Pergamon

0960-0760(95)00135-2

J. Steroid Biochem. Molec. Biol. Vol. 54, No. 5/6, pp. 257–264, 1995

0960-0760/95 $9.50 + 0.00

Direct Expression of Pig Testicular 3α/β (20β)-Hydroxysteroid Dehydrogenase in *Escherichia coli*

Shizuo Nakajin,* Takashi Nakajima, Mihoko Uchida, Shuji Ohno and Masato Shinoda

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan

The cDNA coding for pig testicular 3α/β (20β)-hydroxysteroid dehydrogenase was expressed in *Escherichia coli* by placing it under the control of an isopropylthiogalactoside (IPTG) inducible *tac* promoter. Production of 3α/β (20β)-HSD was demonstrated by Western blotting and by catalytic activity with 5α-dihydrotestosterone as a substrate for 3α/β-HSD, and progesterone and 17α-hydroxyprogesterone as substrates for 20β-HSD in the presence of NADPH. The 3α/β (20β)-HSD enzyme was detected in a soluble fraction of the lysate of *E. coli* added to IPTG to induce the synthesis of the protein. Its molecular weight was estimated to be 30.5 kDa by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Recombinant 3α/β (20β)-HSD was purified to apparent homogeneity as determined by SDS–PAGE by column chromatography using DEAE–cellulose. The purified enzyme reduced not only steroids but also prostaglandins and other carbonyl compounds including aldehydes, ketones and quinones as demonstrated in native enzymes purified from pig testes. The amino terminus of the purified enzyme was serine which was coded next to the ATG start codon, and the sequence of the amino terminal 24 residues was identical with the coding amino acid in the cDNA; whereas, the amino terminus of the native 3α/β (20β)-HSD was not detected suggesting that the N-terminal amino acid was blocked.

J. Steroid Biochem. Molec. Biol., Vol. 54, No. 5/6, pp. 257–264, 1995

INTRODUCTION

We previously reported that a high level of 20β-hydroxysteroid dehydrogenase (20β-HSD) activity was present in pig testes during the neonatal stage [1] and that the enzyme had been purified and characterized [2–4]. The purified testicular 20β-HSD catalyzed the conversion of 5α-dihydrotestosterone (5α-DHT) to both 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol in the presence of NADPH [5]; that is, mammalian testicular 20β-HSD also had 3α/β-HSD activity for 5α-DHT as demonstrated by prokaryotic 3α,20β-HSD from *S. hydrogenans* [6, 7]. However, the enzymatic properties of testicular 3α/β (20β)-HSD were different from the properties of prokaryotic 3α,20β-HSD with respect to the specificity of the catalytic reaction and the cofactor requirement [5]. Recently, cDNA encoding 3α/β (20β)-HSD has been isolated from a neonatal pig testis cDNA library and sequenced [8]. Interestingly, the pig 3α/β (20β)-HSD has been found to be 85% homologous to human carbonyl reductase [9, 10]. Furthermore, pig testicular 3α/β (20β)-HSD exhibits carbonyl reductase-like activity [8]. In this paper, we describe the direct expression of pig testicular 3α/β (20β)-HSD in *Escherichia coli* and compare the enzyme activities of the recombinant and the native testicular enzyme. The development of a large scale expression system of this enzyme contributes to the progress of the characterization of the reaction mechanism of the multifunctional enzyme, 3α/β (20β)-HSD.

*Correspondence to S. Nakajin.
Received 19 Dec. 1994; accepted 13 Apr. 1995.

Abbreviations: 3α/β (20β)-HSD, 3α/β (20β)-hydroxysteroid dehydrogenase; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 5α-DHT, 5α-androstan-17β-ol-3-one; progesterone, 4-pregnene-3,20-dione; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnen-3-one; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

EXPERIMENTAL

Plasmid construction

The construction of an expression plasmid for 3α/β (20β)-HSD from neonatal pig testis was carried out as follows; a cloning vector pBS 52 [8] inserting a full length cDNA which was coded testicular 3α/β (20β)-HSD was linearized by *Ssp* I digestion, and the linearized plasmid was then used as the template for PCR [11]. The primers used were 5′-GGGAATTCATGTCTTCCAACACTCGAG-3′ as a forward primer for the amino terminal side and 5′-GGGAATCAGGACACAAATTATGCAT-3′ as a reverse primer for the carboxy terminal side to extend the DNA linker including the *Eco*RI restriction site in the 5′ and 3′ end of the cDNA. The amplified cDNA was digested with the restriction enzyme *Eco*RI and then purified by agarose gel electrophoresis [12]. The expression vector, pKK223-3, was also digested with *Eco*RI and dephosphorylated with alkaline phosphate. The isolated cDNA was ligated with *Eco*RI cut vector, and the construction was designated pKK223-20β. The expression plasmid, pKK223-20β, inserting 3α/β (20β)-HSD cDNA was transformed to *E. coli* JM 105, and the transformants were selected in LB-agar including ampicillin (50 μg/ml). The plasmid with the correct insertion of the cDNA was selected by mapping the digested plasmid DNA with *Bam*HI and *Xho*I. A clone was isolated that included an expression plasmid constructed with the correct insertion. The general techniques for plasmid construction were performed as described by Sambrook *et al.* [12].

