Expression of human dopamine β-hydroxylase in *Drosophila* Schneider 2 cells

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Human dopamine β-hydroxylase (DBH) has been expressed in transformed *Drosophila* Schneider 2 (S2) cells with yields of > 16 mg/l. Most of the activity was found in the culture fluid. Similarly, human neuroblastoma cells also secrete native DBH into the medium, but at a much lower level than recombinant *Drosophila* cells. We have purified native and recombinant human DBH by a modified purification procedure using SP-Sepharose, lentil lectin–Sepharose and gel-filtration chromatography and carried out studies to compare the two enzymes. Two variants of human DBH that differ by a single amino acid (either serine or alanine) at position 304 were expressed in *Drosophila* cells, purified, and found to have no significant difference in enzyme activity. The molecular mass of human DBH monomer has been determined from SDS/PAGE to be 73 kDa, but the recombinant DBH from *Drosophila* is smaller at 66 kDa. The difference may be due to glycosylation as deglycosylated enzymes from both sources are identical in size (61 kDa). The K_m of tyramine for native and recombinant human enzymes are virtually the same but higher than bovine DBH by about 3-fold. Likewise, the inhibition of native and recombinant human DBH by fusaric acid and SKF102698 is not significantly different but IC_{50} values are 2–3-fold higher than that for the bovine enzyme. These results strongly support the conclusion that recombinant human DBH from *Drosophila* S2 cells can be used in place of human neuroblastoma-derived DBH for drug screening, characterization of the enzyme's physicochemical properties, and determination of structure–function relationships. The *Drosophila* expression system has thus provided a convenient source for large quantities of human DBH enzyme.

INTRODUCTION

Dopamine β-hydroxylase (DBH) catalyses the conversion of dopamine into noradrenaline (norepinephrine) in sympathetic neurons. Overactivation of the sympathetic nervous system and elevated noradrenaline release has been shown to contribute to the pathophysiology of hypertension and conjestive heart failure [1,2]. Inhibition of DBH reduces the levels of noradrenaline and simultaneously increases dopamine levels [3]. Therefore, not only are the effects of noradrenaline reduced but the countering effects of dopamine, i.e. lowering of blood pressure, are enhanced. This dual effect of DBH inhibition renders this enzyme an attractive molecular target for the treatment of cardiac heart failure.

The human DBH gene encodes a 603-amino-acid polypeptide which includes a 25-amino-acid N-terminal signal sequence. Two forms of the enzyme that differ by a single amino acid change at position 304 [either alanine (304A) or serine (304S)] were cloned from a cDNA library [4]. The protein contains four potential N-glycosylation sites and 14 cysteine residues. The cysteines form six intramolecular disulphide bonds. A cysteine near the N-terminus (Cys-140) forms a disulphide with a C-terminal cysteine (Cys-582) and thereby the molecule folds back upon itself. The remaining intramolecular disulphides cluster in two regions of the molecule around His-Xaa-His motifs that may be sites for binding of copper, which is required for enzymic activity [5]. In addition to the intramolecular disulphide bonds, each DBH polypeptide forms two intermolecular bonds resulting in disulphide-linked dimers which interact by a non-covalent mechanism to form tetramers [5].

DBH is found within storage vesicles as both membrane-associated and soluble forms [6–9]. Since there are no obvious hydrophobic regions other than the N-terminal signal sequence, the mechanism by which DBH is associated with membrane is unknown. The detection of uncleaved signal sequences in some enzyme preparations purified from vesicle membranes has led some investigators to suggest that membrane association may be due to the uncleaved signal sequence [10–12]. However, when expressed in heterologous cells using an efficiently cleaved signal-peptide from tissue plasminogen activator (tPA) to replace the native signal-peptide, DBH is found to still associate with the vesicle membranes [13]. Another hypothesis is that DBH may be modified with phosphatidylserine and thereby be partially membrane-associated [9,14]. The membrane-associated form of DBH is made of subunits having slightly higher molecular masses than those found in the soluble form. Also, the larger subunit appears to be a precursor of the smaller subunit [15,16]. The soluble form of DBH contains only the smaller subunit [17].

Upon stimulation, the catecholamine storage vesicles release their contents that include noradrenaline, adrenaline, dopamine, and the soluble form of DBH. In addition to the storage vesicle-associated DBH, constitutively secreted DBH is also found in cultured cells [8,18]. The constitutively secreted DBH may be modified by sulphation of an N-linked glycan residue [19].

In order to discover potent and non-toxic DBH inhibitors that can be developed into therapeutically useful agents, large supplies of the enzyme are needed. Recently, expression of bovine DBH as a fusion protein in *Drosophila* cells was reported. In this case, the tPA prepro-sequence was substituted for the native signal sequence [13]. Both membrane-associated and intracellular soluble enzyme were detected, but at very low levels. The tPA signal was efficiently cleaved, resulting in bovine DBH with an N-terminal extension of the 12 amino acids of the tPA pro-sequence. We have used a similar strategy to express the human DBH. Like the previous authors we also observed low levels of

Abbreviations used: DBH, dopamine β-hydroxylase; DMPD, *N*,*N*-dimethyl-1,4-phenylenediamine; FBS, fetal bovine serum; S2, Schneider 2; tPA, tissue plasminogen activator.

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intracellular DBH chimera. In addition we discovered that most of the enzyme was secreted into the culture fluid. Our report is the first to describe purification of milligram amounts of DBH. The large amounts of DBH purified from *Drosophila* cells has permitted the detailed physicochemical characterization and investigation of DBH inhibitors. Availability of an expression system that can produce milligram amounts of DBH that can be readily purified opens up the possibility of detailed structure–function studies of wild-type and mutant enzymes. In particular, characterization of copper-binding ligands and other residues critical for catalysis can be accomplished. In all aspects, the recombinant chimeric enzyme was similar to human DBH secreted from cultured neuroblastoma cells.

