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Expression and purification of a recombinant form of human aromatase from *Escherichia coli*

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Abstract

Aromatase converts androgen to estrogen, a hormone that plays an important role in the development of breast cancer. Aromatase inhibitors have been shown to be a useful endocrine regimen for estrogen-dependent breast cancer. Structure–function studies of aromatase can generate critical structural information for designing highly potent and specific inhibitors. However, aromatase structure–function studies have been hampered by a lack of purified protein. In this report, we describe the construction and expression of a recombinant derivative of human aromatase in *Escherichia coli* using the pET vector system, and the purification of the enzyme by means of nickel-agarose affinity chromatography. We examined the expression of the full-length, Del-38, C-6xHis-tagged Del-38, and NC-6xHis-tagged Del-38 forms of aromatase. The recombinant aromatase without the first 38 amino acids from the amino-terminus (i.e. Del-38) was found to have a higher activity than the full-length enzyme. Moreover, the addition of two separate hexameric histidine tags at both the amino and the carboxyl-termini (i.e. NC-6xHis-tagged Del-38) increased the binding affinity of the recombinant enzyme to the nickel-agarose. The expressed aromatase (i.e. NC-6xHis-tagged Del-38 aromatase) was eluted from the nickel-agarose with 80 mM EDTA. The total aromatase activity of the 80 mM EDTA-eluted fractions was significantly higher than the detergent-solubilized protein extract, indicating a renaturation process during the nickel-agarose affinity chromatography. Purified aromatase exhibited a single band when analyzed by SDS–PAGE, and activity up to 5.8 nmol/mg/min was obtained using the tritiated water release assay. The K_m value for androstenedione was determined to be 62 ± 24 nM by enzyme kinetic analysis. The recombinant aromatase preparation was also characterized by reduced CO-difference spectral analysis, reaction product extraction assay, and inhibition studies using two aromatase inhibitors (letrozole and anastrozole). The results indicate that the recombinant aromatase from *E. coli* has catalytic properties identical to those of the enzyme expressed in human tissue and will be very useful for further structure–function studies of aromatase.

Keywords: Aromatase; *E. coli* expression; Cytochrome P450 expression and purification; Estrogen synthetase; Aromatase inhibitors

1. Introduction

Aromatase (CYP19), which catalyzes the formation of estrogens from androgens, has received considerable attention because of the importance of estrogen in many reproductive and metabolic processes, and the protective role of estrogen against Alzheimer's disease [1] as well as against colon cancer [2]. Human aromatase is expressed primarily in the ovaries, placenta, adipose tissue, brain, muscle, and skin fibroblasts. In pathological situations, abnormal expression of aromatase has been detected in a significant number of breast tumors [3–6], as well as in uterine [7], testicular [8], and adrenal [9,10] tumors. Over-expression of aromatase has also been reported in men with gynecomastia [11]. Aromatase inhibitors have been found to be valuable to treat these estrogen-dependent and aromatase-mediated diseases, including breast cancer [12].

Previous aromatase inhibitor development has been based primarily on structure–activity relationship studies. To provide useful information about the active site of aromatase for designing aromatase inhibitors with a high specificity and potency, we and other groups [13–23] have carried out structure–function and mechanism studies of

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Abbreviations: δ-ALA, δ-aminolevulinic acid; Del-38, an amino (N)-terminal 38 amino acid sequence deleted form of aromatase; IPTG, isopropyl-β-D-thioglucopyranoside; 19 al A, 19-al-4-androstene-3,17-dione; 19 ol A, 19-hydroxy-4-androstene-3,17-dione; 19 nor A, 19-nor-4-androstene-3,17-dione; NTA, Ni-nitrilotriacetate; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride.