Expression of recombinant 3α/β (20β)-HSD

The *E. coli* JM 105 transformed by pKK223-20β was seeded into LB medium [12] containing 50 μg/ml ampicillin and incubated at 37°C until the absorbance at 600 nm reached about 0.2. Expression of 3α/β (20β)-HSD was induced by adding 0.1 mM IPTG. Incubation was continued up to a definite time at 37°C with vigorous shaking. After incubation, the *E. coli* cells were harvested by centrifugation at 300 *g* for 20 min at 4°C, resuspended with 4 vol. (w/v) of ice-cold 50 mM Tris–HCl (pH 8.0) that included 1 mM PMSF and 10% sucrose and then treated with lysozyme (1 mg/ml) and 1% Triton X-100. These samples were used for SDS–PAGE and Western blotting. A sonic disintegrator was used for another preparation method of the bacterial lysate. *E. coli* cells harvested by centrifugation were washed once with 4 vol. (w/v) of ice-cold 3 mM potassium phosphate buffer (KPB), pH 7.4, including 0.1 mM EDTA and 0.1 mM DTT. The cells were resuspended with the same buffer and were sonicated with the sonic disintegrator (Taytec VP-30) in an ice bath using 4 min pulses with a 10% duty cycle at maximum power, and then they were centrifuged at 105,000 *g* for 60 min. The supernate obtained was used for the enzyme assay and purification of the enzyme.

Purification of 3α/β (20β)-HSD

After the addition of IPTG (1 mM) for the expression of 3α/β (20β)-HSD, *E. coli* JM 109 harboring pKK223-20β in 500 ml × 4 LB medium including ampicillin (50 μg/ml) was cultured at 37°C overnight with vigorous shaking. The *E. coli* cells were harvested

Table 1. *Substrate specificities of recombinant 3α/β (20β)-HSD purified from E. coli and comparison with the testicular enzyme*

Substrate	Concentration (mM)	Recombinant S.A.* (nmol/min/mg)	Relative velocity	
			Recombinant (%)	Testicular (%)
5α-Dihydrotestosterone	0.05	55.1	59	90
5β-Dihydrotestosterone	0.05	35.1	38	29
Testosterone	0.05	8.7	9	0
Progesterone	0.02	3.9	4	10
17α-Hydroxyprogesterone	0.02	15.0	16	10
9,10-Phenanthrenequinone	0.01	139	150	362
Menadione	0.25	116	125	63
4-Nitrobenzaldehyde	0.5	92.7	100	100
Phenylglyoxal	1.0	83.6	90	87
Capric aldehyde	0.2	53.3	57	3
Hydridantin	0.1	120	129	385
Cyclohexanone	10	118	127	96
4-Nitroacetophenone	0.1	71.0	77	20
Camphorquinone	0.05	226	242	103
Prostaglandin E_1	1.0	15.3	17	0
Prostaglandin E_2	1.0	15.9	17	3

*Specific enzyme activity.

Enzyme activity was measured with 44.5 μg of purified enzyme in the presence of 80 μM NADPH in 1.0 ml of 60 mM sodium phosphate buffer (pH 6.5) at 25°C. The decrease in absorbance was monitored at 340 nm. The activity with 4-nitrobenzaldehyde was set as 100%, allowing direct comparison with activity of testicular enzyme.

by centrifugation and sonicated with the sonic disintegrator. The supernate obtained by the centrifugation at 105,000 *g* for 60 min was dialyzed in 3 mM KPB (pH 7.4) including 0.1 mM EDTA and 0.1 mM DTT. The dialyzed sample was then applied to a DEAE-cellulose (DE-52) column (2.6 × 38 cm) which had been equilibrated with 3 mM KPB (pH 7.4)–0.1 mM EDTA–0.1 mM DTT. Elution was carried out with a linear concentration gradient obtained from 3–100 mM KPB (pH 7.4) including 0.1 mM EDTA and 0.1 mM DTT (750 ml each). Aliquots were assayed for enzyme activities with 5α-DHT as a substrate. As a control experiment, the supernate for sonicated *E. coli* cells harboring only a plasmid pKK-223 was applied to the DEAE-cellulose column in a manner similar to that above.