MATERIALS AND METHODS

Enzymes and reagents

DBH from bovine adrenal gland was purchased from Sigma (St. Louis, MO, U.S.A.; catalogue no. D-1147), resuspended in 0.125 M sodium acetate (pH 5.2) and stored at −25 °C. Catalase (65000 units/mg) was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The crystalline suspension was diluted in 0.125 M sodium acetate to give a solution of 1 mg/ml just before use.

Tyramine hydrochloride, 3-hydroxytyramine, ascorbic acid and fumaric acid were purchased from Sigma. SKF102698 was obtained from the Institute of Organic Chemistry at Syntex. 1-Heptanesulphonic acid and tetrabutylammonium phosphate were purchased from Aldrich (St. Louis, MO, U.S.A.). A LiChroCART 125-4 RP-18 column was purchased from EM Separations Technology. SP-Sepharose, lentil lectin–Sepharose and Superdex 200 were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Protein determination reagents were purchased from Bio-Rad (Richmond, CA, U.S.A.).

Construction of expression plasmids

The human DBH gene (1.8 kb) was generated by PCR from human adrenal gland cDNA (Clontech, Palo Alto, CA, U.S.A.) using a 5′-end *Eco*RI tail primer 5′-CTCGAATTCTAAATAT-GCGGGAGGCAGCCTTCATGTAC-3′ and 3′-end *Xho*I tail primer 5′-CACACTCGAGTCAGCCTTTGCCCCCACCAAT-GCTG-3′. The gene was first cloned into the *Eco*RI and *Sal*I site of the baculovirus transplacement plasmid, pSyn XIV-VI+X3 [18]. The cloned gene was then sequenced and compared with the sequences previously published [4,20]. Mutations that were judged to arise from errors in the PCR reaction were repaired.

The nucleotide sequence that encodes the human tPA prepro-sequence was synthesized with a 5′ *Eco*RI and a 3′ *Bgl*II site. This fragment was then substituted for the coding region of the native sequence encoding the signal sequence of the human DBH as described by Gibson et al. [13]. The resulting plasmid was designated pSyn:tDS. The serine at amino acid position 304 in the DBH gene was mutated to alanine with the mutagenesis primer 5′-AAGCCGGCCTTGCGTTCGGGGGTCCAG-3′ using Pharmacia's U.S.E. mutagenesis kit. The mutated plasmid was designated pSyn:tDA and was sequenced to confirm the change of serine to alanine at position 304. The fragments containing the tPA–DBH fusion genes in pSyn:tDS and pSyn:tDA were released by digesting with *Eco*RI and *Sma*I and cloned into the *Eco*RV site of the *Drosophila* expression vector pMK33/pMtHy (a gift from Dr. Michael Koelle, Massachusetts Institute of Technology) [21]. The new plasmids were designated pM/H:tDS and pM/H:tDA, respectively.

Cell culture/Schneider 2 (S2) cell transfection

S2 cells were adapted to serum-free EX-CELL 400 medium (JRH Biosciences, Lenexa, KS, U.S.A.) and cultured in shake flasks at 27 °C without CO_2 at a cell density of 1.5×10^6 to 3×10^7 cells/ml. The cells were seeded at $(1–2) \times 10^6$ cells/well in a 6-well dish the day before transfection and allowed to loosely adhere. A sample (5 μg of plasmid DNA/20 μg of lipofectin) (DOTMA; Gibco-BRL, Grand Island, NY, U.S.A.) was transfected into S2 cells using the lipofectin method [22]. Cells were transfected for 1.5 h and stopped by addition of EX-CELL 400 medium containing 10 % (v/v) fetal bovine serum (FBS). Cells were allowed to grow for 48 h without selection after which time the medium was replaced with EX-CELL 400 medium containing 500 μg/ml hygromycin B (Boehringer-Mannheim). Extensive cell death of non-transfected cells was evident after about 1 week. If cells appeared to be unhealthy, FBS was added to 2.5 %. Cells were then passed in EX-CELL 400 medium containing 500 μg/ml hygromycin B as needed. Shake-flask cultures were initiated when a volume of 10–20 ml at a density greater than 1.5×10^6 cells/ml was obtained.

Growth of human SK-N-SH neuroblastoma cells in a cell cube

SK-N-SH cells were grown and maintained in a 21250 cm^2 Corning Costar Cell Cube. This device consists of a series of parallel plates to which the cells attach and conditioned medium is perfused over them. A 2 l Applikon Bioreactor served as a conditioning device and medium was pumped through the cube at 250–800 ml/min, depending on cell densities. Temperature was controlled at 37 °C. The pH was maintained at 7.3 by sodium bicarbonate/CO_2 addition and the dissolved oxygen was controlled at 50 % saturation by O_2 supplementation.

The cells were grown in Gibco BRL CHO-S-SFM II serum-free medium. Once the production phase was reached, the medium was harvested in 1.5–2.0 l batches. The bioreactor was replenished with fresh medium and this process was repeated on a daily basis for 3 weeks.

Purification of human DBH from SK-N-SH culture medium: SP-Sepharose chromatography

SK-N-SH culture medium from the cell cube was used to purify secreted human DBH. This purification step was performed at 4 °C. The pH of 14 l of the culture with DBH activity greater than 5 units/l was adjusted to 5.55 with 5 % acetic acid and was then filtered with Gilman Suporcap-50 filters (0.45 μm pores). The filtered solution was diluted 3-fold with cold Millipore nanopure water and loaded at 24 ml/min on to a column containing 350 ml of SP-Sepharose equilibrated with 20 mM sodium acetate, pH 5.6. The column was washed extensively with 20 mM sodium acetate, pH 5.6, until the absorbance at 280 nm returned to the baseline. Elution was started with a linear gradient of 0–0.15 M NaCl in 20 mM sodium acetate, pH 5.6, at 32 ml/min for 45 min, followed by an isocratic elution at 0.15 M NaCl for 15 min, and then a gradient of 0.15–0.3 M NaCl for 10 min. Fractions over 1 min (32 ml) were collected and DBH activity assayed by the colorimetric method in a 96-well microplate as described below. The fractions containing most activity were pooled and DBH activity measured by the HPLC assay as described below. The pooled fractions were then mixed with 0.2 vol. of 250 mM KH_2PO_4, pH 6.5, 2.5 M NaCl, the pH was adjusted to 6.5 with NaOH, and material further purified on the lentil lectin–Sepharose column.

