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New approaches to chromatographic purification of bovine dopamine-β-hydroxylase

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Abstract

The use of the traditional scheme for the isolation of bovine dopamine-β-hydroxylase (bDBH) from bovine adrenal medulla resulted in active but not pure bDBH, containing about 50% of admixtures. Immobilized metal chelate affinity chromatography on agarose modified with iminodiacetic acid residues and charged with cobalt ions was applied in the final stage to obtain more than 90% pure and active bDBH. Final purification of bDBH using step elution with 0–0.5 *M* methyl-D-mannoside in buffer solution from concanavalin A–Sepharose was studied. The determination of bDBH in various samples was performed using size-exclusion chromatography.

1. Introduction

Dopamine-β-hydroxylase (DBH) of bovine (b), human and other origins has been intensively studied for the last 20 years [1–3]. The interest in this enzyme is connected with its role in the catalysis of the hydroxylation of dopamine into noradrenaline in the final step of the biosynthesis of this important neurotransmitter [4]. As a result of numerous studies, it was shown that the water-soluble form of this enzyme consists of four subunits and it contains at least one copper atom per subunit and 5% of carbohydrate residues [2,3]. Its molecular mass was shown to be about 290 000 [5].

There are many approaches to the isolation of bDBH, which have been reviewed [6]. An optimized method for bDBH purification from bovine adrenal medulla has been published [7]. It is based on the studies of Ljones et al. [8] and involves aqueous buffer extraction of enzyme from medulla and partial precipitation with polyethylene glycol, followed by chromatography on DEAE-cellulose and concanavalin A (Con A)–Sepharose. The enzyme obtained using this isolation scheme is active and homogeneous according to polyacrylamide gel electrophoresis (PAGE) in the presence and in absence of sodium dodecyl sulfate (SDS).

We have used this approach with slight modifications and finally obtained active but not pure bDBH, containing about 50% of admixtures. In

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order to obtain pure and active bDBH for further structural investigations, we have studied the application of immobilised metal chelate affinity chromatography (IMAC) for the final purification of bDBH, and various methods for its separation on Con A–Sepharose. We also investigated the possible use of size-exclusion chromatography (SEC) for the analysis of bDBH-containing fractions and final samples.

2. Experimental

2.1. Materials

All salts and PEG-6000 were purchased from Merck (Darmstadt, Germany). DEAE-cellulose, α-methyl-D-mannoside, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), phenylmethylsulfonyl fluoride (PMSF), benzamidine and commercial bDBH, the latter as lyophilized powder with specific activity 2.2 units (U) per mg protein (one unit will convert 1.0 μmol of tyramine into octopamine per minute at pH 5.0 at 37°C) were purchased from Sigma (St. Louis, MO, USA). DEAE-Sepharose and Con A–Sepharose were purchased from Pharmacia (Uppsala, Sweden). Iminodiacetate (IDA)–agarose was synthesized according to the literature [9].

2.2. Isolation of partially purified bDBH

Partially thawed bovine adrenal medullae were extracted with 8 m*M* potassium phosphate buffer (pH 7.2) (B-1) to which small amounts of PMSF and benzamidine (final concentrations 20 and 400 μ*M*, respectively) were added. After centrifugation for 30 min at 15 000 *g*, the supernatant was subjected to ultracentrifugation at 100 000 *g* for 90 min and the precipitate and the upper lipid layer were discarded. Then solid PEG-6000 was added to the supernatant to a final concentration of 8% (w/w). After stirring for 30 min, the mixture was centrifuged for 30 min at 15 000 *g*. The supernatant was discarded and the sediment was dissolved in 20 m*M* potassium phosphate buffer (pH 7.5) (B-2) containing the above concentrations of PMSF and benzamidine (the volume of B-2 is one fifth of that of B-1) followed by similar precipitation with 10% PEG-6000, stirring for 15 min and centrifugation for 30 min at 15 000 *g*. The sediment was again dissolved in B-2, stirred for 30 min and centrifuged at 15 000 *g* for 30 min. The supernatant obtained was separated on DEAF-cellulose (or DEAE-Sepharose) with a linear gradient from 0 to 0.25 *M* NaCl in the above buffer. The fractions having enzymatic activity were pooled, then the proteins were precipitated by addition of PEG-6000 up to 12% (w/w). After stirring for 30 min and centrifugation at 15 000 *g* for 30 min, the precipitate was dissolved in 50 m*M* potassium phosphate buffer containing 0.2 *M* NaCl (pH 6.5) (B-3). All the above operations were performed at 4°C.