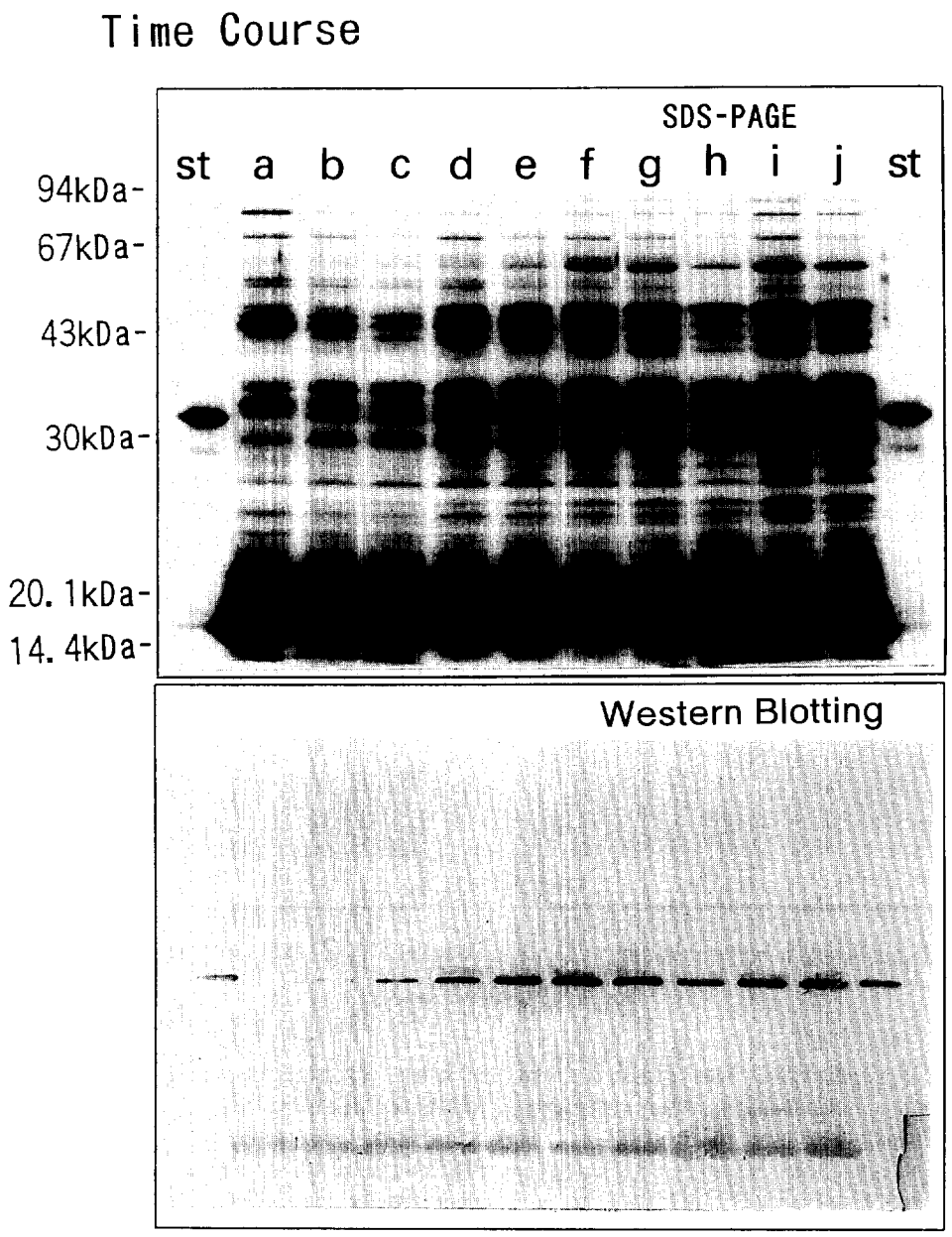

Fig. 1. Detection of the expressed 3α/β (20β)-HSD enzyme protein in *E. coli* lysate by SDS-PAGE and Western blotting. The upper panel is an SDS-PAGE stained with Coomassie blue, and the lower one is a Western blotting. st, purified 3α/β (20β)-HSD from pig testes. Lane a, lysate prepared from bacteria before addition of IPTG ($A_{660} = 0.2$); lanes b–j, lysate prepared from bacteria at 0.5, 0.8, 1.1, 1.4, 1.7, 2.1, 2.4 and 3.0 h after the addition of IPTG, as an inducer. For SDS-PAGE, all bacterial samples were suspended with 4 vol. (w/v) of 50 mM Tris-HCl (pH 8.0), including 1 mM PMSF and 10% sucrose, and were treated with lysozyme (1 mg/ml) and 1% Triton X-100. The Western blotting was carried out in 0.1 M Tris–0.192 M glycine–0.02% SDS containing 20% methanol from SDS-PAGE gel to a nitrocellulose membrane (Hybond C). For immunostaining, pig 3α/β (20β)-HSD IgG (rabbit) and anti-IgG (donkey) conjugated with horseradish peroxidase were used as the primary and secondary antibodies, respectively. The color reaction was carried out with 0.4 mg/ml diaminobenzidine–0.009% H_2O_2 as the substrate of peroxidase.

