-mill (Bühler, Tübingen, Germany) for 20 min at 4°C. After removing the debris by centrifugation, the crude

extract was immediately used or stored at −70°C. All procedures were performed on ice or at 4°C.

2.2. Immobilization of 17β-estradiol 17-hemisuccinate

Here, the preparation of the 17β-estradiol-affinity column with a nine atom spacer and a macromolecular spacer will be described.

Sepharose 4 Fast Flow (Pharmacia-Amersham, Uppsala, Sweden) was first activated with epibromohydrin (EBH) according a procedure described by Nishikawa and Bailon [26]. Diaminodipropylamine (DADPA) was coupled to the activated Sepharose 4 Fast Flow and the gel was successively washed with 0.2 *M* $NaHCO_3$, 0.5 *M* NaCl, pH 8.0 and then 0.2 *M* $NaHCO_3$ pH 8.5. The obtained DADPA-substituted gel was ready for coupling of 17β-estradiol 17-hemisuccinate obtained from Sigma (St. Louis, MO, USA).

For introduction of a macromolecular spacer EBH-activated gel was used. It was reacted with ethylenediamine (Sigma) and further with succinic anhydrid (Sigma). The obtained gel with terminal carboxy groups was activated by N,N′-disuccinimidyl carbonate [27] for BSA immobilization. BSA was dissolved in 0.2 *M* $NaHCO_3$ buffer, pH 8.5, containing 8 *M* urea reacted with the activated gel for 6 h.

The gels containing either DADPA-spacer or BSA-spacer were washed successively with 1 *M* NaCl; 0.2 *M* $NaHCO_3$, 0.5 *M* NaCl; water and finally with acetonitrile.

Three ml of each gel was washed with 2–3 volumes of 70% acetonitrile and suspended in 5 ml of 70% acetonitrile. Five mg of 17β-estradiol 17-hemisuccinate dissolved in 1 ml of acetonitrile was added to the gel suspension. Twenty mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were added and agitated for 4 h at room temperature. Then another 20 mg portion of the EDAC was added and agitation was continued overnight. Immobilization efficiency was controlled by RP-HPLC using a C_{18} column (Macherey–Nagel, Düren, Germany). The substituted Sepharose suspension was filtered and washed with 100 ml of 70% acetonitrile and 100 ml of water at room temperature.

All chemicals have been purchased from Merck (Darmstadt, Germany), if not indicated otherwise.

2.3. Chromatography

Chromatography on heparin–Sepharose 6B (Pharmacia-Amersham) was carried out at 4°C in the packed bed mode. When crude yeast extracts were processed, first the sample was desalted by Sephadex G-25 with TMDG 0.1 buffer before loading onto an equilibrated heparin column. When eluates from estradiol-affinity chromatography were used, the samples were diluted with TMDG 0.1 to reach a final urea concentration of 0.1 *M*. Bound ERα protein was eluted with 0.5 *M* NaCl.

One ml of 17β-estradiol–17-hemisuccinate–Sepharose 4 Fast Flow was washed with 500 ml 80% ethanol and equilibrated with TMDG 0.1 buffer prior to use. Ten ml eluate from heparin-affinity chromatography was incubated with the gel at 4°C for 3 h under gentle shaking. The gel was then packed into a column and washed with TMDG 0.1 buffer containing 0.5 *M* NaCl and subsequently with TMDG 0.1 buffer. Bound ERα protein was desorbed by incubation of the gel with TMDG 0.1 buffer containing 1.0 *M* urea and 50 n*M* 17β-estradiol overnight at 4°C. For this procedure the gel has been again unpacked. After desorption, the gels were decanted and the remaining interstitial fluid was harvested by centrifugation.

2.4. Estrogen ligand binding assay

The specific ligand binding capacity of each preparation of ERα and all chromatography fractions was measured by a ligand binding assay using [^{3}H]17β-estradiol as described by Wittliff et al. [15]. Twenty μl of ERα samples were incubated with 50 μl of 5.5 n*M* of [^{3}H]17β-estradiol (DuPont, Boston, MA, USA). Nonspecific binding was measured by parallel incubation with 3 μg/ml diethylstilbestrol for 16 h at 4°C. Casein was added to the samples as carrier protein to a concentration of 0.5–1 mg/ml, when eluates from chromatography were processed. After the completion of the incubation, the receptor-bound [^{3}H]17β-estradiol and the excess of unbound [^{3}H]17β-estradiol were separated by adding dextran-

coated charcoal (DCC). After 15 min incubation at 4°C, DCC was removed by centrifugation. Aliquots of 100 μl of the supernatant were removed and pipetted into 3 ml of scintillation fluid (Eison, Loughorough, UK) and counted. The specific binding was calculated by subtracting nonspecific binding from total binding.

2.5. Enzyme immunoassay (EIA)

EIA was performed using a monoclonal antibody-based technique with reagents from Abbott Laboratories (Abbott Park, IL, USA) according to the procedure provided by the manufacturer.