Lentil lectin chromatography

A lentil lectin–Sepharose column containing 30 ml of gel was prepared and equilibrated with 50 mM KH_2PO_4, pH 6.5, 0.5 M NaCl. The purification was carried out at 4 °C. The pH-adjusted pool from the SP-Sepharose column was loaded on to the lentil lectin column at 1.2 ml/min and then washed extensively with 50 mM KH_2PO_4, pH 6.5, 0.5 M NaCl until the absorbance at 280 nm returned to baseline. The column was eluted with a linear gradient of 0–5.5 % (w/v) methyl α,D-mannopyranoside in 50 mM KH_2PO_4, pH 6.5, 0.5 M NaCl at 1.5 ml/min for 50 min, followed by an isocratic elution with 5.5 % methyl α,D-mannopyranoside in 50 mM KH_2PO_4, pH 6.5, 0.5 M NaCl for 20 min. Fractions were collected every 2 min (3 ml) and assayed by the colorimetric method in a 96-well microplate reader. The fractions containing most enzymic activity were pooled and concentrated with an Amicon stirred cell using a YM30 membrane. Methyl α,D-mannopyranoside was removed by buffer exchange with 50 mM KH_2PO_4, pH 6.5, 0.1 M NaCl. The concentrated enzyme solution was divided into aliquots and stored at −25 °C until required for further use.

Gel-filtration chromatography

Human DBH from the lentil lectin column was further purified using Superdex 200 at room temperature. Human DBH (0.4 ml) was loaded at 0.5 ml/min on to a Superdex 200 column equilibrated with 50 mM KH_2PO_4, pH 6.5, 0.1 M NaCl. The same buffer was used for elution at 0.5 ml/min. Fractions were collected every 0.5 min and assayed by the colorimetric method. The fractions containing most enzymic activity were pooled, concentrated using Centricon-10 spin columns, divided into aliquots and stored at −25 °C until required for further use.

Purification of recombinant 304A and 304S human DBH from *Drosophila* cell media

Purification of recombinant human DBH containing 304A was carried out in the same way as human DBH using a smaller SP column. The culture medium (1.8 l) was filtered, diluted and purified by SP-Sepharose, followed by lentil lectin–Sepharose and gel-filtration chromatography. Recombinant 304S DBH was purified similarly by SP-Sepharose and lentil lectin–Sepharose chromatography from 1.3 l of the harvested culture medium.

Enzyme assay by colorimetric method

DBH activity was measured by a continuous colorimetric assay using *N*,*N*′-dimethyl-1,4-phenylenediamine (DMPD) as electron donor [23]. The assay was performed in a cuvette (1 ml reaction vol.) or a microplate (200 μl reaction vol.) at pH 5.2, 30 °C in 0.125 M sodium acetate containing 10 mM fumarate, 0.5 μM $CuSO_4$, 0.1 mg/ml catalase (6500 units), 10 mM tyramine and 5 mM DMPD. A typical reaction in the 1.0 ml vol. used 0.02 unit of enzyme, which was added into the above reaction mixture without DMPD and was then incubated for 5 min. DMPD was added to initiate the reaction. The absorbance at 515 nm was monitored continuously using a Perkin Elmer Lambda 2 UV/VIS spectrometer. Data acquisition and analysis were carried out using the PECSS software from Perkin Elmer. An absorbance coefficient of 5200 $M^{-1} \cdot cm^{-1}$ for DMPD was used for calculation of DBH activity. A blank control without enzyme was used as background and the slope was subtracted from the measured slopes for enzyme reactions. For protein purification fractions, the assay in the 96-well microplate was used to detect DBH activity. Typically, 15 μl of each fraction was added into 200 μl of the reaction mixture at room temperature and absorbance at 510 nm read after 5 min on a Dynatech plate reader.

HPLC assay

An HPLC assay was used to measure DBH activity using tyramine and ascorbate as substrates. The method is based on the separation and quantification of tyramine and octopamine by reverse-phase HPLC [24]. The assay was performed at pH 5.2 and 37 °C in 0.125 M sodium acetate containing 10 mM fumarate, 0.5–2.0 μM $CuSO_4$, 0.1 mg/ml catalase (6500 units), 1.0 mM tyramine and 4 mM ascorbate. One unit is equal to 1 μmol of octopamine formation/min under such conditions. As the substrates tyramine and ascorbate are not at saturating concentrations in the HPLC assay, we noticed lower activities in the HPLC assay compared with the colorimetric assay using saturating substrate concentrations. In a typical assay, 0.5–1.0 m-units of enzyme were added to the reaction mixture and then a substrate mixture containing catalase, tyramine and ascorbate was added to initiate the reaction (final volume 200 μl). Samples were incubated at 37 °C for 30–40 min. The reaction was stopped by addition of 50 μl of 25 mM EDTA. The samples (150 μl) were loaded into a Gilson autosampler and analysed by HPLC using UV detection at 280 nm. PC-1000 software (Thermo Separations products, Fremont, CA, U.S.A.) was used for integration and data analysis. The HPLC run was carried out at a flow rate of 1 ml/min using a LiChroCART 125-4 RP-18 column and isocratic elution with 10 mM acetic acid containing 10 mM 1-heptanesulphonic acid, 12 mM tetrabutylammonium phosphate and 10 % (v/v) methanol.

For Cu^{2+}-dependence experiments, reaction mixtures containing various $CuSO_4$ concentrations at pH 5.2 were prepared and the assay carried out as described above. DBH activities at various pH values were measured using lentil lectin–Sepharose-purified enzymes. The pH of the reaction mixture containing 1.6 M sodium acetate, 1.0 mM tyramine, and 4 mM ascorbate was measured and adjusted using 1 M NaOH or 10 % acetic acid. Catalase was added to the mixture to give a final concentration of 0.1 mg/ml. DBH containing $CuSO_4$, to give a final concentration of 2 μM, was added to initiate the reaction at 37 °C, which was stopped by addition of EDTA and analysed by HPLC as described above. The relative activities at various $CuSO_4$ concentrations and pH values were then converted into the corresponding specific activities as measured by the colorimetric method.