The above solution of crude bDBH was adsorbed at 20–25°C on a column of Con A–Sepharose (gel volume 5–7 ml) at a flow-rate of 10 ml/h. Then the column was washed with B-3 at a flow-rate of 40 ml/h. The elution of bDBH was performed with 0.5 *M* methyl-α-D-mannopyranoside (Me-Man) in B-3 three times in a "batch" version according to Ref. [7].

The above solution containing bDBH was subjected to desalting and exchange of the buffer with 10 m*M* BES buffer (pH 7.0) by diafiltration on an Amicon (Beverley, MA, USA) Diaflo ultrafiltration cell with an XM-30 membrane (Amicon) followed by concentration of bDBH solution to the required volume.

The yields of the final product relative to 200 g of adrenal medulla were varied from 15 to 25 mg of partially purified bDBH with specific activity 3–4 U/mg as a result of six independent isolations.

2.3. Purification by IMAC

A glass column (volume 30 ml) packed with IDA–agarose support (50 μmol of iminodiacetic groups per 1 ml of sorbent) was used. After being charged with Co^{2+}, the support was washed with 0.1 *M* sodium acetate buffer (pH 4.0) containing 0.2 *M* sodium chloride and equilibrated with the same buffer at pH 7.5

(buffer 1). The solution of partially purified bDBH (9 mg in 7 ml of buffer 1) with specific activity 3.6 U/mg was then loaded on to a column at a flow-rate of 20 ml/h. The non-retained fraction starting from 28 ml (total volume 20 ml, see Fig. 1) was dialysed against distilled water overnight and the solution containing bDBH was lyophilized. About 3 mg of bDBH with 90% purity (see Fig. 2, lane 3) was obtained with a specific activity of 14 U/mg. Elution with buffer 1 but at pH 6.0 (Fig. 1, step I) released a small amount (not more than 5% of total activity) of bDBH and the main impurities with M_r around 50 000 and 28 000 (see Fig. 2, lane 1). Finally, desorption of the other impurities (or the minor ballast proteins) could be carried out with buffer 1 containing 50 m*M* of imidazole.

2.4. Chromatography

Low-pressure chromatographic purifications were performed on standard equipment for gradient separation produced by Pharmacia. A Superose 12 column (40 × 10 mm I.D.) was purchased from Pharmacia.

HPLC was performed on a Familic 300 S isocratic pump equipped with Uvidec 100-Y UV detector (both from JASCO, Tokyo, Japan)

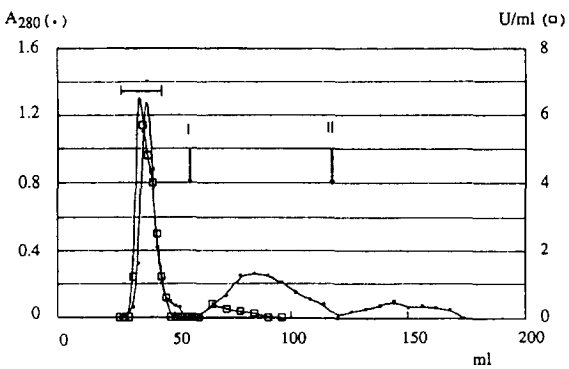

Fig. 1. Chromatography of initial bDBH sample on a Co^{2+}–IDA–agarose column (6.5 × 2.5 cm I.D.). Equilibrating and starting buffer, 0.1 *M* sodium acetate buffer (pH 7.5) containing 0.2 *M* NaCl (buffer 1); flow-rate, 0.3 ml/min; sample loaded, 9 mg in 7 ml of buffer 1. (I) Elution with buffer 1 at pH 6.0; (II) elution with buffer 1 containing 50 m*M* imidazole. □ = Enzymatic activity; ● = absorbance.