2.6. Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) and Western blot

Protein samples were analyzed by SDS-PAGE and Western blot according to the Laemmli procedure [28] with slight modification. Broad range molecular mass marker proteins from Bio-Rad (Richmond, VA, USA) were used for estimating apparent molecular mass. Precast Tris–glycine gradient gels (4–20% polyacrylamide) from Novex (San Diego, CA, USA) were loaded with the respective samples and electrophoresed for 2 h at 125 V and then silver stained or transferred to an Immobilon P membrane (Millipore, Bedford, MA, USA) and incubated with antibody AER314 (NeoMarkers, Fremont, USA). As staining antibody antimouse IgG conjugated with alkaline phosphatase was used. Proteins were visualized by incubation for 10 min with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate dissolved in a buffer containing 5 m*M* $MgCl_2$, 100 m*M* Tris, pH 9.5 and 100 m*M* NaCl.

2.7. Protein determination

Protein was estimated by the modified Bradford method [29] using the Bio-Rad protein assay reagent with BSA as standard.

2.8. DNA bandshift assay

Single stranded oligonucleotides, 5′-GATCTCAGGTCACAGTGACCTGA-3′and 3′-AGTCCAGTGTCACTGGACTCTAG-5′, containing the consensus sequences for the estrogen responsive element (ERE) were annealed and ligated as described by Kadonaga and Tjian [30]. Labeling of ERE was carried out similarly as described by Wittliff et al. [15]. Briefly, the ligated ERE was labeled by filling in the ends with Klenow enzyme (Boehringer, Mannheim, Germany) using ^{32}P-dCTP (Amersham, UK). The yeast extract and eluates from different chromatography purification steps were tested for the DNA binding ability. Four μl of sample were combined with 0.6 mU poly(dI–dC)·poly(dI–dC) (Sigma) in 20 m*M* Tris–HCl, pH 7.4, containing 50 m*M* KCl, 10% glycerol, 2 m*M* DTT and protease inhibitors (0.1 m*M* TPCK, 0.1 m*M* TLCK, 1 m*M* PMSF). When a supershift was carried out, antibodies AER311 and AER304 (Neomarkers) were used: After incubation for 15 min on ice, ^{32}P-labeled ERE (50 000 cpm in 2 μl) was added and a second incubation was performed for 30 min to allow association at room temperature. BSA as carrier protein was added when total protein in the sample was lower than 100 μg/ml. Reaction mixtures combined with 1 μl bromphenol blue marker were loaded onto 5% nondenaturing polyacrylamide gels (acrylamide:methylenebisacrylamide, 30:1) and electrophoresis was performed with 0.5×TBE (50 m*M* Tris–HCl, 41 m*M* boric acid, 5 m*M* EDTA, pH 8.3) as running buffer at 110 V at room temperature for 3 h. The gel was dried under vacuum and autoradiographed on Kodak X-ray film (Eastman Kodak, Rochester, NY, USA).

3. Results

3.1. Fermentation

A brief induction study in shake culture was carried out with the transformed yeast clones prior to mass propagation in the 14 l bioreactor. The studies showed that 6 h induction gave maximum ERα expression (Fig. 1). These conditions have been used for fermentation. Specific yield decreased with induction time, since biomass production and expression of the heterologous protein is not strictly interrelated.

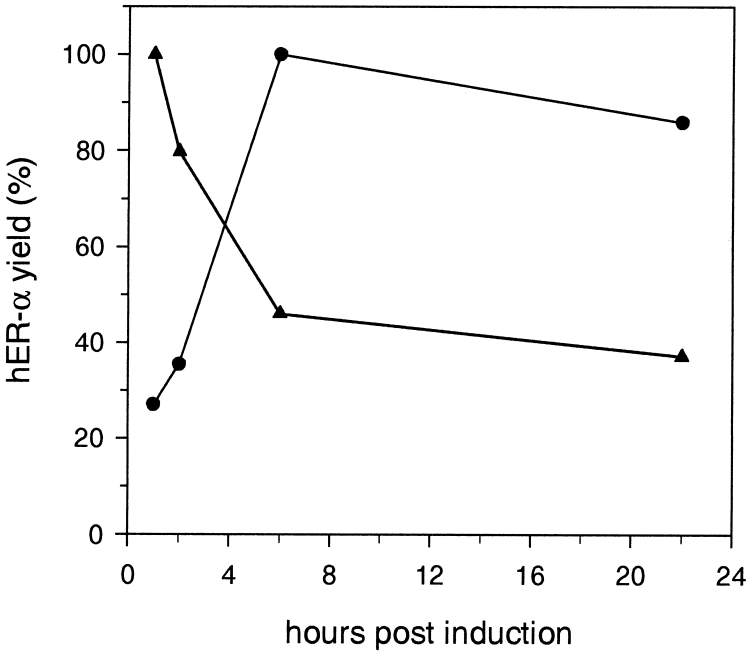

Fig. 1. Expression of ERα in yeast dependent on induction time. (●) Denotes ERα content; (▲) indicates ERα specific yield, expressed as relative values. The highest value is set to 100%.

3.2. Heparin-affinity chromatography

Heparin-affinity chromatography has been previously described for purification of naturally occurring estrogen receptor [24]. We have used a commercially available heparin-affinity sorbent heparin–Sepharose 6B, for purification of ERα overexpressed in *S. cerevisiae*. The harvested yeast cells have been washed and disintegrated as described in the Experimental section. The clarified extract has been desalted by Sephadex G-25 and then loaded onto the heparin–Sepharose 6B column. Tenfold purification of the receptor (Table 1) could be achieved by heparin-affinity chromatography.