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aromatase by site-directed mutagenesis using a mammalian cell expression method. Although important information regarding the structure of the aromatase active site has been generated using this approach, the mammalian cell expression system cannot produce adequate amounts of the enzyme for a detailed structure–function analysis. Expression of functionally active human aromatase in insect cells [24–26] and in yeast [27] has been reported. To date, these expression methods have not been demonstrated to be practical approaches for the large-scale expression of aromatase. In addition, it has been difficult to purify the recombinant aromatase from the enzyme-expressing mammalian cells, insect cells, or yeast. A number of attempts from this and other laboratories have been devoted to the expression of human aromatase in *Escherichia coli*. We [19] have learned that significant amounts of aromatase can be generated using the *E. coli* expression method; however, the recombinant protein is catalytically inactive and present mainly in the inclusion bodies. Previous protein sequence analysis from our laboratory has revealed that the N-terminal sequence of aromatase P450 carries the membrane-anchoring hydrophobic region [28]. By deleting this region, it was thought that the solubility of the enzyme might increase while retaining biological function. Therefore, Del-38 was expressed in *E. coli*. While this enzyme form still remains in the inclusion body, it was found to be catalytically active under a reconstitution condition (with NADPH-cytochrome P450 reductase). Amarneh and Simpson [26] have reported the expression of a catalytically active Del-41 form of aromatase, utilizing the baculovirus vector system.

This report describes several significant achievements in the *E. coli* expression of human aromatase. We have produced functionally active aromatase, established conditions to solubilize aromatase present in the inclusion bodies, and developed methods to purify the recombinant enzyme. More importantly, our analyses have confirmed that the catalytic properties of the recombinant form of aromatase are identical to those of the enzyme found in mammalian tissue. This *E. coli*-expressed aromatase is a very important tool for further structure–function studies of human aromatase.

2. Materials and methods

2.1. Materials

The restriction enzymes *Bam*HI, *Nde*I, and *Bgl*II were purchased from the New England BioLabs Co. *Taq* DNA polymerase and NTA agarose were from Qiagen Inc. Bacterial media for *E. coli* were from Difco. IPTG, NADPH, δ-ALA, and PMSF were purchased from the Sigma Chemical Co. [1β-^{3}H]Androstenedione (specific radioactivity 25 Ci/mmol) was purchased from NEN^{TM} Life Science Products, Inc. pET3b vector and *E. coli* host strain BL21(DE3) pLysS were from Strategene. SDS–PAGE molecular weight standards were from Bio-Rad Laboratories. DNA primers were synthesized at Integrated DNA Technologies, Inc. Rabbit polyclonal IgG against the His-tag was obtained from Santa Cruz Biotechnology Inc.

2.2. Buffers

Buffer A was 10 mM potassium phosphate (pH 7.4) containing 0.1 mM EDTA. Buffer B consisted of 67 mM potassium phosphate (pH 8.0), 20% glycerol, 1 mM PMSF, 0.1 mM dithiothreitol, 0.1% Tween 20, and 0.2 M NaCl. Buffers C and D were the same as buffer B, except that they contained 1 and 2 mM EDTA, respectively. Buffer E was composed of 160 mM potassium phosphate (pH 7.4), 20% glycerol, 80 mM EDTA, and 0.5 M NaCl.

2.3. Construction of plasmids

The four plasmids constructed were used to express the full-length, Del-38 (38 amino acid residues of the N-terminus were deleted), C-terminus 6xHis-tagged Del-38, and NC-terminus 6xHis-tagged Del-38 forms of human aromatase. The plasmid containing the full-length insert was prepared by Chen *et al.* [19]. The inserts for the other three plasmids were prepared using PCR. Two forward primers (5′-GGCATATGAATTATGAGGGCACATCC-3′ and 5′-GGCCATATGCATCATCATCATCATCATAATT-ATGAGGGCACATCCTCAATA-3′) and two reverse primers (5′-AGATCTGTGTTCCAGACACCTGTC-3′ and 5′-CCGAGATCTCTAATGATGATGATGATGGTGGTG-TTCCAGACACCTGTCTGAGTTTCTTGG-3′) were used. The forward PCR primers contain an *Nde*I restriction site including the translation initiation ATG codon, and the reverse primers have a *Bgl*II site at the 3′ end immediately after the stop codon, to facilitate cloning of the resulting amplified products into the expression vector, pET3b. The longer forward and reverse primers also contain a sequence to encode for the 6xHis-tag. The PCR products were sequenced to ensure that no sequence error was introduced during PCR amplification. PCR cycle conditions were 26 cycles with each cycle including 94° for 1 min, 55° for 1 min, and 72° for 2 min. The reaction concluded with an 8-min elongation at 72°. The gel-purified PCR products were digested with *Nde*I/*Bgl*II and ligated into the *Nde*I/*Bam*HI-digested pET3b vector to produce the expression plasmids pET3b aromatase, pET3b Del-38 aromatase, pET3b C-6xHis-tagged Del-38, and pET3b NC-6xHis-tagged Del-38.

