Biochimica et Biophysica Acta, 481 (1977) 706—711

BBA 68114

PURIFICATION OF L-GLUTAMATE DECARBOXYLASE BY AFFINITY CHROMATOGRAPHY

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(Received October 4th, 1976)

Summary

L-Glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) from rat brain synaptosomal extract was partially purified by affinity chromatography. On further purification by DEAE-Sephadex A 50 and Sephadex G-200, L-glutamate decarboxylase was purified to greater extent. It was found that a single affinity chromatography by appropriate elution gave a highly purified protein giving a single band of high specific activity on polyacrylamide gradient gel slab electrophoresis with minimal contamination.

Substrate specificity of the purified enzyme was modified in the presence of 6-azauracil or phenylalanine resulting in decreased specificity to L-glutamate and increased specificity to L-aspartate.

Introduction

Several attempts have been made to purify mammalian L-glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) by conventional stepwise fractionation methods [1,2] to about 700-fold purification. The fact that in such preparations recovery was surprizingly low prompted us to apply a new procedure for purification of the enzyme.

Recently affinity chromatography has been developed and has been applied in the field of purification of macromolecules. In the present paper, it is demonstrated that such a method offers more advantages in purification of the enzyme than has been reported so far.

As is well known, L-glutamate decarboxylase in mammalian tissue is located mostly in nerve endings of the brain and catalyzes decarboxylation of L-glutamate to yield γ-aminobutyric acid which regulates nerve transmission at inhibitory nerve endings. Other ω-amino acids like β-alanine may also participate as inhibitory chemical transmitters which could arise from pyrimidine catabolite [3] but also from decarboxylation of L-aspartate [2]. Therefore, enzyme

regulation by certain drugs or physiological substances on L-glutamate decarboxylase provides a possible control of ω-amino acid distribution in the nervous system.

Material and Methods

Rat brains without midbrains were homogenized in 0.25 M sucrose solution and centrifuged at 1000 × *g* for 20 min to remove nuclei and cell debri. The supernatant was centrifuged at 23 000 × *g* for 30 min to obtain crude mitochondrial P_2 fraction. The pellet was suspended in 0.25 M sucrose density gradient consisting of five layers, i.e. 20%, 16%, 12%, 8% and 2% Ficoll in 0.25 M sucrose. It was then centrifuged in a swing rotor at 25 000 rev./min for 60 min. The interlayers between 2% and 8% Ficoll in 0.25 M sucrose were collected as a synaptosomal fraction. Morphological confirmation on electron microscopy and distribution of marker enzymes were described elsewhere [4]. Application of syringe suction of the synaptosomal fractions after discontinuous centrifugation for subfractionation of crude mitochondrial P_2 fraction was reported previously [5]. Synaptosomal fractions were washed in 0.25 M sucrose and centrifuged down at 150 000 × *g* for 60 min to remove Ficoll and sucrose. The pellet was suspended in an aliquot of distilled water and subjected to freezing and thawing to obtain a crude extract of L-glutamate decarboxylase from synaptosomal fractions. The water extract of synaptosomes was directly applied to a column set up for affinity chromatography.

Affinity binding of the ligand to the Sepharose matrix was performed by a conventional method described by Cuatrecasas et al. [6,7]. Namely, CH-Sepharose 4B was swollen in 0.5 M NaCl at a ratio of 200 ml per dry gel and DL-α-methylglutamate was adjusted to pH 4.7 to make 0.25 mM ligand concentration in the reaction mixture. Under slow stirring at room temperature, 40 mg of *N*-cyclohexyl-*N'*-[2-(4-β-morpholinylethyl)]-carbodiimidemethyl-*p*-toluolsulfonate from Fluka Chem. Co., dissolved in 2 ml of water to each 1.2 gram dry gel, was added drop by drop. pH was kept between 4.5 and 6.0 during the addition of the carbodiimide and the mixture was stirred for 20 h. The gel was packed in a 0.9 × 30 cm column and washed successively with NaCl, Tris/phosphate buffer pH 8.4, NaCl, pyridine buffer pH 4.7 then equilibrated with the eluting buffer, 0.05 M potassium phosphate buffer pH 7.2. The standard eluting buffer contained 10^{-3} M aminoethyl isothiuronium bromide and 10^{-4} M pyridoxal phosphate. Materials adsorbed to the matrix with ligand in the column were gradually eluted by increasing the NaCl concentration in a standard buffer.

Mammalian L-glutamate decarboxylase was assayed by a modification of the method of Fonnum [8] with 0.05 μCi of DL-[1-^{14}C]glutamate or L-[U-^{14}C]-aspartate from Bio-Rad in 5 mM substrate in 0.5 M potassium phosphate buffer with the same components of standard eluting buffer for column chromatography in a closed Warburg vessel. $^{14}CO_2$ released at 37°C in 30 min was collected in ethanolamine/methyl cellosolve, 1 : 2, in a center well after enzyme reaction had been terminated by the addition of 2.5 M H_2SO_4 from the side arm during another 60 min incubation. Radioactivity of $^{14}CO_2$ was counted in a solvent consisting of Triton X-100/toluene with scintillants, 1 : 2,

in the Packard Tricarb scintillation counter.

Electrophoresis was performed by Pharmacia polyacrylamide gradient gel slab electrophoresis, PAA 4/30, in 0.25 M Tris/glycine buffer, pH 8.4, with 20 mg/l of glutathione, at 170 V for 4 h, with Bromophenol Blue as a front marker. Gel was stained in 1% Amido Black 10B in 7% acetic acid for 60 min at room temperature and destained in a gel destainer GD-4, Pharmacia at 12 V for 60 min.