For kinetic and inhibition experiments, assay reactions were performed according to the procedure for HPLC as described above using lentil lectin–Sepharose-purified enzymes. The reactions were quenched by the stop solution containing 25 mM EDTA and 240 μM 3-hydroxytyramine (internal standard). The remaining percentage activity was calculated based on the control without inhibitor, corrected using internal standards and fitted to a non-linear 4-parameter dose–response curve (eqn. 1) to obtain the IC_{50} values using Grafit from Erithacus Software (London, U.K.).

$$\text{Percentage activity} = \frac{\text{Max} - \text{Min}}{1 + \left(\frac{X}{IC_{50}}\right)^h} + \text{Min} \quad (1)$$

Max is the fitted maximum response, Min the fitted minimum response and h is the Hill coefficient.

Protein analysis

Protein concentration was determined by the Bradford method

(Bio-Rad kit using BSA as protein standards). Protein gel electrophoresis was performed using an SDS/10 %-polyacrylamide ready-made gel from Novex. Location of protein bands on the SDS/PAGE gel was determined using a scanning densitometer from PDI Systems (Huntington Station, NY, U.S.A.).

N-terminal sequences of native and recombinant human DBH were determined by amino acid sequencing of the denatured protein. Protein gel electrophoresis was carried out as described above, and protein bands were transferred to Immobilon-P membrane (Millipore). The DBH band on the membrane was cut out and submitted for amino acid sequencing.

Deglycosylation of DBH

Native and recombinant human DBH were mixed with 0.5 % SDS and 5 % (w/v) 2-mercaptoethanol and boiled for 4 min. The mixture was then treated with N-Glycanase in the presence of protease inhibitors (pefabloc SC, 3 mM; pepstatin A, 38 μg/ml; leupeptin, 38 μg/ml; aprotinin, 6 μg/ml) for 3 h at room temperature and then 16 h at 37 °C. SDS sample loading buffer was added and the mixture was boiled for 3 min. Samples were analysed on a Novex pre-made SDS/10 %-polyacrylamide gel using the following molecular-mass standards, also from Novex: myosin, 250 kDa; BSA, 98 kDa; glutamic dehydrogenase, 64 kDa; alcohol dehydrogenase, 50 kDa; carbonic anhydrase, 36 kDa; and myoglobin, 30 kDa. A sample without N-Glycanase was used as a control for comparison.

RESULTS

Expression of DBH

Insect cells (Sf-9) were co-transfected with baculovirus virion DNA (Baculogold; PharMingen, San Diego, CA, U.S.A.) and either pSyn:tDA or pSyn:tDS. The resulting recombinant viruses were plaque-purified and the expression of DBH upon infection of Sf-9 cells with recombinant virus determined. No activity was detected in pellets of cells infected with the recombinant viruses, but low levels of activity (approx. 0.2 unit/l) were detected in the culture fluid.

Since expression of DBH in the baculovirus expression system was low, the DBH genes were cloned into a *Drosophila* expression vector, pMK33/pMtHy (a gift from Dr. Michael Koelle, Massachusetts Institute of Technology). The vector contains the hygromycin resistance gene which is under the control of the *Drosophila copia* long terminal repeat. Chimeric DBH genes with the tPA prepro-sequence replacing the native signal sequence were put downstream from the metallothionein promoter present in the expression vector. The resulting vectors were designated pM/H:tDA (alanine at position 304) and pM/H:tDS (serine at position 304). *Drosophila* S2 cells that had been adapted to grow in serum-free medium (EX-CELL 400) were transformed with the plasmids. The cells were placed in hygromycin-containing medium. Hygromycin-resistant cells began to grow in about 3 weeks following extensive cell death. The hygromycin-resistant cells were grown in either shake flasks or bioreactors. DBH activity was detected in both the cell lysates and the culture fluid, although most of the activity (95 %) was in the culture fluid. *Drosophila* cells growing in bioreactors were fed a mixture of nutrients (yeast extract, glucose, glutamine and lipids) that has previously been shown to enhance Sf-9 cell growth [25]. As a result of the nutrient feeding the cell density increased from 2×10^7 to 1×10^8 cells/ml (Figure 1a). Concomitantly, the amount of DBH secreted into the culture fluid was increased by more than 2-fold (Figure 1b). The level of DBH activity is clearly dependent on cell density and is usually > 30 units/l at a cell density of 1×10^8 cells/ml, as assayed by the HPLC method.