A NaCl gradient elution was studied for heparin–Sepharose 6B. The concentration range of NaCl where ERα has been observed was very wide (Fig. 2). The main fraction of ERα could be eluted between 0.4–0.6 *M*. A considerable reduction of ligand binding activity has been observed in these ERα fractions eluted with 0.5 *M* NaCl. When the eluates were desalted by gel filtration, ligand binding activity could be partially recovered.

In a parallel experiment, ERα samples were treated the same way as described above except for elution. A heparin solution was used for this purpose. The Western blots of these fractions are shown in Fig. 3. Competitive elution with 4 mg/ml free heparin in the elution buffer was compared to NaCl elution. Competitive elution gave higher specific activity measured as ligand binding activity and higher recovery measured by EIA (Table 1).

3.3. 17β-Estradiol-affinity chromatography

17β-Estradiol 17-hemisuccinate was coupled to Sepharose through different spacers as described in the Experimental Section.

Gels must be efficiently washed with organic solvent to remove uncoupled ligands, since free 17β-estradiol 17-hemisuccinate is a rather hydrophobic molecule and will compete with immobilized ligand for receptor binding. Two ml of the gel was washed at room temperature with 300–1000 ml of 80% ethanol for 20–48 h. Washing efficiency was tested by chromatographing a sample of yeast extract containing ERα. After 300 ml of 80% ethanol washing, a substantial amount of free estradiol

Table 1
Purification of ERα by heparin-affinity chromatography; ERα contents were determined by EIA

Sample	Protein (μg/ml)	Total binding (fmol)	Specific binding (fmol/mg)	Purification (fold)	Yield (%)
Yeast extract, eluted with heparin					
Yeast extract.	2210.6	4737.6	714.4		
Eluate (4 mg/ml)	126	4947.5	6544.3	9.2	104.4
Yeast extract, eluted with NaCl gradient					
Yeast extract	2210.6	4737.6			
Eluate 0.3 *M* NaCl	187.4	1218.5	714.4		25.7
Eluate 0.4 *M* NaCl	285.1	1454.6	4334.3		30.7
Eluate 0.5 *M* NaCl	215.3	1597.1	3401.8	6.9	33.7
			4945.3		total 90.1
Liganded yeast extract, eluted with NaCl gradient					
Yeast extract	2210.6	4737.6	714.4		
Eluate 0.3 *M* NaCl	204.7	1138.1	3706.8		24
Eluate 0.4 *M* NaCl	301.1	1361.0	3013.8		28.7
Eluate 0.5 *M* NaCl	226.1	1438.9	4242	5.9	30.4
					total 83.1

absorbed to the column could be observed. This became evident by a high amount of ERα in the flow-through (Fig. 4) measured by SDS-PAGE and Western blotting. ERα preferably bound to free ligand and passed the column. After 1000 ml ethanol washing, the Western blot experiments showed a significant reduction of ERα in the flow-through indicating that most of free estradiol could be washed out by the organic solvent (lanes 2 and 5 in Fig. 4). The column with low molecular mass spacer

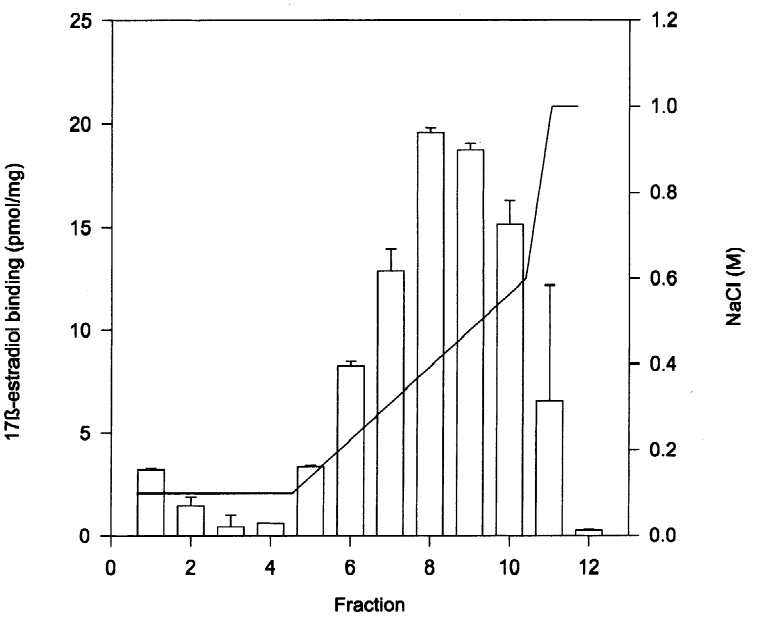

Fig. 2. Heparin-affinity chromatography of estrogen receptor on heparin–Sepharose 6B. Ten ml of yeast extract was applied directly to the heparin column (3 ml gel volume). The receptor proteins were eluted with a NaCl gradient (line) from 0.1 *M* to 1.0 *M* in TDMG 0.1 buffer. The [^{3}H]17β-estradiol ligand binding assay was used for evaluation of receptor elution.

