Purification of aromatic L-amino acid decarboxylase from bovine brain with a monoclonal antibody

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Aromatic L-amino acid decarboxylase was purified from bovine brain for the first time by affinity chromatography using a monoclonal antibody to the enzyme, and it was compared with the decarboxylase purified from bovine adrenal medulla by the same procedure. The monoclonal antibody was produced from a hybridoma established for the enzyme highly purified from bovine adrenal medulla. The M_r values of brain and adrenal-medulla enzyme were both estimated to be approx. 100 000 by gel-permeation chromatography. SDS/polyacrylamide-gel electrophoresis revealed a single band with an apparent M_r of 50 000. Western immunoblot analysis showed that the antibody recognized each enzyme. With regard to substrate specificity, pH-dependence and effect of pyridoxal 5'-phosphate as a cofactor, both enzymes were similar.

INTRODUCTION

Aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) [1] is the enzyme which decarboxylates L-dopa (dihydroxyphenylalanine) and L-5-hydroxytryptophan to produce catecholamines and serotonin (5-hydroxytryptamine), respectively, in various mammalian tissues. Catecholaminergic and serotonergic cells such as adrenal glands and brain are rich in this enzyme, but it is widely distributed in various other tissues, including non-neuronal ones, especially liver and kidney [2]. Aromatic L-amino acid decarboxylase is also found in sera of various animals such as guinea pigs, rats, monkeys, mice and humans [3,4].

Several groups have described the purification of AADC to homogeneity from various tissues of many species: pig and guinea-pig kidneys [5–8], human pheochromocytoma [9,10] and *Micrococcus peritremis* [11]. However, purification of the enzyme from brain has not yet been reported, although it has been recently reported that a single gene codes for AADC in both neuronal and non-neuronal tissues [12]. In the present paper, we describe the isolation of a monoclonal antibody to AADC by using highly purified AADC from bovine adrenal medulla as an antigen, and the purification and characterization of AADC from bovine brain and bovine adrenal medulla by monoclonal-antibody affinity chromatography.

EXPERIMENTAL

Preparation of monoclonal antibody to AADC

AADC, highly purified from bovine adrenal medulla by the method of Ichinose *et al.* [10], was used as the antigen for monoclonal-antibody production. The hybridoma secreting monoclonal antibody to AADC was prepared by the procedure of Köhler & Milstein [13]. Monoclonal antibody to the enzyme was selected by a combination of enzyme-linked immunosorbent assay,

Western immunoblotting and immunoprecipitation assay. A large amount of the monoclonal antibody to AADC was isolated from mouse ascitic fluids by DEAE-Affi-Gel Blue (Bio-Rad) chromatography as described by Bruck *et al.* [14].

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli [15]. A gradient slab gel (4–15% polyacrylamide) was used. Gels were stained with Coomassie Brilliant Blue R-250.

Western immunoblotting and immunoprecipitation

Immunoblotting was carried out as described by Towbin *et al.* [16]. Immunoprecipitation assay was performed as follows: 80 μ l of a 50% (v/v) suspension of anti-mouse IgG–Sephadex 4B in phosphate-buffered saline (PBS), consisting of 10 mM-sodium phosphate buffer and 150 mM-NaCl (pH 7.4), was incubated with 100 μ l of various amounts (0, 0.1, 1, 10 and 100 μ g) of monoclonal-antibody solutions at 37 °C for 1 h. After the incubation, 1 ml of PBS was added to each incubation mixture, which was then centrifuged at 1000 *g* for 5 min. The resulting sediments were washed with 1 ml of PBS and incubated with appropriate amounts of AADC preparations for 1 h in an ice bath. The AADC activities of the supernatants were measured.

Assay of L-dopa and L-5-hydroxytryptophan decarboxylase activities, and protein determination

These decarboxylase activities were assayed on the basis of measurement of enzymically formed dopamine and serotonin, respectively, by h.p.l.c., with electrochemical detection after isolation by Amberlite CG-50 column by the method of Nagatsu *et al.* [17,18], as slightly modified by Ichinose *et al.* [10]. AADC activity was assayed with L-dopa as a substrate, except for K_m and V_{max} determinations.