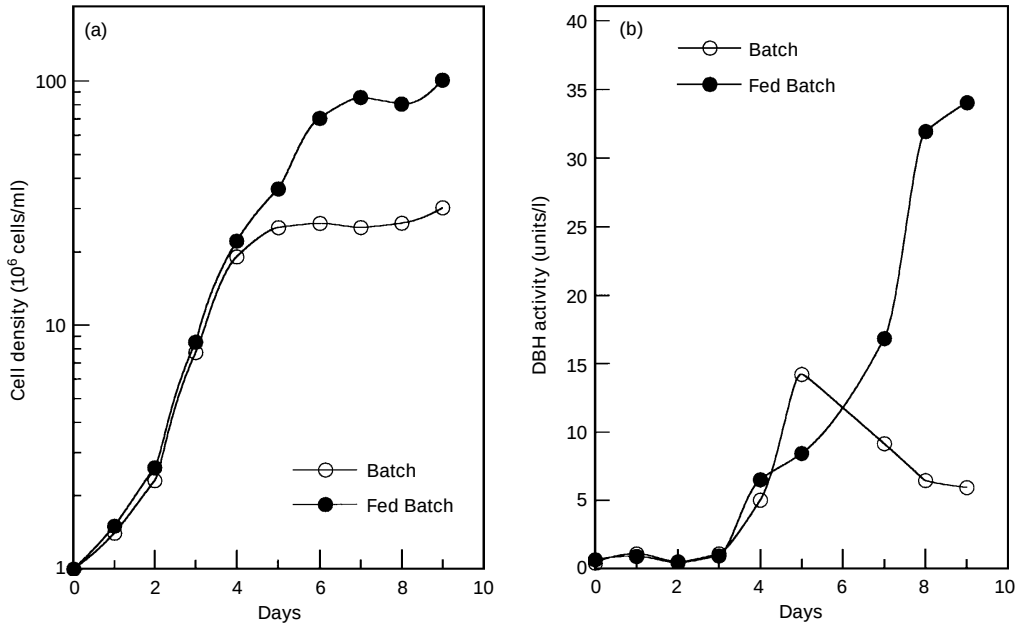

Figure 1 Growth curve of *Drosophila* S2 cells and the corresponding DBH activity profile

Drosophila S2 cells containing pM/H:tDA plasmid were grown in a bioreactor with and without nutrient feed (**a**) as described in the Materials and methods section. DBH activity (**b**) was monitored by the HPLC assay as described in the Materials and methods section. One unit equals 1 μmol of octopamine formation/min at pH 5.2, 37 °C at substrate concentrations of 1 mM tyramine and 4 mM ascorbate.

Table 1 Purification of native and recombinant human DBH

Enzyme specific activities (μmol/min per mg) were also determined by colorimetric assay. The colorimetric assay was performed at pH 5.2, 30 °C with 10 mM tyramine and 5 mM DMPD as described in the Materials and methods section. Lentil lectin–Sepharose purified native and recombinant human enzymes were used for activity assays. Values are as follows: bovine, 2.0 (determination of one enzyme preparation); native human, 15.2 ± 3.9 (average of two preparations); recombinant 304A human, 9.0 ± 4.1 (average of four preparations); recombinant 304S human, 10.3 (determination of one preparation). Bovine DBH was purchased from Sigma.

Step	Volume (ml)	Protein (mg)	Specific activity* (μmol/min per mg)	Yield (%)	Purification (fold)
Human DBH from SK-N-SH culture medium					
Culture medium	14000	1640	0.04	100	1
SP-Sepharose	1300	66	1.1	111	28
Lentil lectin–Sepharose	15	13	2.4	48	60
Recombinant DBH (304A) from *Drosophila* S2 culture medium					
Culture medium	1800	396	0.23	100	1
SP-Sepharose	216	78	1.0	85	4.3
Lentil lectin–Sepharose	20	30	1.7	53	7.4
Recombinant DBH (304S) from *Drosophila* S2 culture medium					
Culture medium	1320	898	0.05	100	1
SP-Sepharose	230	69	0.5	77	10
Lentil lectin–Sepharose	9.4	24	0.9	48	17

* Activity was assayed at 1 mM tyramine and 4 mM ascorbate at pH 5.2, 37 °C by the HPLC method as described in the Materials and methods section.

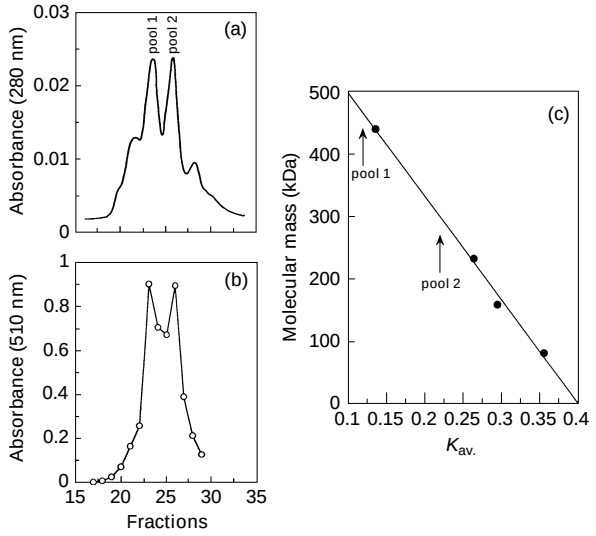

Figure 2 Chromatography of native human DBH from SK-N-SH cells on Superdex 200

(**a**) Protein elution profile with protein detection at 280 nm. (**b**) DBH activities of the corresponding fractions were measured by colorimetric method at 510 nm as described in the Materials and methods section. Plot (**c**) is the apparent-molecular-mass determination of the two pools. Protein standards were run under the same conditions and have the following molecular masses (kDa): transferrin, 81; aldolase 158; catalase, 232; ferritin, 440. $K_{av.}$ values were calculated according to O'Connor et al. [17]. Arrows indicate the two protein peaks with DBH activity, and the apparent molecular masses are 299 and 466 kDa.

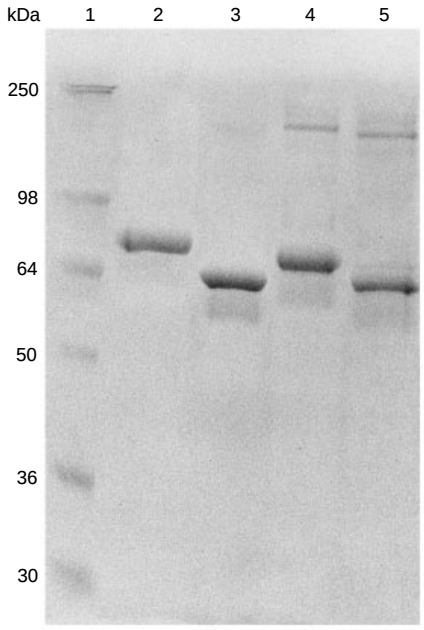

Figure 3 SDS/PAGE of native and recombinant (304A) human DBH before and after deglycosylation

Lane 1, molecular-mass standards; lane 2, native human DBH from SK-N-SH cells as purified by Superdex 200 (pool 2); lane 3, same as lane 2 after deglycosylation; lane 4, recombinant human DBH from *Drosophila* S2 cells as purified by Superdex 200 (pool 2); lane 5, same as lane 4 after deglycosylation.