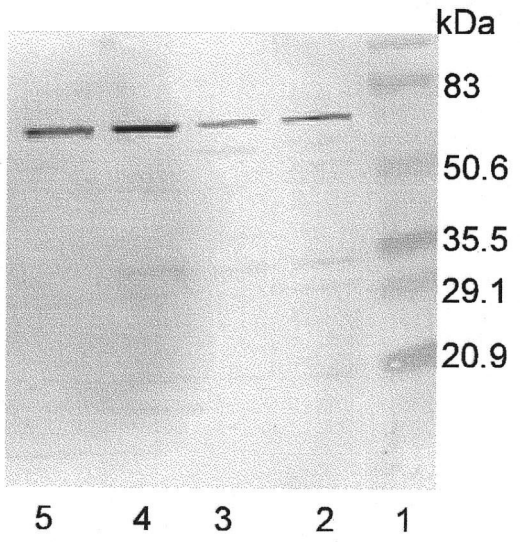

Fig. 3. Western blot of the fractions of heparin-affinity chromatography. Yeast extract was applied onto the heparin–Sepharose 6B. After washing with TMDG 0.1, pH 7.4 buffer, the receptor proteins were eluted with 4 mg/ml free heparin or 0.5 *M* NaCl in loading buffer. Lanes: 1, molecular mass markers; 2, yeast extract; 3, flow-through; 4, receptor eluate with free heparin; 5, receptor eluate with 0.5 *M* NaCl. On each lane 2.2 μg protein were applied, except lane 4 (1.5 μg). kDa=kilodaltons.

Table 2
Elution conditions of ERα from 17β-Estradiol–BSA–Sepharose; elution efficiency was estimated from bound receptor

Elution agent (in TMDG 0.1, pH 7.4)	Recovery of bound hERα
0.5 *M* NaCl	<1%
0.5 *M* NaCl, 0.5 *M* NaSCN	<25%
0.5 *M* NaCl, 0.5 *M* NaSCN, 10% DMF	<25%
0.5 *M* NaCl, 0.5 *M* NaSCN, 10% DMF, 50 n*M* 17β-Estradiol	50–75%
1 *M* Urea, 50 n*M* 17β-Estradiol	>75%
5 *M* Urea, 50 n*M* 17β-Estradiol	>90%

did not bind ERα in sufficient amount and bound material could not be eluted. Therefore only experiments with the macromolecular spacer were further pursued.

Different conditions for ERα elution from estradiol-affinity columns were tested. These condition are listed in Table 2. Salts, such as NaCl up to 0.5 *M*

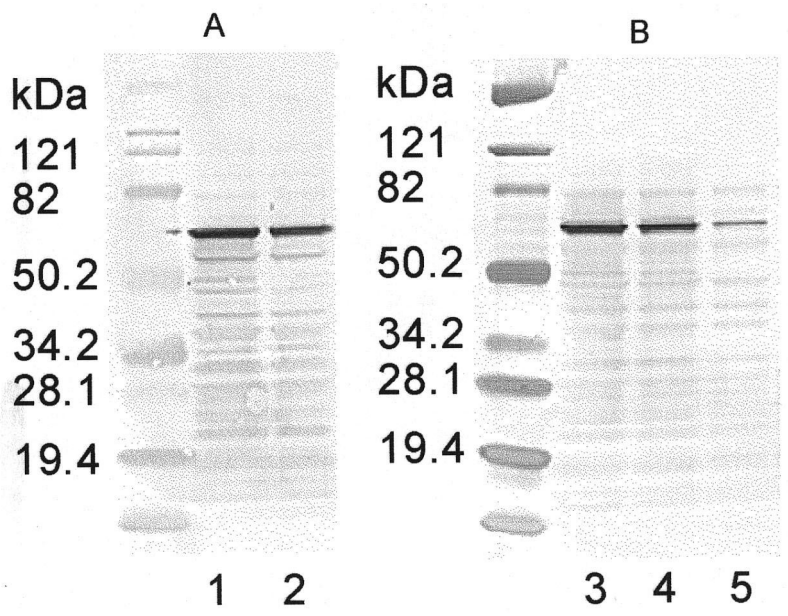

Fig. 4. Influence of extensive gel washing on ERα binding to 17β-estradiol–BSA–Sepharose. Western blot analysis, lanes: 1, 3 and 4, yeast extract; 2, fraction containing unbound ERα after gel (2 ml) was washed with 300 ml of 80% ethanol, and 5, after washing with 800 ml of 80% ethanol.

in the elution buffer could not elute ERα (data not shown). A considerable amount of ERα was eluted with chaotropic salts; 0.5 *M* NaSCN supplemented with 10% dimethylformamide (DMF) and 50 n*M* 17β-estradiol was used. 17β-Estradiol competitive elution with 17β-estradiol combined with 5 *M* urea gave the same elution efficiency as competitive elution without urea. (Figs. 5 and 6).