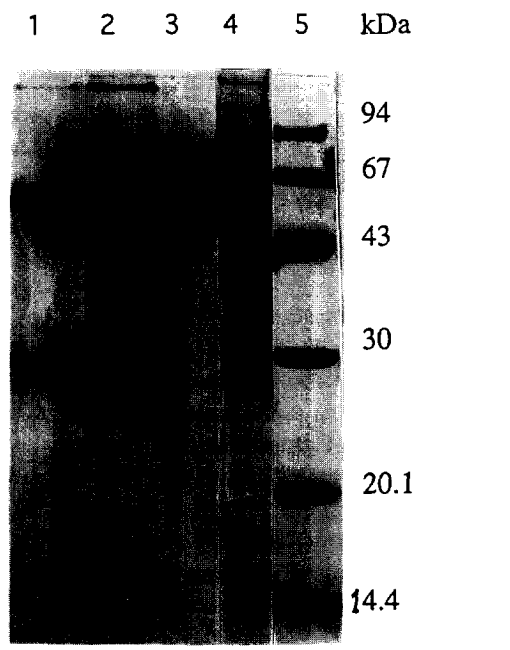

Fig. 2. SDS-PAGE patterns of various bDBH samples. Lanes: 1 = fraction eluted at pH 6.0 from Co^{2+}–IDA column; 2 = initial bDBH sample (fraction eluted from Con A–Sepharose); 3 = purified bDBH after Co^{2+}–IDA column; 4 = bDBH from Sigma; 5 = protein standards. M_r values are given on the right.

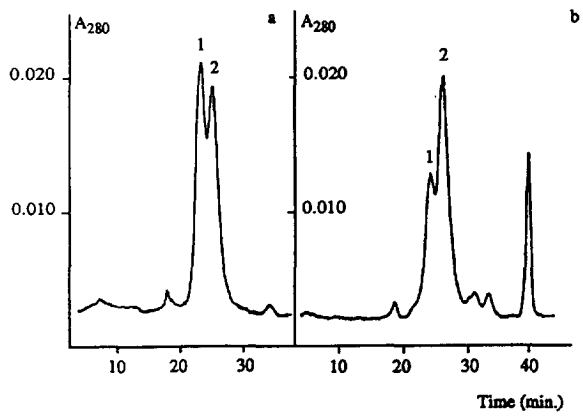

Fig. 3. Chromatography of bDBH eluates from Con A–Sepharose on a Superose 12 HR column (40 × 10 mm I.D.). Mobile phase, 50 m*M* potassium phosphate buffer (pH 7.0) containing 0.15 *M* NaCl; flow-rate, 0.4 ml/min; sample loaded, 25 μl; sample concentration, 2 mg/ml; (a) Pooled and desalted "batch" eluate from Con A–Sepharose obtained with 0.5 *M* Me-Man in B-3; (b) desalted eluate after overnight incubation with 0.5 *M* Me-Man in B-3.

operated at 229 or 280 nm, a Rheodyne Model 7125 injector (20-ml loop) and an M-745 B computing integrator (Waters, Milford, MA, USA). The column used was an Ultropack TSK 4000 SW (600 × 7.5 mm I.D.) (LKB–Pharmacia, Uppsala, Sweden) with 20 m*M* potassium phosphate buffer (pH 7.5) as the eluent at a flow-rate of 0.7–1.0 ml/min.

2.5. Assay methods

The determination of the enzymatic activity of DBH is based on continuous spectrophotometric measurement of the oxidation of Fe^{2+} to Fe^{3+} according to Ref. [10] in relation to a bDBH sample from Sigma. Protein concentration was determined either by measuring the absorbance at 280 nm using a solution with $A^{1\%} = 12.4$ or with a Spector [11] with calibration by bacterial RNase.

2.6. Electrophoresis

SDS-PAGE of bDBH-containing samples was performed according to the standard Laemmli procedure [12] using low-molecular-mass protein standards of M_r 94 000, 67 000, 43 000, 30 000, 20 000 and 144 000 from Pharmacia.