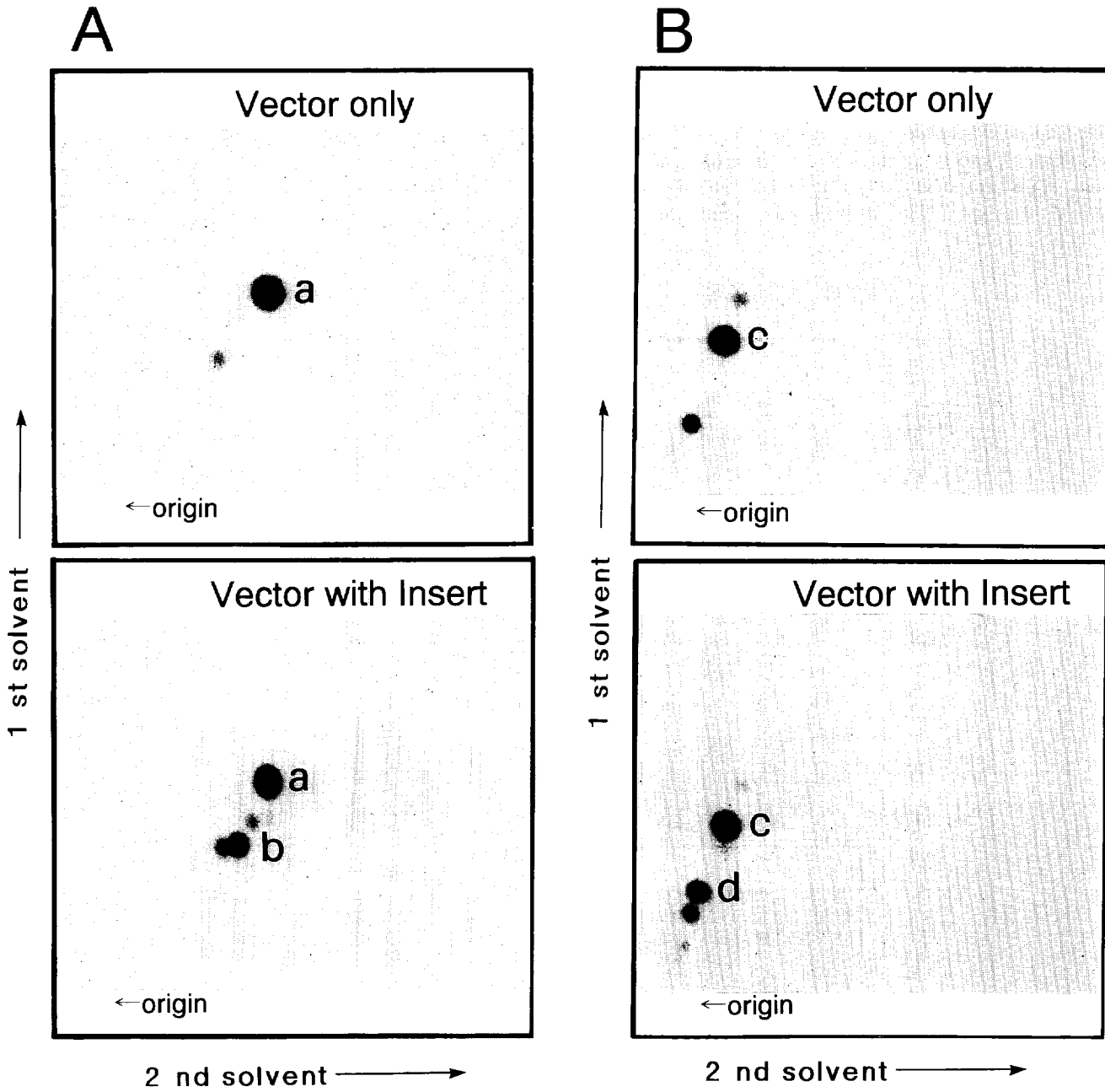

Fig. 2(A,B)—*legend opposite.*

Pig testicular 3α/β (20β)-HSD was purified from testes obtained from a neonatal pig after castration according to a previously reported method [2].

Enzyme assay

An enzyme assay for 3α/β-HSD and 20β-HSD was carried out according to a previous report using radioactive substrates, 5α-DHT, progesterone and 17α-hydroxyprogesterone, which were labeled by ^{14}C at the C4 [1]. Substrate specificity was examined under conditions described previously [8]. Reaction mixtures consisted of 60 mM sodium phosphate (pH 6.5), 44.5 μg of purified 3α/β (20β)-HSD, 80 μM NADPH, and various amounts of substrates (dissolved in 10 μl of ethyl alcohol) as indicated in Table 1 and were incubated in quartz cuvette (1 cm path length) in a total volume of 1 ml at 25°C. The assay of 3α/β (20β)-HSD activity for substrate specificity was spectrophotometrically carried out by monitoring the change in absorbance at 340 nm with time. Furthermore, gas–liquid chromatography was used as previously described to identify the product, the 3α- and/or 3β-hydroxy steroid corresponding to 5α-DHT [5].

Amino acid sequence

The amino terminal amino acid sequences of purified 3α/β (20β)-HSDs were determined by Edman degradation using a gas-phase protein sequencer (Applied Biosystems, model 477A).

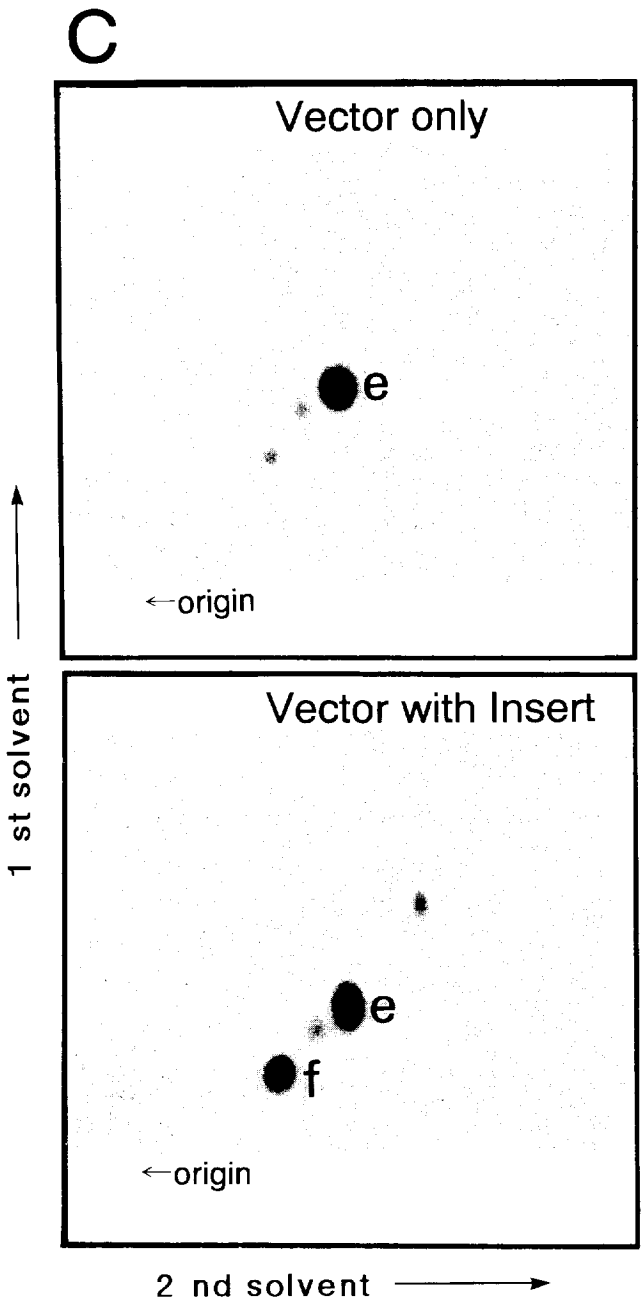

Fig. 2(C).