An elution mode depending on temperature has been also used for elution of bound receptor on the 17β-estradiol–BSA–Sepharose. After washing, the loaded gel was divided into two parts and incubated with free estradiol combined with NaSCN and DMF at 4°C overnight and at 40°C for 30 min. Western blots of eluate composition showed no significant difference (data not shown).

Finally a complete purification cycle with the optimized sequence and elution conditions was performed. After harvesting, washing and disintegration of the yeast cells, gel filtration, heparin- and estradiol-chromatography was used as a purification procedure. The desalted extract was loaded onto the heparin–Sepharose. ERα was eluted with 0.5 *M* NaCl. ERα containing fractions were directly loaded onto estradiol-affinity column. The main objective of the heparin column was to eliminate those substances that may damage the estradiol gel as described in the Experimental Section. Additionally, five- to tenfold purification could be achieved and the eluate could be directly incubated with 17β-estradiol 17-hemisuccinate–BSA–Sepharose. One *M* urea combined with 50 n*M* of 17β-estradiol was used to elute bound hERα from the estradiol-affinity gel. SDS-PAGE and Western blot shown in Fig. 7 indicated that the same purification factor could be obtained as described by the previous experiments.

Bandshift assay of the eluates showed that the purified receptor retained the ERE binding activity, although a denaturing agent was used (Fig. 8). Competitive elution with 17β-estradiol of course resulted in ligand occupied receptor.

4. Discussion

Yeast transformed with the plasmid YEpE13 has been used as vehicle to produce active ERα. The receptor as it was present in the extract after

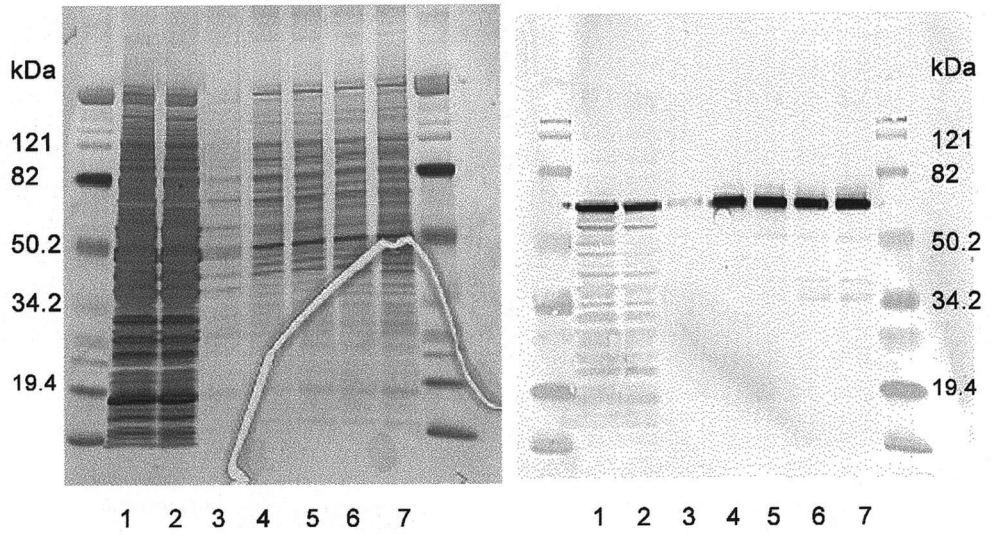

Fig. 5. Influence of urea in the elution buffer on elution efficiency. SDS-PAGE and Western blot analysis of ERα fractions from 17β-estradiol–BSA–Sepharose. Twenty ml of yeast extract were incubated with 3 ml of 17β-estradiol 17-hemisuccinate–BSA–Sepharose 4FF gel. After washing with TMDG 0.1, pH 7.4, and TMDG 0.5, pH 7.4, the gel was divided into four parts and they were incubated with TMDG 0.1, pH 7.4 containing 100 n*M* 17β-estradiol and 0 *M*, 1 *M*, 3 *M*, 5 *M* urea, respectively, for hER elution. Lanes 1, yeast extract; 2, nonbound fraction; 3, wash fraction; 4–7, eluates with the buffer containing 0 *M*, 1 *M*, 3 *M* and 5 *M* urea, respectively. Protein applied: from lanes 1–7, 18.8, 14.5, 0.5, 0.36, 0.52, 0.49, 0.8 μg.