3. Results and discussion

3.1. Procedure for isolation of bDBH

The scheme for the purification of bDBH from bovine adrenal medulla is well established [7]. We applied this procedure with slight modifications including the addition in the first two stages of inhibitors of proteases in small amounts (see Experimental) and the application of ultracentrifugation. The latter seems to improve the purification of the initial extract from small particulates and thus from traces of the membrane form of bDBH. It also facilitates the separation of the lipids forming the upper layer which can be discarded, otherwise the lipids present make difficult the centrifugation stages on PEG precipitation and subsequent chromatography.

The additional stages should improve the quality of the final product, but the bDBH obtained was not homogeneous according to SDS-PAGE (see Fig. 2, lane 2). The variations in the sources of bovine adrenal glands, their storage conditions, storage temperature of adrenal medulla, application of DEAE-Sepharose instead of DEAE-cellulose and pooling of a smaller number of fractions always result in active (3–4 U/mg) but not pure (about 50% of admixtures) bDBH. According to reduced SDS-PAGE, the final bDBH sample contains the band of bDBH (in the zone of M_r 67 000) and two main bands of admixtures (one in the zone of M_r 50 000 and the other in the zone of M_r 28 000, substances 1 and 2, respectively; see also Fig. 3a, peak 2, substance 1). The molecular mass of bDBH obtained by reduced SDS-PAGE agreed with recently published results [13]. The other points of concern were desalting and concentration while chromatography on Con A–Sepharose will be discussed separately.

Application of an ultrafiltration cell with vertical flow results in the formation a of thick polarization layer and a decreased filtration rate and thus in the loss of 20–30% of the final product, since washing of the membrane surface leads to dilution again. The application of slower dialysis followed by lyophilization resulted in a much better recovery.

Hence the application of the known purification procedure [7] resulted in active but not pure bDBH. It is worth mentioning that the purity of the commercial bDBH sample from Sigma and obtained according to the modified procedure [7] contained even more admixtures than our initial sample (see Fig. 2, lane 4) with specific activity 2.2 U/mg.

Perhaps such results are connected with the fact that some minor aspects of the isolation procedure were omitted in published reports.

3.2. Purification of bDBH on Con A Sepharose

The final purification step on a Con A-modified support, e.g. on Con A–Sepharose, is based

on its affinity to carbohydrate residues present in the bDBH molecule. The main admixtures present in the final bDBH preparation also exhibit affinity to Con A–Sepharose, which seems to be even higher than that of bDBH. For example, incubations of Con A–Sepharose with bDBH bound to it three times for 15 min with 10 ml of 0.5 *M* Me-Man in B-3 (so-called batch version) resulted in an increase in the relative content of substance 1 from 30% (first eluate) to 60% (third eluate) according to SEC data on the Superose 12 column. If the thus-treated Con A–Sepharose is incubated overnight with fresh 0.5 *M* Me-Man in B-3, 15% of the total protein is eluted which contains about 80% of substance 1 (see Fig. 3a and b, peak 2).

The use of step elution from the Con A–Sepharose column with increasing concentration (0.1, 0.2, 0.3, 0.4 and 0.5 *M*) of Me-Man in B-3 at a flow-rate of 10 ml/h results in elution at 0.1 *M* Me-Man of about 50% of the total proteins, containing together with the main portion of bDBH about 30–40% of substance 1. The following elution leads to the same tendency as in the batch version, when the proportion of substance 1 in the mixture increases with increase in the Me-Man concentration.

Our attempts to vary the initial treatment of Con A–Sepharose did not result in an improvement in the percentage of bDBH in the final mixture. The successful approach to final purification of bDBH was found to be with the use of IMAC.

3.3. Isolation of bDBH by IMAC

IMAC was introduced for the selective binding and fractionation of proteins by Porath et al. [14]. Recently, metal affinity separations have been developed and become much more powerful [15,16]. The interactions between particular surface amino acids and immobilized metal ions provide the basis for metal affinity protein separations. The chromatographic elution of proteins in IMAC can be achieved in some instances by reducing the pH or by increasing the concentration of a competing ligand such as imidazole in the buffer. If these approaches fail, choosing a different metal ion such as Cu^{2+}, Ni^{2+}, Zn^{2+} or Co^{2+} may become relevant.