2.4. Expression of aromatases in E. coli and preparation of the membrane-bound fractions

The *E. coli* BL21(DE3) strain was used for the expression of the recombinant protein. A 1-L culture was prepared starting from a single colony, and the bacteria were grown at 37° in Luria broth medium containing 120 μg/mL

of ampicillin and 34 μg/mL of chloramphenicol. The flasks were shaken at 270 rpm for about 3 hr until the optical density measurement at 600 nm reached about 0.6–0.8. δ-ALA was then added to a final concentration of 80 μg/mL. After a 30-min incubation, T7 RNA polymerase expression was induced by the addition of IPTG to a final concentration of 0.5 mM. Growth was allowed to continue for 24 hr at 28°, at a shaking rate of 120 rpm. Cells were harvested by centrifugation at 2500 *g* for 10 min at 4°, washed with buffer A, resuspended in buffer A, and stored frozen at −80° overnight.

After thawing, PMSF was added to a concentration of 2 mM, the cells were disrupted by sonication on ice (Sonifier: Branson Sonifier 450, 4 × 1 min), and an equal volume of buffer A containing 40% glycerol was added. The suspension was centrifuged at 100,000 *g* for 60 min at 4°. After centrifugation, the pellet was resuspended in buffer A containing 20% glycerol, and sonicated again. Then the combined suspension was centrifuged again at 100,000 *g* for 60 min at 4°. The bright red gelatinous pellet is the membrane-bound fraction, and contains the membrane-bound proteins including aromatase.

2.5. Purification of aromatase expressed in E. coli from the membrane-bound fractions

Aromatase in the membrane-bound fractions was solubilized with detergent (0.1% Tween 20) and purified by metal-ion affinity chromatography. The bright red gelatinous pellets were resuspended in buffer B (the same volume of buffer A as for the previous step), stirred at 4° for 3 hr, and centrifuged at 30,000 *g* for 60 min at 4°.

The NC-hexahistidine-tagged recombinant form of aromatase was purified on a Ni-NTA agarose column. The supernatant containing the soluble recombinant proteins was loaded onto a 2.5-mL Ni-NTA-agarose column at a flow rate of about 20 mL/hr. The Ni-NTA-agarose column was pre-equilibrated with buffer B. After adsorption of aromatase, the column was washed with about 20 column volumes of buffer C. Then the contaminating proteins were eluted with buffer D (20 column volumes), and the recombinant protein (aromatase) was eluted with buffer E (10 column volumes). The enzyme activity remained stable when the purified enzyme was stored frozen at −80°.

2.6. Reduced CO-difference spectral studies

Aliquots of aromatase preparations (0.18 mg/mL) were added to two cuvette (1 mL each), and the baseline of light absorption between 500 and 400 nm was recorded in a spectrophotometer DU 640 (Beckman). Carbon monoxide was bubbled slowly into the sample cuvette for about 30 s. Reduction of the sample was achieved by adding a few grains of sodium dithionite. The reduced CO-difference spectrum between the sample and the blank cuvette was recorded. The extinction coefficient, $\varepsilon_{450-490} = 91\ mM^{-1}\ cm^{-1}$ [29], was used to calculate the concentration of cytochrome P450 in the cuvette.