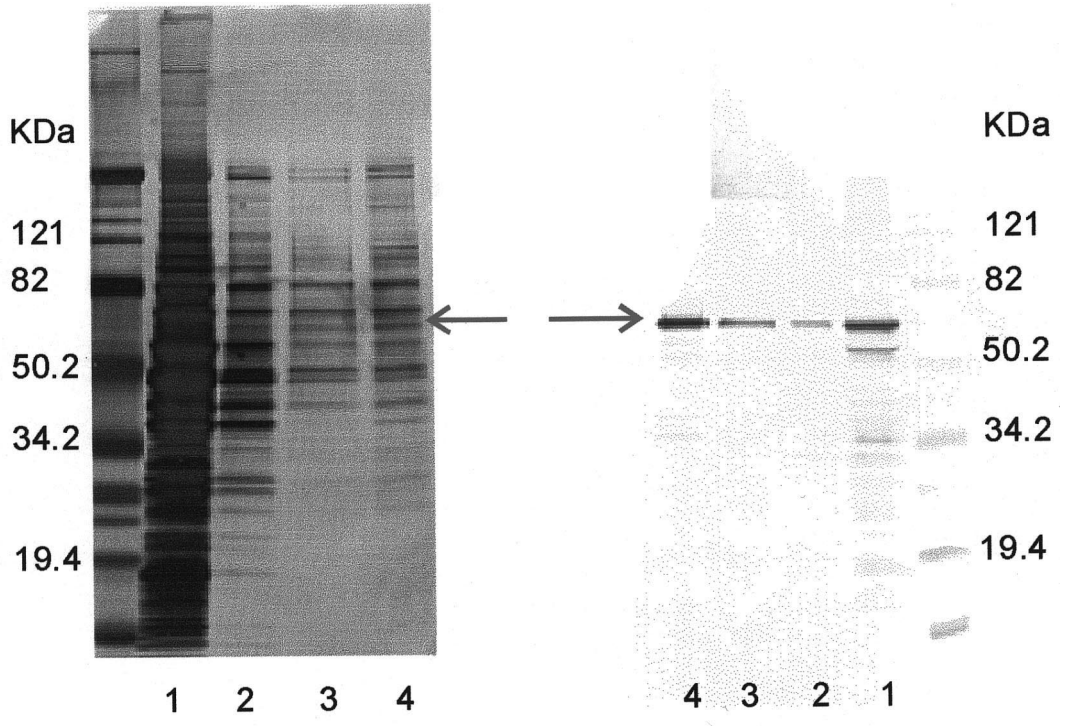

Fig. 6. Comparison of various additives is the elution buffer using 17β-estradiol 17-hemisuccinate–BSA–Sepharose 4FF as sorbent. Fractions were analyzed by SDS-PAGE and Western blot analysis. Yeast extract (lane 1) was incubated with the affinity gel. Bound ERα protein was eluted with TDMG 0.1, pH 7.4, containing 100 n*M* 17β-estradiol (lane 2); 100 n*M* 17β-estradiol, 0.5 *M* NaSCN and 10% DMF (lane 3); 100 n*M* 17β-estradiol, 5 *M* urea (lane 4), respectively. Protein applied: from lanes 1–4, 22.0, 0.41, 0.32, 0.35 μg.

disintegration of the yeast cells showed ligand binding activity and the capability to bind to the vitelogenin hormone responsive element assessed by ERα induced bandshift and antibody mediated supershift.

Scale up from shake-culture into bioreactor did not change the biological properties of the overexpressed protein. The residence time of ERα in the cells after induction is identical in shake-culture and fermentation. In both scales yeast has been cultivated under selection pressure and therefore loss of plasmids could be prevented. The material produced in pilot scale allowed conduction of parallel experiments to study various elution conditions and combinations of chromatography steps.

Although the concentration of ERα in the harvested biomass is still low compared to other recombinant proteins, the expressed receptor level is 100-fold higher than ERα in normal cells and reaches the same or higher levels as ‘‘super’’ expressing breast cancer cells. With yeast the amount of material is unlimited in contrast to the natural sources. Chromatography using heparin–Sepharose is a useful prepurification step for ERα. The main purification step is 17β-estradiol 17-hemisuccinate–BSA–Sepharose 4 Fast Flow affinity chromatography. After incubation of crude yeast extract directly with this affinity-sorbent release of 17β-estradiol into the running buffer was observed. The action of compounds, such as reducing substances and biochemical components with proteolytic and esterase activity present in the extract may be responsible for the attack of susceptible bonds between the 17β-estradiol and matrix. However, when yeast extract is first chromatographed on heparin–Sepharose, the release of free 17β-estradiol decreased. Nearly no ERα in the flow-through of the estradiol-affinity chromatography could be identified by Western blots. The yeast components and the additives producing the above mentioned effect have been eliminated.