Protein was measured by the method of Bradford [19], with γ -globulin as the standard.

Determination of K_m and V_{max} .

K_m and V_{max} values were determined by the method of Wilkinson [20] from the Lineweaver-Burk plots.

RESULTS

Characterization of monoclonal antibody to AADC from bovine adrenal medulla

A hybridoma clone secreting monoclonal antibody to AADC was established from a cell of a mouse immunized

Fig. 1. Western immunoblot of AADC partially purified from bovine adrenal medulla

Standard proteins (a) and AADC [30–50% satd.-(NH₄)₂SO₄ fraction] (b) were separated on SDS/polyacrylamide slab gels, transferred to a nitrocellulose sheet, and stained with Amido Black and monoclonal antibody to AADC from bovine adrenal medulla respectively. Standard proteins, from top to bottom, are: phosphorylase b (M_r 92 500), bovine serum albumin (66 200), ovalbumin (45 000) and carbonic anhydrase (31 000).

with highly purified AADC from bovine adrenal medulla. The monoclonal antibody, which was designated as NI-86, was IgG₁.

Fig. 1 shows a Western immunoblot of partially purified AADC from bovine adrenal medulla. Bovine adrenal medulla was homogenized in 4 vol. of a solution containing 0.25 M-sucrose, 1 mM-dithiothreitol and 0.1 mM-EDTA. The homogenate was centrifuged at 100 000 *g* for 1 h. The supernatant was passed through a glass-wool column to remove floating lipids. Protein in the supernatant was sedimented between 30 and 50% satd. (NH₄)₂SO₄. On SDS/polyacrylamide-slab-gel electrophoresis of the enzyme preparation, many protein bands were stained with Coomassie Brilliant Blue R-250. However, after transfer on to a nitrocellulose sheet, only one of the protein bands corresponding to pure human

Fig. 3. Elution profile of bovine brain AADC from a monoclonal-antibody affinity column

Fractions containing the highest AADC activity from a DEAE-Sephacel column were applied to an antibody affinity column. Washing and elution were performed as described in the Experimental section. ●, AADC activity; ○, protein.

Fig. 2. Immunoprecipitation of AADC activities from bovine adrenal medulla (a), bovine brain (b) and pig kidney (c)

Increasing amounts of monoclonal antibody to AADC from bovine adrenal medulla coupled with anti-(mouse IgG)-Sephacel 4B were incubated with partially purified enzyme preparations [30–50% satd.-(NH₄)₂SO₄ fraction] from bovine adrenal medulla, bovine brain and pig kidney, with total activities of 0.73, 0.71 and 0.38 nmol/min respectively. After antigen-antibody complexes were pelleted by centrifugation, the supernatants were assayed for AADC activities.

Table 1. Purification of AADC from bovine brain (a) and adrenal medulla (b) by monoclonal-antibody affinity chromatography

Tissue	Step	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
(a) Brain	Homogenate	1001	11 000	0.091	1	100
	100 000 g supernatant	612	2556	0.239	2.63	61
	DEAE-Sephacel	519	302	1.72	18.9	52
	Monoclonal-antibody affinity chromatography	90	2.08	43.3	476	9.0
(b) Adrenal medulla	Homogenate	4177	3797	1.1	1	100
	100 000 g supernatant	2782	1112	2.5	2.2	67
	DEAE-Sephacel	2548	72.8	35	31.8	61
	Monoclonal-antibody affinity chromatography	358	1.8	190	173	8.6

AADC, with M_r 50 000 [10], was stained with the monoclonal antibody. No protein band was stained in control experiments, where the monoclonal antibody was replaced by the same amount of normal mouse IgG (results not shown).