Purification of native and recombinant human DBH

Recombinant human 304A and 304S forms of DBH expressed in *Drosophila* S2 cells and native human DBH from neuroblastoma cells were purified using cation-exchange and lentil lectin chromatography. The purification scheme resulted in similar recoveries and specific activities of DBH from the two different sources. Examples of purification of each of these enzymes are given in Table 1(a). The specific activity of bovine DBH was considerably lower than the human enzymes presumably due to lower purity. No significant differences among the recombinant human, 304A or 304S, and neuroblastoma-derived DBH was evident (Table 1).

Further purification of both native human and recombinant human DBH by gel filtration gave rise to two protein peaks which had about equal amounts of enzyme activity (Figures 2a

Table 2 N-Terminal amino acid sequences of native and recombinant human DBH

Sequences of native and recombinant human DBH were determined from the denatured proteins after SDS/PAGE run and protein transfer on to the membrane. The sequences in bold type belong to tPA signal sequence.

Recombinant tPA-DBH construct	**MDAMKRGLCCVLLLCGAVFVSPSQEIHARFRRGAR**SAPRESPLPYHI
Sequence of recombinant DBH found in S2 culture medium	**GAR**SAPRESPLP
Published human DBH sequence*	MREAAFMYSTAVAIFLVILVAALQGSAPRESPLPYHI
Sequence of DBH from SK-N-SH culture medium	SAPRESPL

* Taken from Lamouroux et al. [20].

and 2b). Comparison of the column resident times (K_{av}) of the two DBH peaks with molecular-mass markers (Figure 2c) indicated that the apparent molecular masses of the two forms of DBH were 299 and 466 kDa. Previous reports show that human DBH dimers and tetramers can be resolved by size-exclusion chromatography [17]. However, the apparent molecular masses of the dimer and tetramer were 189 and 560 kDa, substantially higher than the actual molecular masses of 147 and 289 kDa. The two forms that we observed may also be dimers and tetramers since analysis of both peaks by denaturing SDS/PAGE resulted in a single molecule with the molecular mass expected of the monomer. When pool 1 or pool 2 was re-chromatographed under the same conditions using Superdex 200, a single peak with DBH activity was observed. This indicates that the dimers and tetramers are not in rapid equilibrium under the conditions of the experiment. This observation is consistent with reports that human DBH dimers and tetramers are not rapidly interconverted [26,27]. In contrast, bovine DBH dimers and tetramers have been reported to undergo rapid equilibrium [28].

Figure 3 shows the SDS/PAGE gel of human native and recombinant DBHs that have been purified by Superdex 200. The purity of native human DBH from SK-N-SH cells was $> 90\,\%$, as judged from the protein gel quantification by densitometer, whereas recombinant DBH was $> 80\,\%$ pure. The molecular masses, as determined by densitometer scanning of the SDS gel, were 73 kDa for native human DBH and 66 kDa for recombinant human DBH.

N-terminal amino acid sequences

The N-terminal sequences for both native human DBH from SK-N-SH cells and recombinant human DBH expressed in *Drosophila* cells are presented in Table 2. The published full-length N-terminal sequence and the constructed sequence with tPA signal are also included for comparison. The tPA prepro-sequence (bold type in Table 2) in recombinant human DBH was cleaved, leaving three amino acids attached to the N-terminus of recombinant DBH. The cleavage site was identical to that utilized by mammalian cells [29]. The same cleavage site was also observed for the secreted HIV-1 gp120 envelope glycoprotein which was expressed in *Drosophila* S2 cells using a construct containing tPA signal sequence [30]. The N-terminus of the native DBH from neuroblastoma cells was identical to that reported for intracellular soluble human DBH [20].

Deglycosylation of native and recombinant human DBH

Deglycosylation was performed to ascertain if the apparent disparity in molecular masses for the native and recombinant

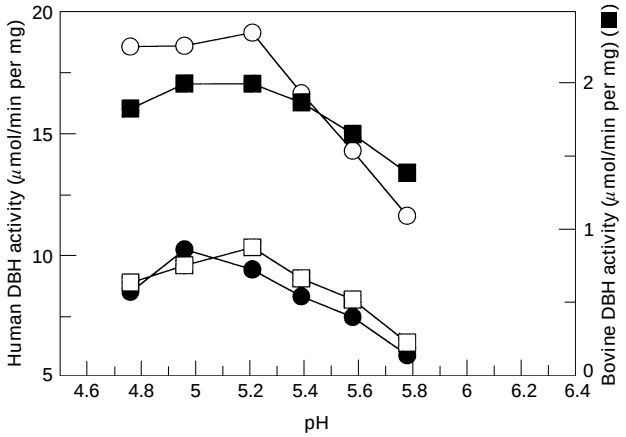

Figure 4 DBH activity dependence on pH

Native human (○), recombinant human 304A (●), recombinant 304S (□), and bovine DBH (■) were assayed under the HPLC assay conditions, using ascorbate and tyramine as substrates, at various pH values as described in the Materials and methods section. The specific activities as measured by colorimetric assay were used for the plot.

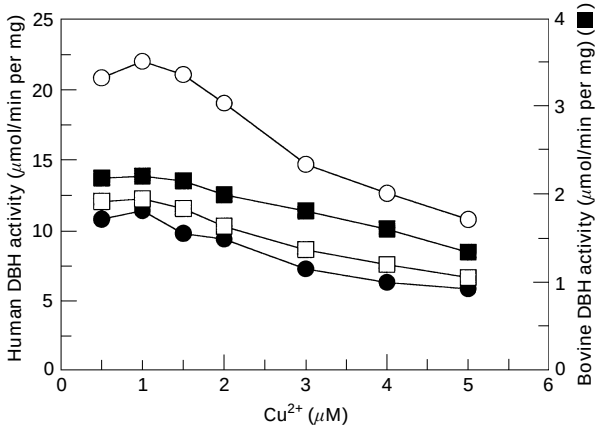

Figure 5 DBH activity dependence on Cu^{2+} ions

Native human (○), recombinant human 304A (●), recombinant 304S (□), and bovine DBH (■) were assayed under the HPLC assay conditions, using ascorbate and tyramine as substrates, at various Cu^{2+} concentrations at pH 5.2 as described in the Materials and methods section. The specific activities by colorimetric assay were used for the plot.