The interactions of bDBH and the main admixtures with Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} chelated to IDA–Sepharose were strong. However, with Co^{2+} this interaction was more reversible and we chose the experimental conditions for elution of bDBH in starting buffer with low retention ($V_e \approx 2V_0$). Under these conditions, the main admixtures were eluted only by reducing the pH from 7.5 to pH 6.0. The final bDBH product (90% purity) was recovered almost quantitatively according to the total activity data with a fourfold increase in specific activity and a 30% yield of total protein.

It is possible that this approach to bDBH purification may be applied instead of chromatography on Con A–Sepharose in a bDBH isolation scheme.

3.4. Analysis of bDBH-containing fractions by SEC

Owing to the different molecular masses of bDBH and the admixtures, SEC can be applied to the analysis of various bDBH-containing fractions. This was achieved using separation on a Superose 12 column of various eluates from the Con A–Sepharose column (Fig. 3a and b). The identification of bDBH as peak 1 was effected after its micropreparative isolation, measurements of enzymatic activity and analysis by reduced SDS-PAGE. A better resolution for analytical purposes was obtained by using a more efficient Ultrogel TSK 4000 SW column with 20 m*M* Potassium phosphate buffer (pH 7.5) as the eluent.

This analysis in the HPLC mode was applied to control the separation on an IDA–Co^{2+}–agarose column. In Fig. 4, the chromatographic profiles of (a) the partially purified bDBH sample, (b) the solution of pure bDBH passing through and (c) the fraction eluted from the column at pH 6 may be compared. It can be seen that the product obtained according to our isolation procedure contains two peaks (Fig. 4a, peak 1 with retention time t_R 22.1 min and peak 2 with t_R 24.8 min) where the proportion of

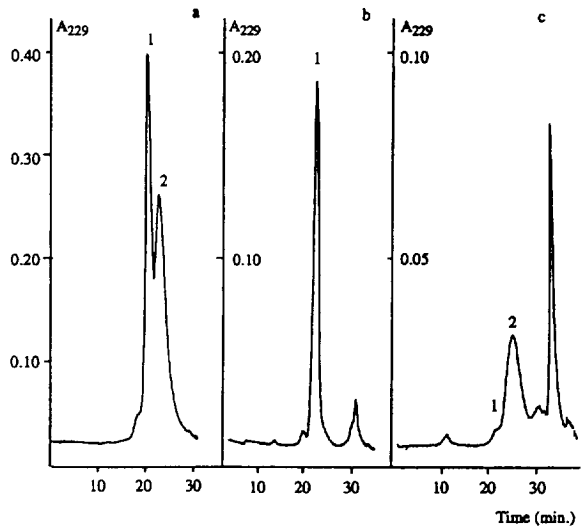

Fig. 4. Chromatography of bDBH eluates from Co^{2+}–IDA–agarose column on an Ultropac TSK 4000 SW column (600 × 7.5 mm I.D.). Mobile phase, 20 m*M* potassium phosphate buffer (pH 7.5); flow-rate, 0.7 ml/min; sample loaded, 20 μl; sample concentration, 0.2 mg/ml. (a) Initial bDBH sample obtained after isolation; (b) purified bDBH obtained by passage through Co^{2+}–IDA–agarose column; (c) solution eluted from the Co^{2+}–IDA–agarose column.

bDBH (peak 1) is about 45% according to the integration data. After purification on the IDA–Co^{2+}–agarose column, bDBH passed through whereas all admixtures were retained (compare Fig. 4a–c). Thus we obtained fairly pure bDBH (Fig. 4b; the proportion of the main peak is more than 90%), which corresponds to SDS-PAGE data.

After calibration with standard proteins of the above SEC columns, it was found that the apparent M_r of bDBH was 300 000, which was close to the published value [5], whereas that of the basic admixture was about 200 000.

Hence SEC on Superose 12 and Ultragel TSK SW4000 columns may be applied for the control of the various stages of the bDBH isolation procedure and for the determination of its molecular mass.

4. Conclusions

IMAC on a Co^{2+}-modified IDA–agarose column can be applied for the final purification of bDBH isolated from bovine adrenal medulla according to a slightly modified procedure [7]. Purification on Con A–Sepharose does not result in pure bDBH in spite of various approaches to performing the elution process. SEC can be applied for the control of various stages of the bDBH isolation process.

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