2.7. SDS–PAGE and western blot

The samples were analyzed by SDS–PAGE using 12% gels. Protein bands were visualized by Coomassie blue staining or electrophoretically transferred to nitrocellulose membranes (0.45 μm) (Bio-Rad Laboratories). After transfer, the membranes were blocked with 5% non-fat milk in PBS for 1 hr at room temperature, and then probed for 1 hr at room temperature with rabbit polyclonal IgG against the His-tag (1:1000) that was diluted with 5% non-fat milk. Following incubation with horseradish peroxidase (HRP)-linked goat-antirabbit IgG antiserum, the 6-His-tag containing proteins were detected using a SuperSignal Chemiluminescent substrate for detection of HRP (Pierce Inc.).

2.8. Aromatase assays

Aromatase activity was determined using the tritiated water method [30]. The tritiated water release assay for human aromatase was validated in our laboratory by a product isolation assay [13]. The solubilized and purified enzymes were reconstituted with bovine liver microsomes (final concentration: 40 μg/mL) and NADPH (150 μM) in 67 mM potassium phosphate buffer (pH 7.4). After the reconstituted system was incubated with 100 nM [1β-^{3}H]androstenedione for 10 min at 37°, the reaction mixture was removed and extracted with an equal volume of chloroform. The mixture was then centrifuged at 100 *g* for 10 min at room temperature, and the aqueous upper layer was mixed with charcoal-dextran to remove any trace amount of unreacted substrate. In the second extraction, the sample was vortexed and subsequently centrifuged at 15,000 *g* for 5 min at room temperature. Aliquots of supernatant were assessed for radioactivity using a liquid scintillation counter. Protein concentrations were determined by the method of Bradford [31].

2.9. Metabolite analysis

The assay was performed using [1β-^{3}H]androstenedione as a substrate. The reaction mixture was extracted with an equal volume of chloroform. A 500-μL aliquot of the chloroform phase containing steroid substrate, product, and intermediates was withdrawn, and the chloroform was removed by lyophilization. The residue was dissolved in 100 μL acetonitrile, and a 50-μL aliquot was mixed with 10 μL of internal standards (the concentration of each internal standard was 200 μM). The reaction intermediates were separated by reverse phase high-performance liquid chromatography on a C_{18} column (218TP54; VYDAC), using a solvent system of acetonitrile:water (25:75, v/v) at a flow rate of 1 mL/min. The retention times of 19 ol A, 19 al A, 19 nor A, and androstenedione were determined based

on the absorbance of the internal standards at 214 nm and eluted at 8, 12, 17, and 20 min, respectively. The radioactivity associated with each peak was used to calculate the amount of each steroid. The level of the product estrone was estimated from the amount of tritiated water formed.

3. Results and discussion

3.1. Construction of the expression plasmids

Four plasmids for the expression of full-length, Del-38, C-6xHis-tagged Del-38, and NC-6xHis-tagged Del-38 forms of aromatase, respectively, were designed and prepared as described in Section 2. The cDNAs were cloned into the pET3b *E. coli* expression vector which carries the bacteriophage T7ϕ 10 promoter (pϕ 10) and ϕ terminator (Tϕ). The recombinant protein without the first 38 amino acids from the N-terminus was found to be catalytically active. Moreover, the addition of two separate hexameric histidine tags to the N- and C-termini increased the binding of the recombinant enzyme to the nickel-agarose (discussed further below).

3.2. Expression of the recombinant forms of aromatase

The *E. coli* cultures transformed with the four individual constructs were induced with IPTG. In this expression system, aromatase cDNAs were transcribed by T7 RNA polymerase whose expression was induced by IPTG through a *lac UV5*:T7 RNA polymerase fusion gene present in the expression host BL21(DE3) pLysS. The bacteria were grown in the presence of the heme precursor, δ-ALA (80 mg/L). SDS–PAGE and western immunoblot analyses of the membrane fractions of the aromatase expression bacteria revealed the presence of distinct bands with molecular weights corresponding to those of the expressed aromatase in the IPTG-induced but not in the non-induced cells. The *E. coli* inclusion bodies solubilized with 0.1% Tween 20 had aromatase activities of 0.12, 5.79, 8.48, and 9.22 pmol/min/mg for the full-length, Del-38, C-6xHis-tagged Del-38, and NC-6xHis-tagged Del-38 forms of aromatase, respectively. The result demonstrated that the Del-38 form is more active than the full-length aromatase. Placing the histidine tags at the N- and C-termini did not affect the aromatase activity.