Immunoprecipitation analysis was performed by adding partially purified AADC [30–50 % satd.-(NH_4) $_2$ SO $_4$ fraction] to increasing amounts of monoclonal antibody coupled with anti-(mouse IgG)-Sephacel 4B. As shown in Fig. 2, the monoclonal antibody immunoprecipitated the activities of AADC preparations from bovine brain (Fig. 2b) and pig kidney (Fig. 2c), as well as the activity from bovine adrenal medulla (Fig. 2a). For 50% precipitation of AADC from bovine adrenal medulla, bovine brain and pig kidney with total activities of 0.73, 0.71 and 0.38 nmol/min, 0.46, 0.77 and 3.16 μ g of the monoclonal antibody was needed respectively. These results indicate that the monoclonal antibody cross-reacts with AADC from bovine brain and pig kidney, owing to common antigenic determinants, suggesting its possible application as a ligand in affinity chromatography for the purification of AADC from bovine brain and pig kidney. The monoclonal antibody did not directly inhibit AADC activities.

Purification of AADC from bovine brain and bovine adrenal medulla

All procedures were carried out at 4 °C. The (NH_4) $_2$ SO $_4$ fraction (30–50 % satn.), prepared from the tissue (250 g of bovine adrenal medulla or bovine brain stem) as described above, was dissolved in 100 ml of buffer A, consisting of 20 mM-Tris/HCl (pH 7.3), 8 % (w/v) sucrose, 1 mM-dithiothreitol and 1 mM-EDTA. After dialysis against buffer A, the fraction was applied to a column (5.0 cm internal diam. $\times$ 25 cm) of DEAE-Sephacel previously equilibrated with buffer A. The column was washed with 0.05 M-NaCl in buffer A and the enzyme was eluted by a linear gradient (1500 ml) of 0.05–0.4 M-NaCl in buffer A. Fractions containing the highest AADC activity were concentrated by precipitation with (NH_4) $_2$ SO $_4$ and dialysed against PBS containing 1 M-NaCl, 1 mM-dithiothreitol and 2 mM-pyridoxal 5'-phosphate (PLP).

The dialysis residue was applied to a monoclonal-antibody affinity column previously equilibrated with the same solution as the eluate from the DEAE-Sephacel

Fig. 4. SDS/polyacrylamide-slab-gel electrophoresis of AADC from bovine brain purified by monoclonal-antibody affinity chromatography

(a) Standard proteins, from top to bottom: phosphorylase b (M_r 92 500), bovine serum albumin (66 200), ovalbumin (45 000) and carbonic anhydrase (31 000). (b) AADC preparation from a DEAE-Sephacel column, before loading on a monoclonal-antibody affinity column. (c) AADC from a monoclonal-antibody affinity column.

column was dialysed against. The affinity column (1 cm $\times$ 5 cm), in which Affi-Gel 10 (Bio-Rad) conjugated with AADC-directed monoclonal antibody was packed, was connected to a pre-column (2 cm $\times$ 15 cm) in which Affi-Gel 10 conjugated with rabbit serum was packed. The pre-column, which was used to prevent non-specific proteins from binding to the affinity column, was removed after application of the dialysis residue. The affinity column was extensively washed with PBS containing 1 M-NaCl, 10 % (v/v) glycerol, 0.1 % Tween 20,

Table 2. Kinetic parameters of AADC from bovine brain (a) and adrenal medulla (b) for substrates

Tissue	Substrate	K_m (M)	V_{max} (nmol/min per mg of protein)
(a) Brain	L-Dopa	1.4×10^{-4}	43.3
	L-5-Hydroxytryptamine	1.2×10^{-4}	13.7
(b) Adrenal medulla	L-Dopa	1.6×10^{-4}	190
	L-5-Hydroxytryptamine	8.3×10^{-5}	63.3

1 mM-dithiothreitol and 2 mM-PLP, and then AADC was eluted with a solution containing 50 mM-acetic acid, 10% (v/v) ethylene glycol, 1 mM-dithiothreitol and 2 mM-PLP. The eluate was immediately neutralized with 1 M-Tris/HCl (pH 8). Fig. 3 shows a typical elution pattern of enzyme from bovine brain. The resultant enzyme was eluted as a sharp peak. The purification procedures for the enzymes from bovine brain and adrenal medulla are summarized in Table 1.