Fig. 2. Detection of 20β-HSD (reductase) activity and 3α/β-HSD activity (reductase) of expressed protein in *E. coli*. Radioautograms obtained by two-dimensional TLC of radioactive metabolites from progesterone (A), 17α-hydroxyprogesterone (B) and 5α-DHT (C) incubated with bacterial lysate in the presence of NADPH. Development of the two-dimensional TLC was carried out with benzene:acetone (8:2 v/v) as a first solvent, and ethyl acetate–*n*-hexane (7:3 v/v) for panels A and B and benzene–ethyl acetate (2:1 v/v) for panel C as a second solvent. Vector only: incubation with the lysate prepared from bacteria transformed with the expression vector. Vector with insert: incubation with the lysate prepared from bacteria transformed with the expression vector inserted into testicular 3α/β (20β)-HSD cDNA. The assigned spots are (a) progesterone, (b) 20β-hydroxy-4-pregnen-3-one, (c) 17α-hydroxyprogesterone, (d) 17α,20β-dihydroxy-4-pregnen-3-one, (e) 5α-DHT, (f), 5α-androstane-3α (or β), 17β-diol.

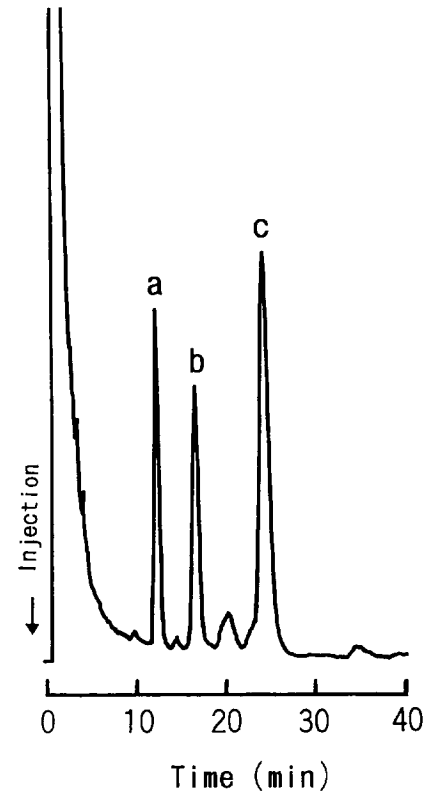

Fig. 3. Gas–liquid chromatography of steroid metabolites of 5α-dihydrotestosterone. A methylene dichloride extract was silylated with TMS–imidazole at room temperature. Peak assignments: (a), 5α-androstane-3α,17β-diol; (b) 5α-androstane-3β,17β-diol; (c) 5α-DHT.

Analytical methods

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) was carried out by the method of Laemmli [13] using 10% acrylamide gels. The molecular weight markers used for SDS–PAGE were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

The protein concentrations were estimated by the method of Lowry *et al.* [14] using crystalline bovine serum albumin (Armour Pharmaceutical Co., Fraction V) as a standard. During the column chromatography, the concentration of the protein was estimated by measuring the absorbance at 280 nm.

Western blotting was carried out as previously described [15].

Chemicals

Plasmid vector, pKK223-3, and *E. coli* JM 105 were obtained from Pharmacia P-L Biochemicals Inc. (Milwaukee, WI).

Restriction enzymes, *Eco*RI, *Ssp*I, *Bam*HI and *Xho*I, alkaline phosphatase (calf intestine) and a ligation kit were purchased from Takara Shuzo Co. Ltd.

(Kyoto, Japan). AmpliTaq DNA polymerase was from Perkin-Elmer Cetus Instrument (Norwalk, CT). Various additional materials and chemicals were obtained from sources previously reported [1, 2, 5, 8]. Other reagents were of the best grade available and were obtained from Wako Chemicals (Tokyo, Japan).

RESULTS AND DISCUSSION

Construction of the expression plasmid

To prepare 3α/β (20β)-HSD cDNA for insertion into the expression plasmid, the PCR technique was used. Amplification of the 3α/β (20β)-HSD sequence, which was incorporated in the cloning vector pBS52, was performed by PCR. The forward and reverse primers were designed to create *Eco*RI restriction sites at the 5′-site of the ATG start codon and at the 3′-site of the 10 nucleotides downstream from the TAA stop codon. The amplified cDNA which was purified by agarose gel electrophoresis, was ligated with the expression plasmid pKK223-3, which had a dephosphorylated *Eco*RI cut, and the constructed plasmid pKK223-20β was transformed to *E. coli* JM 105. A clone that included an expression plasmid constructed with the correct insertion was isolated, and the expression of the protein was placed under the control of IPTG.