A variety of bacterial growth temperatures and times were compared. The culture conditions for the highest expressed activity were 24 hr of incubation at 28°. The condition resulting in the best level of protein expression, but no activity, was 37° for 3 hr.

The *E. coli* expression system produces aromatase as insoluble inclusion bodies, which needed to be solubilized and refolded. To obtain active aromatase, the refolding procedure is very important. Twenty percent glycerol used during the extraction and purification is necessary for the membrane protein refolding. In addition, treatment with detergent (discussed further below) for 3 hr or longer at pH 8.0 seems to promote the refolding process.

Among the solubilization conditions examined, 0.1% Tween 20 and 0.5% CHAPS were found to be the best for maintaining aromatase activity during the recombinant protein extraction. Solubilization with Triton X-100, Nonidet P-40, and Emulgen 913 led to the loss of aromatase activity.

3.3. Purification of NC-6xHis-tagged Del-38 aromatase

The inclusion bodies were solubilized with 0.1% Tween 20 for 2–3 hr and purified on a bed of metal-chelating resin. Supernatant of the membrane-bound fraction solubilized by detergent was loaded on a column packed with Ni-NTA agarose beads. Most of the soluble recombinant aromatase was bound to the column and minimally eluted in the flow through. The non-specific proteins were removed from the Ni-NTA column through a series of washes in buffer B, followed by buffer C. Some recombinant proteins were eluted along with contaminating proteins in buffer D, but this loss was disregarded in order to increase the overall purity of the recombinant protein. The catalytically active aromatase was eluted with buffer E. Fig. 1 shows a representative SDS–PAGE gel and western blot (using a polyclonal antibody against the 6xHis-tag) in which the membrane-bound and purified NC-6xHis-tagged Del-38 aromatase appear as single bands with an apparent molecular mass of 47 kDa.

The aromatase activity of the purified preparations was measured using the tritiated water release method. Table 1 shows the amount and fold of purification of the recombinant double His-tagged protein at different stages from a typical preparation. The total activity of the recombinant protein was increased gradually during the extraction and purification. When whole cells were sonicated, the enzyme activity was very low (17.14 pmol/min). After Tween 20 solubilization and purification by Ni-NTA chromatography, the recombinant protein activity was much higher (1752.78 pmol/min), indicating a renaturation process during the solubilization and purification steps. While the exact nature of the renaturation process is not known, it is thought that the detergent and glycerol in the buffers

Table 1
Purification of the NC-6xHis-tagged Del-38 form of human aromatase from *E. coli* membrane fraction

	Total protein (mg)	Total activity (pmol/min)	Specific activity (pmol/min/mg)
Membrane-bound fraction	95.2	17.14	0.18
Solubilized proteins	59.2	381.25	6.44
Purified aromatase	2.1	1752.78	834.66

The recombinant aromatase was purified a significant number of times, as described in the text. The results of a typical preparation are shown.