Both the brain and adrenal-medulla enzymes were eluted by gel-permeation chromatography at similar positions, with M_r of approx. 100 000 (results not shown).

An essentially single band, with an apparent subunit M_r of 50 000, was observed in SDS/polyacrylamide-gel electrophoresis of the resultant enzyme from both bovine brain (Fig. 4) and adrenal medulla (results not shown). Therefore brain and adrenal medulla enzymes appear to be homodimers. Moreover, on Western immunoblotting of the resultant enzymes from bovine brain and adrenal medulla, a single band corresponding to the same M_r as the subunit of AADC was stained with the monoclonal antibody (results not shown). The results of SDS/polyacrylamide-gel electrophoresis and Western immunoblotting of the enzymes imply good separation of the enzymes.

The specific activities of both enzymes were low (Table 1) compared with the enzymes purified from other sources by different methods: 8670, 351 and 3069 nmol/min per mg from pig kidney [5–7], 9350 nmol/min per mg from guinea-pig kidney [8], 152.5 nmol/min per mg and 10.3 μ mol/min per mg from human pheochromocytoma [9,10] and 37.7 μ mol/min per mg from *Micrococcus percuteus* [11].

Fig. 5. Effects of pH on the activities of AADCs purified from bovine brain (a) and adrenal medulla (b)

Enzyme activity was assayed as described in the Experimental section, in 30 mM-sodium phosphate (●), -Tris/HCl (■) or -glycine/NaOH (▲).

Fig. 6. Effects of PLP on activities of AADCs purified from bovine brain (a) and adrenal medulla (b)

Activity of AADC was measured as described in the Experimental section, except that the concentration of PLP was varied as indicated.

Properties of AADC from bovine brain and bovine adrenal medulla

Substrate specificity of the purified enzymes from bovine brain and adrenal medulla was examined with L-dopa and L-5-hydroxytryptophan. Michaelis constants (K_m) and maximum velocities (V_{max}) towards each substrate are shown in Table 2. For enzymes from both bovine brain and from bovine adrenal medulla, K_m values for L-5-hydroxytryptophan and L-dopa were similar.

The pH-dependencies of the decarboxylation of dopa by both enzymes are shown in Fig. 5. Optimum pH was 7.2, and the effects of pH were reversible over the pH range 6.0–8.0 for both enzymes.

The effect of PLP as a cofactor of AADC on each enzyme activity is shown in Fig. 6. Each enzyme, which was dialysed against PBS before assay, had some activity in the absence of exogenous PLP, owing to the coenzyme covalently bound to the enzyme [5], but the activity was stimulated by the addition of PLP. High concentrations of PLP inhibited the activity of both enzymes.

DISCUSSION

AADC was purified homogeneously from bovine brain for the first time by affinity chromatography using a monoclonal antibody. Although the specific activity was low, probably owing to inactivation of the enzyme by the acidic elution during affinity chromatography, the protein was homogeneous. On SDS/polyacrylamide gel electrophoresis of AADC purified from bovine brain and from bovine adrenal medulla, a single protein band with an apparent subunit M_r of 50 000 was observed. The position of the band accords well with a previous report on subunit M_r of pure human AADC [10]. Furthermore, on Western immunoblotting of each purified enzyme, a single band corresponding to the same position as the protein band on SDS/polyacrylamide-gel electrophoresis was stained with the monoclonal antibody. Since the M_r of both the bovine brain and adrenal-medulla enzymes was estimated to be approx. 100 000, the enzymes seem to be composed of two homologous subunits, each of M_r 50 000. The results of characterization suggest that bovine brain AADC is similar to that from bovine

adrenal medulla, in good agreement with the results of Christenson *et al.* [5] and Ichinose *et al.* [10]; however, it is still uncertain whether or not both enzymes are identical.

Our results indicate that this purification procedure is useful for preparation of homogeneous AADC on a large scale, which will assist further investigation of its chemical properties.

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