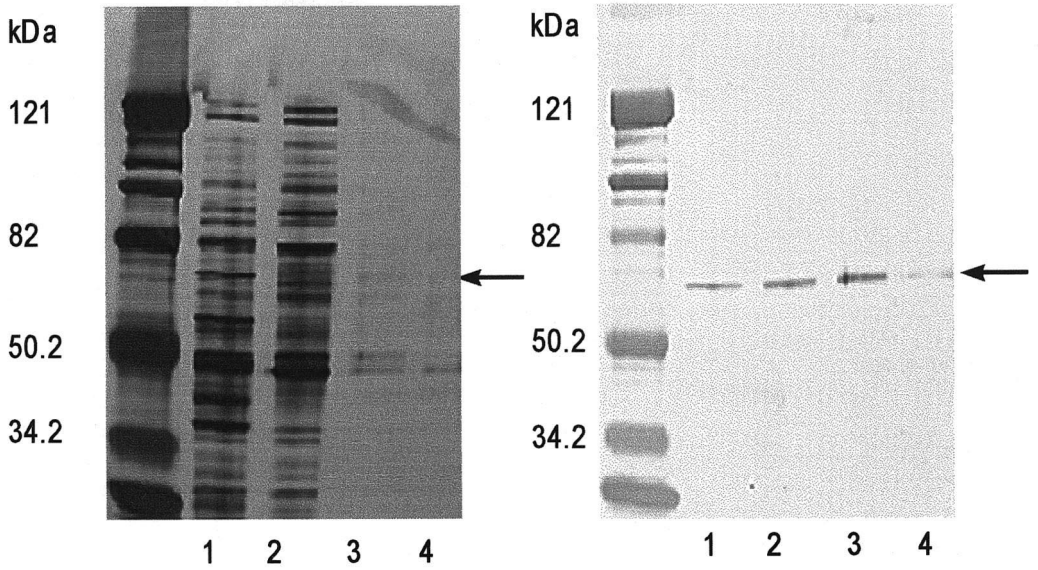

Fig. 7. SDS-PAGE and Western blot of a complete purification cycle. Lanes: 1, yeast extract (protein: 0.65 μg); 2, first heparin-affinity chromatography eluate (protein: 0.60 μg); 3, eluate from 17β-estradiol 17-hemisuccinate–BSA–Sepharose 4FF (protein below detection limit) and 4, second heparin-affinity chromatography eluate (protein below detection limit). ERα bands are indicated by arrows.

When ERα is eluted by competitive elution with free heparin, aggregation resulting in inactivation of estrogen-free ERα can be inhibited. Sica and Bresciani [19] have shown that the heparin–agarose interacts with 17β-estradiol liganded receptor with very high affinity. This property can be exploited to achieve about 100-fold purification of the receptor from calf uterus cytosol. Our results indicate that there is no significant difference between liganded and unliganded receptor for the purification by heparin-affinity chromatography, when yeast extract is processed.

Additional advantages of heparin-affinity chromatography steps are the five- to tenfold purification of ERα, reduction of the volume of yeast extract to less than one third, and furthermore direct combination with estrogen-affinity chromatography without an additional desalting step. The second heparin-affinity chromatography after 17β-estradiol-affinity chromatography is a rapid and efficient method for removing free 17β-estradiol and components present in the elution buffer such as urea, DMF or NaSCN.

17β-Estradiol binds to estrogen receptor with high affinity (dissociation constant$=1\cdot10^{-10}$ M). With Mannich reaction it can be coupled to agarose via the A-ring, but this immobilized estradiol did not give sufficient binding to ER in calf uterus [4]. One of the derivatives of estradiol, 17β-estradiol 17-hemisuccinate, has a lower affinity than estradiol itself but displays sufficient selectivity to be a useful ligand in the preparation of an affinity resin. It is a D-ring derivative which can be easily immobilized via the hemisuccinate group by carbodiimide coupling. Therefore it has been often used as ligand for affinity purification of ERα from cytosol. We first used it for purification of recombinant ERα expressed in yeast.

The most useful estradiol adsorbent was the matrix with a macromolecular spacer arm (BSA) interposed between the Sepharose backbone and the estradiol molecule. We used denatured BSA, since it does bind to the Sepharose backbone at several points, and therefore greatly increases the stability of subsequently immobilized estradiol ligand. Through this immobilization BSA binds more tightly to the matrix and provides a more favorable microenvironment for receptor binding. We observed that using 17β-estradiol 17-hemisuccinate–BSA–Sepharose resulted in better purification of hERα than using 17β-estradiol 17-hemisuccinate–DADPA–Sepharose. This has been also observed by Sica and Bresciani [19].

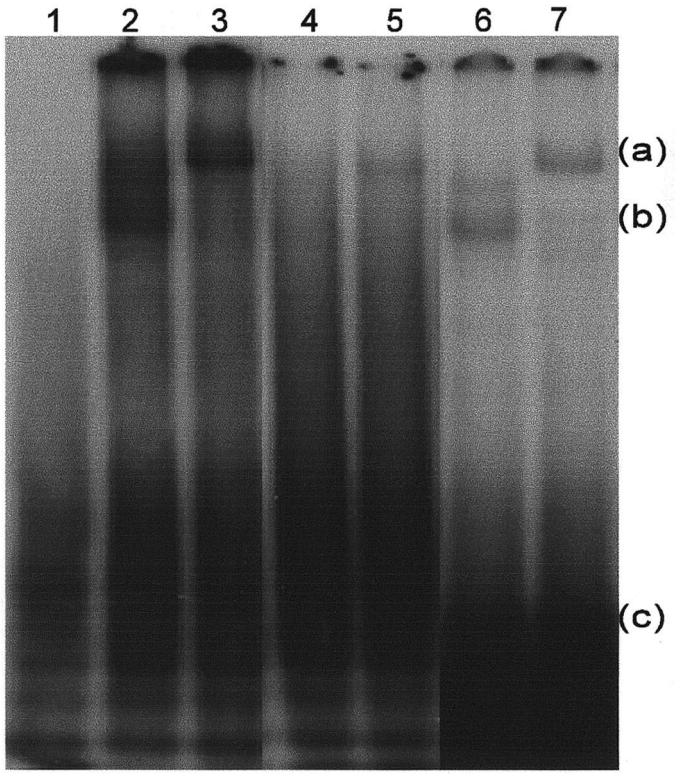

Fig. 8. DNA bandshift analysis of ERα purified by estradiol-affinity chromatography. A DNA fragment containing multiple repeats were labeled with ^{32}P and used for lanes 1–5. A single repeat was used for lanes 6 and 7. Lanes: 1, repeat-ligated ERE alone; 2, 3, 6 and 7, crude ERα fractions; 4 and 5, purified ERα with estradiol-affinity chromatography. Mixtures applied on lanes 3, 5 and 7 contained also antibody. (a) Supershifted ERE–ERα–AER311 complex; (b) ERE–ERα complex; (c) free ^{32}P-labeled ERE.