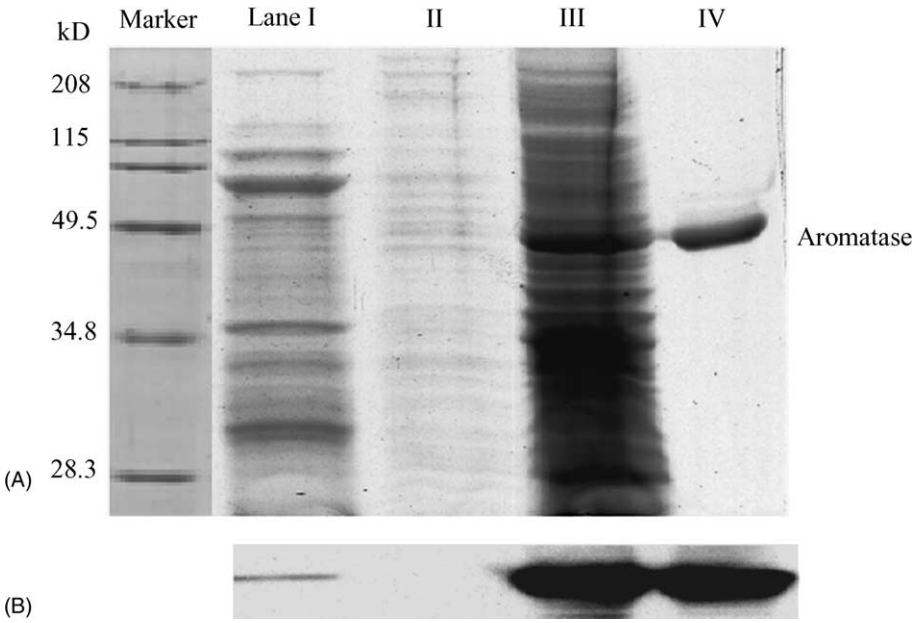


Fig. 1. SDS–PAGE analysis of *E. coli* expressed NC-6xHis-tagged Del-38 aromatase. (A) Coomassie blue-stained 12% SDS–PAGE gel of fractions generated in the purification of NC-6xHis-tagged Del-38 aromatase. (B) Western blot analysis performed using a rabbit polyclonal antibody against the 6xHis-tag at 1:1000 dilution. Lane I, flow through fraction, 3 μg protein; lane II, wash fraction, 2 μg protein; lane III, membrane-bound fraction, 6 μg; and lane IV, purified aromatase, 2 μg.

provide a suitable environment for the recombinant aromatase. Furthermore, the washing steps remove the excess detergent to allow the expressed enzyme to fold properly and eliminate contaminating proteases that can degrade the expressed protein. In addition, attachment of the enzyme to the Ni-NTA agarose beads through the N- and C-terminal histidine-tags may facilitate the folding process. The specific activity of the recombinant protein could be increased up to 5.8 nmol/mg/min by longer equilibration of the solubilized preparations on the Ni-NTA column. However, the yield of aromatase protein decreased significantly after a long equilibration step, probably due to an enhanced interaction of the recombinant enzyme with the Ni-NTA agarose beads.

We had tried to elute the recombinant protein with imidazole, and the eluted protein was found to be homogenous by SDS–PAGE. However, the aromatase activity was lost after imidazole treatment. This reagent inactivates the enzyme, probably through its interaction with the heme prosthetic group.

3.4. Characterization of NC-6xHis-tagged Del-38 aromatase

The reduced CO-difference spectral analysis was performed to demonstrate that the preparation is a functionally active cytochrome P450 (Fig. 2). The K_m value was estimated to be 62 ± 24 nM by enzyme kinetic analysis,

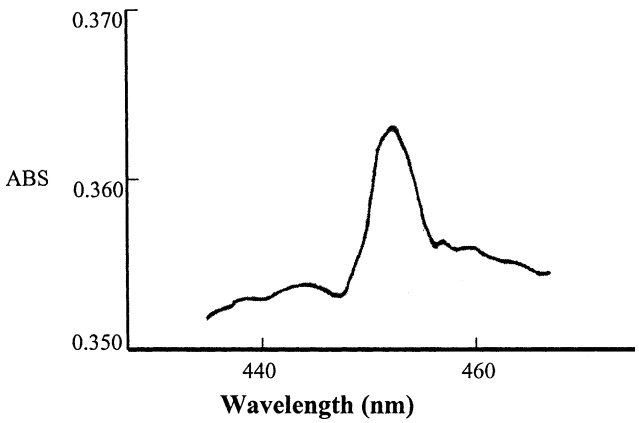


Fig. 2. Reduced CO-difference spectrum of the recombinant aromatase preparation. The analysis was performed with an aromatase preparation (0.18 mg/mL) in 160 mM potassium phosphate (pH 7.4), 20% glycerol, and 0.1% Tween 20. The procedures are those described in Section 2.