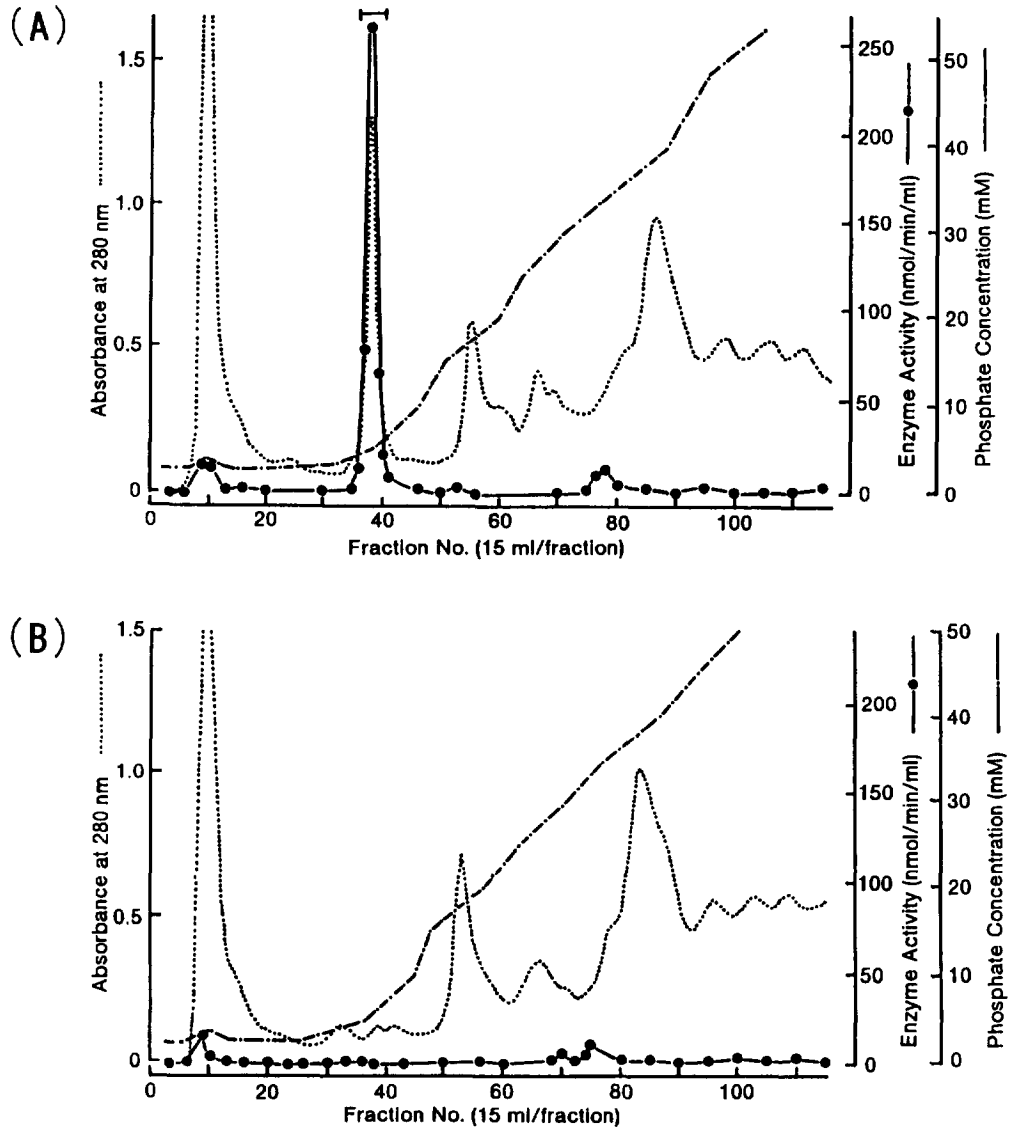

Fig. 4. DEAE–cellulose column chromatography of *E. coli* lysate. Supernatant fluids prepared from the lysate of IPTG induced *E. coli* transformed with the expression vector inserting cDNA (A) and the expression vector only (B) were dialyzed with 3 M KPB (pH 7.4) including EDTA and DTT (0.1 mM each). Dialyzed samples (1.1 g) were applied to a DE-52 column (2.6 × 38 cm) which had been equilibrated with a dialysis buffer. The column was then eluted with a linear concentration gradient of KPB from 3 to 100 mM, and 15 ml of the fraction was collected. The proteins were monitored by measuring the absorption at 280 nm, and aliquots were assayed for enzyme activity.