Table 3 Kinetic and inhibitory properties of bovine, native and recombinant human (304A and 304S) DBH

Bovine DBH was included in the experiments for comparison. K_m (tyramine) and IC_{50} values were determined by the HPLC assay at pH 5.2 and 37 °C as described in the Materials and methods section.

DBH	K_m (tyramine) (mM)	IC_{50} (μM)	
		Fusaric acid	SKF102698
Bovine	0.8 ± 0.1	0.034 ± 0.002	0.12 ± 0.01
Native human	1.8 ± 0.2	0.10 ± 0.01	0.26 ± 0.01
Recombinant human	1.4 ± 0.3	0.086 ± 0.003	0.23 ± 0.01
Recombinant human	2.4 ± 0.4	0.10 ± 0.01	0.33 ± 0.05

human DBHs was due to differences in glycosylation. The protein bands in Figure 3 were scanned by a densitometer and R_F values compared with those of the protein standards to calculate the molecular masses. Deglycosylated native and recombinant DBH had the same molecular mass, 61 kDa, although the native protein is 7 kDa larger than recombinant protein before deglycosylation. Evidently, the *Drosophila* cells glycosylate DBH differently to human cells.

Enzyme properties

The enzyme properties were examined for bovine, native human, and recombinant human 304A and 304S DBHs. No major difference in pH optima among the enzymes was evident (Figure 4). The slight variation of 0.2 pH unit is probably not significant. The examination of dependence of the enzyme activity on Cu^{2+} was also carried out under the HPLC assay conditions (Figure 5). Optimum activities were found at 0.5–1 μM Cu^{2+}. Higher Cu^{2+} concentrations resulted in inhibition of the enzymes. This experiment was done twice with the same results. Table 3 summarizes the results on K_m for tyramine and IC_{50} values of the known DBH inhibitors, SKF102698 and fusaric acid. All three human enzymes had higher K_m values (tyramine) than bovine DBH by 2–3-fold. The inhibition data corresponded well to the K_m values, being 2–3-fold higher for the human than the bovine enzyme. Since bovine DBH is intracellular and the native human and recombinant human DBH are secreted, we have obtained an intracellular human DBH from SK-N-SH cells by sonication and purification by lentil lectin chromatography. Human intracellular DBH was tested against DBH inhibitors and has virtually the same IC_{50} value as human secretory DBH (B. Li unpublished work).

DISCUSSION

Expression of bovine DBH in *Drosophila* S2 cells has been reported previously by Gibson et al. [13], leading to low levels of DBH activity in subcellular fractions which corresponds to a 70–75 kDa protein. We have attempted to express human DBH in both baculovirus Sf-9 and *Drosophila* S2 cells and observed no (Sf-9) or low (S2) levels of intracellular DBH activity. In contrast, the culture medium from *Drosophila* cells contained a much higher level of DBH activity than the subcellular fractions and accounted for more than 95% of the total activity in the expression system. Secretion of DBH into the medium has provided a convenient source for milligram quantities of DBH for screening purposes. Although the *Drosophila* expression system is inducible under the control of metallothionein promoter, DBH has been detected even in the absence of added metal ions to the culturing medium. This is in contrast to the report of Gibson et al., who used a similar expression vector construct. One possible explanation is that in serum-free medium the presence of Cu^{2+} ions is sufficient to induce active transcription without the requirement of additional metal ions. Another difference we have noticed is the cleavage site of the tPA signal sequence. Gibson et al. [13] reported that the tPA signal sequence was cleaved from the intracellular bovine DBH chimeric protein but the tPA pro-domain was retained. In contrast, we observed that both the pre- and pro-sequences from the tPA signal sequence were cleaved from the secreted human DBH. Cleavage of the pro-domain may have resulted in rapid secretion of the DBH and thus fully processed chimeric DBH cannot be detected inside the *Drosophila* cells.

SP-Sepharose, a cation exchanger, was used as the initial step of purification for its ease of scale up and for its high recoveries. The modified purification procedure had resulted in highly purified protein based on SDS/PAGE gel analysis. It is apparent from the gel-filtration chromatography that two species exist, both with DBH activity. This phenomenon was observed for both DBH from a human cell line (Figure 2) and from recombinant *Drosophila* cells. Apparent-molecular-mass determination suggests that they are dimers and tetramers (S. Tsing, unpublished work). As reported previously [26,27], human DBH dimers and tetramers, unlike bovine adrenal DBH, are not in rapid equilibrium since both dimer and tetramer remain as single peaks after rechromatography. The molecular masses of the human DBH from SK-N-SH and *Drosophila* cells, according to amino acid sequences, should be very close (differ by three amino acids). Therefore, the difference in molecular mass of 7 kDa between the two forms is apparently due to differences in glycosylation, as deglycosylated proteins are identical in molecular masses. The molecular mass of 73 kDa for human DBH from SK-N-SH cells agrees well with the reported 72 kDa for DBH from human plasma, and the 73 kDa for soluble DBH from the neuroblastoma cell line SH-SY5Y [8].