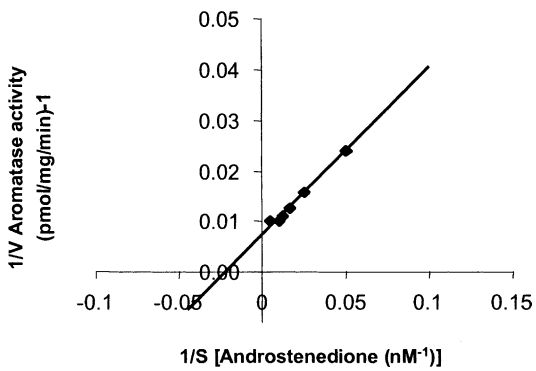
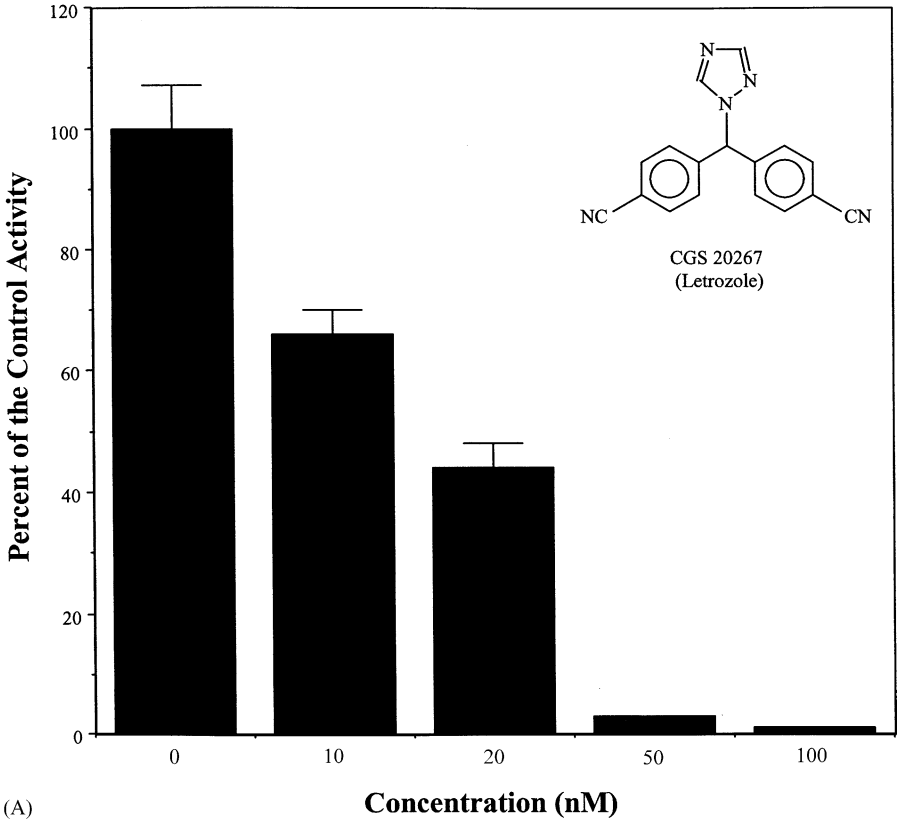
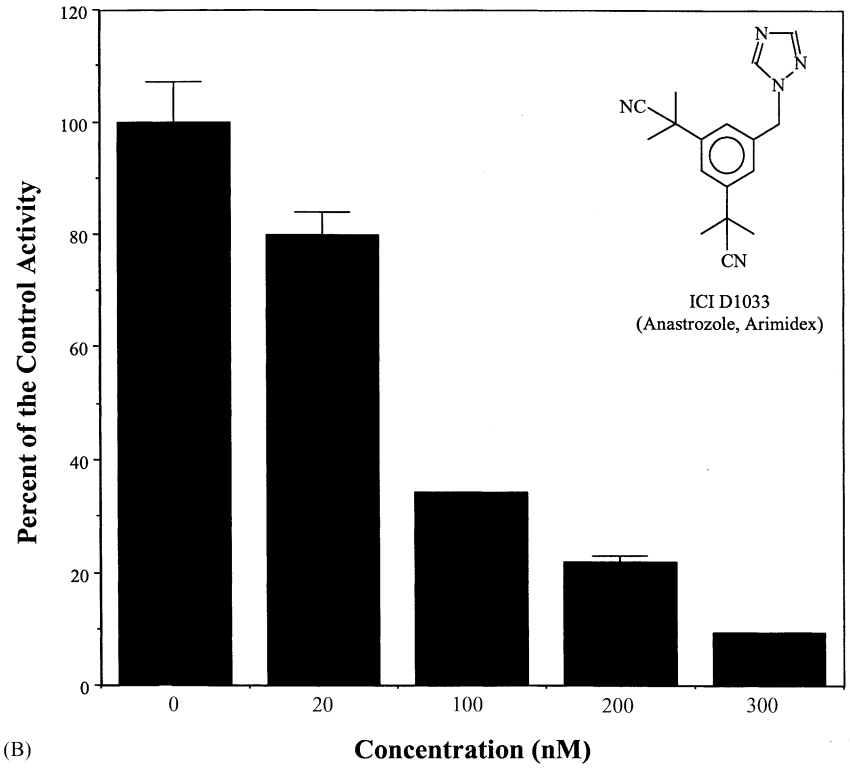