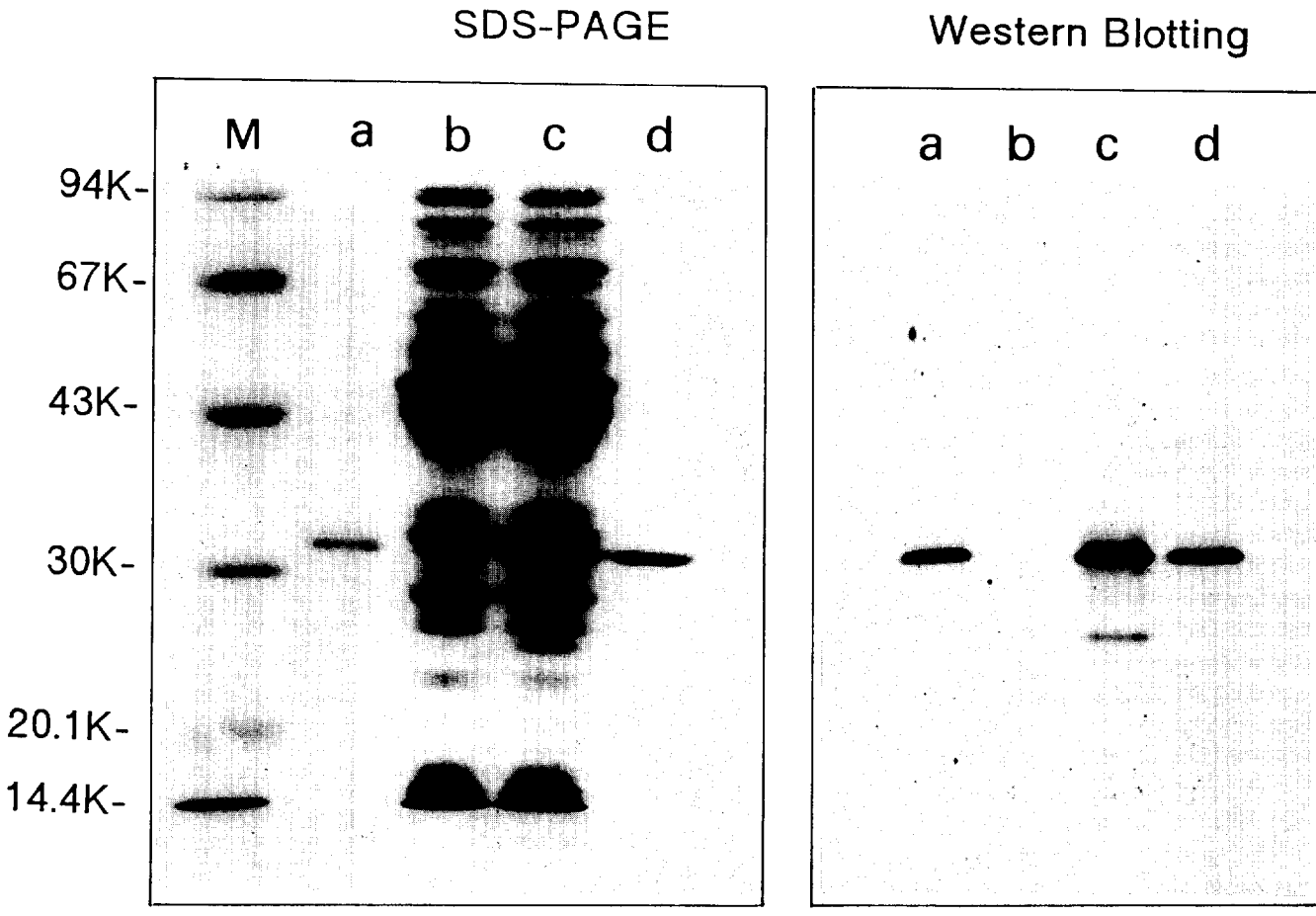

Fig. 5. SDS–PAGE and Western blotting of the recombinant 3α/β (20β)-HSD purified from *E. coli* lysate. Lane M, molecular weight markers; lane a, native 3α/β (20β)-HSD purified from pig testicular cytosol; lanes b and c, the lysate of IPTG induced *E. coli* transformed with the expression vector only and the expression vector inserting cDNA, respectively. Lane d, recombinant 3α/β (20β)-HSD purified *E. coli* lysate.

Expression of 3α/β (20β)-HSD enzyme

The expression product was detected by Coomassie blue stain and Western blot after SDS–PAGE as shown in Fig. 1. The expression product in the lysate of the bacteria had migrated to almost the same position as the native 3α/β (20β)-HSD and increased with the passage of time, after the induction of IPTG; whereas, the bacteria which was not induced by IPTG did not produce an expression product. In addition, the expression product was localized in the supernatant fluid from the bacteria lysate and was not detected in the 105,000 *g* precipitate.

Recombinant 3α/β (20β)-HSD expressed in *E. coli* was catalytically active with some steroids in the presence of NADPH. As shown in Fig. 2(A and B), when a soluble fraction from the bacterial lysate was incubated with progesterone or 17α-hydroxyprogesterone in the presence of an NADPH-generating system, 20β-hydroxy-4-pregnen-3-one or 17α,20β-dihydroxy-4-pregnen-3-one was produced, respectively. In addition, as shown in Fig. 2(C), when 5α-DHT was incubated as a substrate instead of C_{21} steroids, 5α-androstane-3α (or β), 17β-diol was produced by catalysis of the recombinant enzyme. Furthermore, based on the analysis of gas–liquid chromatography of the product after incubation using the purified recombinant enzyme, as shown later, both the 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol were detected as the products from 5α-DHT (Fig. 3). Accordingly, the recombinant 3α/β (20β)-HSD expressed in *E. coli* had 3α/β-HSD activity against 5α-DHT (C_{19} steroid) and 20β-HSD activity against progesterone and 17α-hydroxyprogesterone (C_{21} steroids).

Purification of recombinant 3α/β (20β)-HSD

The recombinant 3α/β (20β)-HSD expressed in *E. coli* JM 105 was purified from the cell lysate using

Table 2. Amino acid sequences of the amino terminal region of testicular and recombinant 3α/β (20β)-HSD

	1	10	20
Deduced from cDNA	MSSNTRVALVTGANKGIGFAIVRDL		
Recombinant enzyme	SSNTRVALVTGANKGIGFAIVRDL		
Native enzyme	ND		

The numbers above the sequence indicate the residue number from the animo terminus.
ND, Amino terminus was not detected by Edam degradation.

DEAE–cellulose (DE-52) column chromatography (Fig. 4). The purified enzyme showed a single band on SDS–PAGE and Western blotting as shown in Fig. 5, with the same migration as that of the native 3α/β (20β)-HSD purified from the testicular cytosol of a neonatal pig. The molecular weight was estimated to be 30.5 kDa by SDS–PAGE. The yield of the recombinant enzyme from the bacterial culture was 20 mg/l of medium and was 3.5% from the bacterial lysate.

Amino terminal sequence of 3α/β (20β)-HSD

The amino terminal sequence of 3α/β (20β)-HSD is shown in Table 2. The amino terminus of the recombinant enzyme was serine and the sequence of the 20 amino terminal residues was identical to that deduced from cDNA. Based on these data, it can be demonstrated that after translation of the ATG start codon to formyl methionine, its methionine was removed by methionine amino peptidase from *E. coli*, as previously reported by Ben-Bassat *et al.* [16]. On the other hand, the amino terminus of native 3α/β (20β)-HSD was not detected by Edman degradation suggesting that the amino terminus of the enzyme was blocked as has been reported for carbonyl reductase [9].