Comparison of the enzyme properties between native and recombinant DBH indicates that the two forms of enzyme are nearly identical in Cu^{2+} and pH optima, in K_m for tyramine and in inhibitory properties. Therefore, the recombinant DBH expressed in *Drosophila* S2 cells is useful in drug screening and can replace the human enzyme from the SK-N-SH cell line. The level of activity in recombinant *Drosophila* is more than 10-fold higher than that found in SK-N-SH cells. Moreover, it is much easier to grow large quantities of *Drosophila* cells than human cells. The combined advantages have made the expressed recombinant DBH a convenient source of enzyme for drug screening. The 2–3-fold higher K_m and IC_{50} values of the human enzymes versus the bovine enzyme reflects an intrinsic difference between the two enzymes, and has little to do with their subcellular locations. Specifically, we have obtained intracellular human DBH from SK-N-SH cells and carried out an inhibition study using a proprietary DBH inhibitor. The resulting IC_{50} was identical to that of human secretory DBH, suggesting that secretion has not altered the kinetic properties of the enzyme.

It has been reported that human DBH exists in two allelic forms with either alanine or serine at position 304. Ishii et al. noticed a significant difference in the specific activities for the 304A and 304S forms based on transient expression of the two enzymes in COS cells [31]. 304A was found to be 10-fold more active than 304S. Their estimated specific activities, however, were based on unpurified preparations. We have purified both the 304A and 304S recombinant DBH from *Drosophila* cells by SP-Sepharose and lentil lectin–Sepharose chromatography. By

all measures tested, 304S is not significantly different from 304A in specific activity or any other enzyme property, suggesting that the change at position 304 has little functional significance.

In conclusion, we have expressed recombinant human DBH in *Drosophila* S2 cells and compared the enzyme with human DBH purified from neuroblastoma cell line SK-N-SH. The secretory enzymes account for more than 95% of the total activity in both cases and differ by 7 kDa in molecular mass, although without any significant differences in enzyme properties. The high level of expression has provided a convenient source of DBH for drug screening.

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REFERENCES

1 Hasking, G. J., Esler, M. D., Jennings, G. L., Burton, D., Johns, J. A. and Korner, P. I. (1986) Circulation **73**, 615–621
2 Stewart, L. C. and Klinman, J. P. (1988) Annu. Rev. Biochem. **57**, 551–592
3 Berkowitz, B. A., Arleth, A. J., Sunh, C. P., Kruse, L. I., DeWolf, W. E., Jr. and Ohlstein, E. H. (1988) J. Pharmacol. Exp. Ther. **245**, 850–857
4 Kobayashi, K., Kurosawa, Y., Fujita, K. and Nagatsu, T. (1989) Nucleic Acids Res. **17**, 1089–1102
5 Robertson, J. G., Adams, G. W., Medzihradszky, K. F., Burlingame, A. L. and Villafranca, J. J. (1994) Biochemistry **33**, 11563–11575
6 Dhawan, S., Duong, L. T., Ornberg, R. L. and Fleming, P. J. (1987) J. Biol. Chem. **262**, 1869–1875
7 Helle, K. B., Pihl, K. E. and Serck-Hanssen, G. (1985) Acta Physiol. Scand. **125**, 423–427
8 Oyarce, A. M. and Fleming, P. J. (1991) Arch. Biochem. Biophys. **290**, 503–510
9 Saxena, A. and Fleming, P. J. (1983) J. Biol. Chem. **258**, 4147–4152
10 Bon, S., Lamouroux, A., Vigny, A., Massoulie, J., Mallet, J. and Henry, J.-P. (1991) J. Neurochem. **57**, 1100–1111
11 Feng. Z., Angeletti, R. H., Levin, B. E. and Sabban, E. L. (1992) J. Biol. Chem. **267**, 21808–21815
12 Taljanidisz, J., Stewart, L., Smith, A. J. and Klinman, J. P. (1989) Biochemistry **28**, 10054–10061
13 Gibson, K. R., Vanek, P. G., Kaloss, W. D. et al. (1993) J. Biol. Chem. **268**, 9490–9495
14 Taylor, C. S. and Fleming, P. J. (1989) J. Biol. Chem. **264**, 15242–15246
15 Sabban, E. L., Greene, L. A. and Goldstein, M. (1983) J. Biol. Chem. **258**, 7812–7818
16 Sabban, E. L., Kuhn, L. J. and Levin, B. E. (1987) J. Neurosci. **7**, 192–200
17 O'Conner, D. T., Frigon, R. P. and Stone, R. A. (1979) Mol. Pharmacol. **16**, 529–538
18 McHugh, E. M., McGee, R., Jr. and Fleming, P. J. (1985) J. Biol. Chem. **260**, 4409–4417
19 Wang, X., Ooi, B. G. and Miller, L. K. (1991) Gene **100**, 131–137
20 Lamouroux, A., Vigny, A., Biguet, N. F., Darmon, M. C., Franck, R., Henry, J.-P. and Mallet, J. (1987) EMBO J. **6**, 3931–3937
21 Koelle, M. R. (1992) Ph.D. Dissertation, Stanford University
22 Felgner, P. L., Gadik, T. R., Holmes, M. et al. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7413–7417
23 Wimalasena, K. and Wimalasena, D. S. (1991) Anal. Biochem. **197**, 353–361
24 Feilchenfeld, N. B., Richter, H. and Waddell, W. H. (1982) Anal. Biochem. **122**, 124–128
25 Nguyen, B., Jarnagin, K., Williams, S., Chan, H. and Barnett, J. (1993) J. Biotechnol. **31**, 205–217
26 Rosenberg, R. C. and Lovenberg, W. (1977) Mol. Pharmacol. **13**, 652–661
27 Frigon, R. P. and Stone, R. A. (1978) J. Biol. Chem. **253**, 6780–6786
28 Saxena, A., Hensley, P., Osborne, J. C., Jr. and Fleming, P. J. (1985) J. Biol. Chem. **260**, 3386–3392
29 Wallen, P., Pohl, G., Bergsdorf, N., Ranby, M., Ny, T. and Jornvall, H. (1983) Eur. J. Biochem. **132**, 681–686
30 Culp, J. S., Johansen, H., Hellmig, B., Beck, J., Matthews, T. J., Delers, A. and Rosenberg, M. (1991) Biotechnology **9**, 173–177
31 Ishii, A., Kobayashi, K., Kiuchi, K. and Nagatsu, T. (1991) Neurosci. Lett. **125**, 25–28

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