Fig. 3. Kinetic analysis of purified NC-6xHis-tagged Del-38 aromatase. The analysis was performed using the tritiated water release method with [1β-^{3}H]androstenedione concentrations from 20 to 100 nM. Kinetic parameters of three purified aromatase preparations were determined and found to be similar. The results of one analysis are shown.



(A)



(B)

Fig. 4. Inhibition analysis of NC-6xHis-tagged Del-38 aromatase by letrozole (A) and anastrozole (B). The inhibitor was added during the enzyme assay, and aromatase activity was measured as described in Section 2. The studies were performed in triplicate, and the results are shown as means ± SEM. Control activity (100%) = 835 pmol/min/mg.

indicating that the binding affinity of the substrate androstenedione to the recombinant enzyme is similar to that of aromatase in human tissue [10,32]. A double-reciprocal plot of the kinetic analysis of a typical recombinant aromatase preparation is shown in Fig. 3. The efficiency of the reaction was evaluated by reaction metabolite analysis that revealed that minimal levels of 19-ol and 19-al intermediates were released during the conversion. Finally, the reaction was suppressed by letrozole and anastrozole, two known aromatase inhibitors (Fig. 4). Letrozole and anastrozole were approved recently by the US Food and Drug Administration for use as first-line agents against estrogen-responsive cancer. The recombinant aromatase was inhibited by these two inhibitors at concentrations identical to those used to suppress human placental aromatase and aromatase expressed in CHO cells [22]. In summary, our functional characterizations indicate that the recombinant aromatase preparation has catalytic properties identical to the enzyme found in human tissue.

We feel that this study is important for the following reasons: for the first time, a functionally active aromatase can be generated and purified from *E. coli*. Deletion of the first 38 amino acids and introduction of two 6xHis-tags at the N- and C-termini of the aromatase facilitate the solubilization and purification of the recombinant aromatase. This *E. coli* expression method provides an easy way to produce significant quantities of aromatase for research because it is easier and cheaper to grow a large quantity of *E. coli* than either insect or mammalian cells. In addition, our study reveals that a renaturation process takes place during the purification of the recombinant enzyme. The development of this purification procedure is essential for the isolation of functionally active aromatase from *E. coli*. Finally, our biochemical characterization indicates that our recombinant aromatase efficiently catalyzes the aromatization of androgens and has catalytic properties identical to aromatase found in human tissue. This recombinant form of aromatase is a very useful tool for evaluating the molecular action of aromatase inhibitors such as letrozole and anastrozole.

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