The estradiol molecules in this gel are separated from the Sepharose backbone by very long and chemically neutral extensions, which have proven very favorable for selective interaction in affinity chromatography [26].

BSA as a huge spacer has also disadvantages. The ion-exchange properties of some amino acids may result in the binding of impurities. Another problem is that BSA itself may bind receptor with weak affinity. These problems can be partially solved by washing with high concentrations of salt since salt does not dissociate the estradiol–receptor complex.

Due to the hydrophobic properties of the backbone of the gel, a fraction of the ligand could be nonspecifically adsorbed on the surface of the gel. It has been shown that one of the most important steps in the preparation of the estradiol-affinity gel and purification of ERα is the removal of the noncovalently adsorbed estradiol before application onto the resin. Although these adsorbed hormone molecules are tightly bound to the matrix, they could be removed by application of yeast extract, because the protein solution alters the partitioning properties of estradiol between solid-phase and aqueous phase. This phenomenon prohibits the binding of ERα to the gel and results in a major loss during purification. This effect may lead to incorrect interpretation that the gel is performing inefficiently. The gel must therefore be extensively washed prior to the chromatography experiments. The resin can be washed with large volumes of organic solvent or with crude yeast extract.

High NaCl concentration (0.8 *M*) did not interfere with the binding of ERα to 17β-estradiol. Incubation of the affinity gel with ERα extract in 0.5 *M* NaCl solution allows sufficient adsorption of ERα. This

reaction is very slow but the formed complex has high affinity. The affinity of a complex and the speed of formation do not relate to each other. Borgna and Ladrec [31] have determined the dissociation rate constant and association rate constant values of ERα by kinetic experiments. Rough calculations suggest that the residence time in packed bed chromatography is too low for efficient adsorption. These finding have been also supported by our experiments. Complete elution of ERα from such a gel is also very difficult. Conventionally, relatively moderate NaCl elution cannot desorb ERα. Very high salt concentration may result in a salt induced dissociation of ERα or salt-inactivation.

Chaotropic salt such as NaSCN and DMF have been used to elute bound ERα from calf cytosol. However it has been shown that this elution protocol is not effective in our case. NaSCN (0.5 *M*) combined with 10% DMF cannot efficiently elute ERα. Most of ERα was retarded on the gel.

Competitive elution with free 17β-estradiol has been reported as a major elution method, although ligand free receptor cannot be produced by this method. When the gel with residual ERα was boiled with SDS buffer, ERα was dissociated as confirmed by Western blots (data not shown). One *M* urea together with competitive elution could desorb ERα from the matrix and 5 *M* urea combined with 50 n*M* free 17β-estradiol resulted in complete elution. It has been noted that urea alone could not dissociate the ERα–hormone complex.

Elution of ERα by increasing temperature has also been studied. Incubation of the gel saturated with ERα at 45°C for 30 min did not enhance elution compared to experiments conducted at 4°C overnight. Exposing ERα to high temperature for long times is critical, since degradation and inactivation may be accelerated.

Low purification yield with estradiol-affinity chromatography has been reported for uterine samples [32–35]. Earlier work on progesterone receptor purification indicated that low yields may result from failure to elute the receptor from the gel due to the high ligand density within the resin [36]. Proteolysis contributes also to low yield because of inactivation. This is illustrated also by Western blot experiments. Much more degradation products of ERα have been found in the flow-through compared to that in eluates. Other factors such as dephosphorylation may also contribute to receptor inactivation. Dephosphorylation of ERα resulting in inactivation and loss of hormone binding has been described in the past [37]. Horigome et al. [38] suggested that an inactivation or stabilization factor may exist in tight association with ERα, which is washed away during chromatography. These factors have been identified as heat shock proteins and other accompanying proteins. On the other hand, estradiol-affinity chromatography can remove inactivated ERα and purify the activated ERα.

In conclusion, the methods described in this paper using heparin- and estradiol-affinity chromatography can be applied for the large-scale purification of ERα expressed in yeast to high purity level. The partially purified receptor serves for the definition of biochemical characteristics of the receptor and for the further understanding of the role of the receptor in gene regulation.

5. Abbreviations and symbols

BSA	bovine serum albumin
DADPA	diaminodipropylamine
DCC	dextran-coated charcoal
DMF	dimethylformamide
DTT	dithiothreitol
EBH	epibromohydrin
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EIA	enzyme immunoassay
ERα	human estrogen receptor-α
ERE	estrogen responsive element
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TLCK	Nα-*p*-tosyl-L-lysine chloromethyl ketone
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone
YEpE13	yeast expression plasmid13 for estrogen receptor-α

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