Enzyme activity of the recombinant 3α/β (20β)-HSD

The specificity of recombinant 3α/β (20β)-HSD for the substrate, including some steroids and other carbonyl compounds, was examined using the compounds listed in Table 2 for comparison with the native enzyme, which exhibits carbonyl reductase-like activity [8]. The purified recombinant enzyme reduced 5α-DHT at a comparatively high rate in the presence of NADPH. Identification of the product was performed by gas–liquid chromatography. As shown in Fig. 3, trimethylsilyl (TMS)-derivatives of 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol were detected as the product. Furthermore, the recombinant enzyme reduced not only steroids but also prostaglandins and other carbonyl compounds, including aldehydes, ketones and quinones. These carbonyl compounds were reduced much more efficiently than the steroids and prostaglandins. The rate of reduction of 4-nitrobenzaldehyde was set at 100%, allowing direct comparison of the results with native 3α/β (20β)-HSD isolated from neonatal pig testes [8]. It was found that the substrate specificity of the recombinant enzyme was similar to that of the native enzyme. However, a slight difference was observed which may be attributable to the difference in the amino terminus region between the native and recombinant enzymes.

In conclusion, a plasmid for direct expression in *E. coli* of functional pig testicular 3α/β (20β)-HSD was constructed from the full-size cDNA for the enzyme. The recombinant 3α/β (20β)-HSD was purified from the transformed *E. coli* cell lysates using DEAE–cellulose (DE-52) column chromatography with a yield of 20 mg/l of culture. The purified recombinant enzyme showed enzyme activities 3α/β-HSD (reductase) and 20β-HSD (reductase), and carbonyl reductase-like activity in the presence of NADPH, as shown in the native enzyme. However, the amino terminus of the recombinant enzyme was distinct from that of the native enzyme.

REFERENCES

1. Nakajin S., Ohno S., Takahashi M., Nishimura K. and Shinoda M.: 20β-Hydroxysteroid dehydrogenase of neonatal pig testis. Localization in cytosol fraction and comparison with the enzyme from other species. *Chem. Pharmac. Bull.* **35** (1987) 2490–2494.
2. Nakajin S., Ohno S. and Shinoda M.: 20β-Hydroxysteroid dehydrogenase of neonatal pig testis. Purification and some properties. *J. Biochem.* **104** (1988) 565–569.
3. Nakajin S., Ohno S., Aoki M. and Shinoda M.: 20β-Hydroxysteroid dehydrogenase of neonatal pig testis. Cofactor requirement and stereospecificity of hydrogen transfer from nicotinamide adenine dinucleotide phosphate, reduced form. *Chem. Pharmac. Bull.* **37** (1989) 148–150.
4. Ohno S., Nakajin S. and Shinoda M.: 20β-Hydroxysteroid dehydrogenase of neonatal pig testis. Reverse catalytic (oxidation) reaction. *Chem. Pharmac. Bull.* **39** (1991) 972–975.
5. Ohno S., Nakajin S. and Shinoda M.: 20β-Hydroxysteroid dehydrogenase of neonatal pig testis. 3α/β-Hydroxysteroid dehydrogenase activities catalyzed by highly purified enzyme. *J. Steroid Biochem. Molec. Biol.* **38** (1991) 787–794.
6. Pockilington T. and Jeffert J.: 3α-Hydroxysteroid: NAD oxidoreductase activity in crystalline preparations of 20β-hydroxysteroid: NAD oxidoreductase. *Eur. J. Biochem.* **7** (1968) 63–67.
7. Gibb W. and Jeffery J.: 3-Hydroxysteroid dehydrogenase activities of cortisone reductase. *Biochem. J.* **135** (1973) 881–888.
8. Tanaka M., Ohno S., Adachi S., Nakajin S., Shinoda M. and Nagahama Y.: Pig testicular 20β-hydroxysteroid dehydrogenase exhibits carbonyl reductase-like structure and activity. cDNA cloning of pig testicular 20β-hydroxysteroid dehydrogenase. *J. Biol. Chem.* **267** (1992) 13,451–13,455.
9. Wermuth B., Bohren K. M., Heinemann G. Wartburg von J-P. and Gabbay K. H.: Human carbonyl reductase. Nucleotide sequence analysis of cDNA and amino acid sequence of the encoded protein. *J. Biol. Chem.* **263** (1988) 16,185–16,188.
10. Forrest G. L., Akman S., Krutzik S., Paxton R. J., Sparkes R. S., Doroshow J., Felsted R. L., Glover C. J., Mohandas T. and Bachur N. R.: Induction of a human carbonyl reductase gene located on chromosome 21. *Biochem. Biophys. Acta* **1048** (1990) 149–155.
11. Saiki R. K., Gelfand D. H., Stoffels, Schart S. J., Higuch R., Horn G. T., Mullis K. B. and Erlich H. A.: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239** (1988) 487–491.
12. Sambrook J., Fritsch E. F. and Maniatis T.: *Molecular Cloning 2nd edition.* Cold Spring Harbor Laboratory Press, NY (1989).
13. Laemmli U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227** (1970) 680–685.
14. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the folin–phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
15. Ohno S., Nakajin S. and Shinoda M.: Ontogeny of testicular steroid dehydrogenase enzyme in pig (3α/β-, 20β- and 20α-). Evidence for two forms of 3α/β-hydroxysteroid dehydrogenase. *J. Steroid Biochem. Molec. Biol.* **42** (1922) 17–21.
16. Ben-Bassat A., Bauer K., Chang S-Y., Myambo K., Boosman A. and Chang S.: Processing of the initiation methionine from proteins. Properties of the *Escherichia coli* methionine aminopeptidase and its gene structure. *J. Bacteriol.* **169** (1987) 751–757.