A commercial preparation of L-glutamate decarboxylase from *Escherichia coli* was obtained from Kyowa Hakko Co. The enzymatic assay of bacterial L-glutamate decarboxylase was done in 0.4 M pyridine buffer according to Hager [9].

Results and Discussion

In an attempt to confirm the establishment of L-glutamate decarboxylase, affinity chromatography between α-methyl glutamate which is a competitive inhibitor of the enzyme, covalently bound to CH-Sepharose 4B and a commercial preparation of L-glutamate decarboxylase from *E. coli* was initially studied. Fig. 1 shows the elution profile of a mixture of the bacterial enzyme, and bovine serum albumin as a protein nonspecific to the matrix. The first elution peak with a standard buffer without NaCl shows a dominant elution of protein nonspecific to the matrix with a minimal elution of the enzyme, demonstrating the lowest specific activity of the enzyme eluted. As the concentration of NaCl in a standard buffer increased, L-glutamate decarboxylase of higher specific activity was eluted. At 0.5 M NaCl the specific activity of the bacterial enzymes attained 0.347 μmol/min per mg protein. The commercial preparation of the bacterial enzyme was 200 U/mg solid. When AH-Sepharose 4B was substituted for CH-Sepharose 4B, the purification efficiency was less than before. However, a similar elution pattern was observed (data not shown).

Crude extract of L-glutamate decarboxylase from nerve endings of rat brain

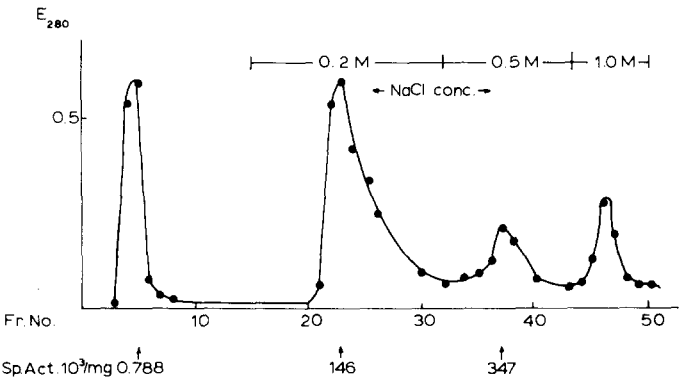

Fig. 1. Affinity chromatography of *E. coli* L-glutamate decarboxylase on Sepharose 4B. DL-α-methyl-glutamate was covalently bound to the matrix as indicated in the text. 10 mg of *E. coli* enzyme and 10 mg of bovine serum albumin were applied to the column, eluted with 0.05 M potassium phosphate buffer, pH 7.2 with or without NaCl as indicated in the figure. 3 ml of fractions were collected. Specific activity is expressed as μmol CO_2 evolved per min per mg protein.

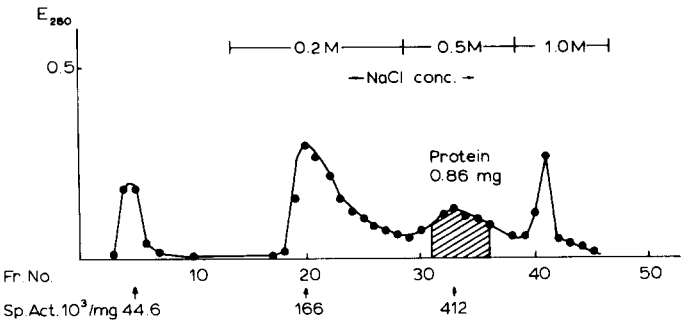

Fig. 2. Affinity chromatography of rat nerve ending L-glutamate decarboxylase on Sepharose 4B. Water extract of nerve ending subfraction from brain homogenate was applied to the column, and eluted as indicated in Fig. 1. For experimental details see the text.

was directly applied to the affinity column whose elution pattern is shown in the Fig. 2. The enzyme with specific activity of 0.412 μmol/min per mg protein was eluted at 0.5 M NaCl concentration. The recovery was 8.6% protein from 10 mg nerve ending extract protein as is summarized in the Table I. This single step provided 80-fold purified L-glutamate decarboxylase from nerve ending extract which corresponded to a several hundred-fold purification of L-glutamate decarboxylase in the brain homogenate. Further purification was attained by DEAE-Sephadex A 50 and Sephadex G 200 column chromatographies. As is shown in Fig. 3, the enzyme purification is raised to a specific activity of 1.095 μmol/min per mg protein by DEAE-Sephadex A 50 in the elution peak of No. 4. Fractions of Nos. 1—3 of the eluates from DEAE-Sephadex A 50 column chromatography were combined and the fourth peak alone was separately chromatographed on Sephadex G-200 as the next purification step. Fig. 4 demonstrates the elution patterns of two chromatographies showing that combined eluates of Fractions 1—3 in DEAE-Sephadex A 50 consisted of two components, while Fraction 4 was eluted as a single peak of a specific activity of 1.124 μmol/min per mg protein.

The fraction eluted by 0.5 M NaCl in Fig. 1 mobilized as a major band shortly after serum albumin with minimal accessory bands which were not visible on the electrophoretic pattern of the eluate obtained without NaCl which is the first elution peak of protein nonspecific to the ligand. The mobility of a highly purified enzyme on the polyacrylamide gel slab electro-

TABLE I

PURIFICATION OF RAT NERVE ENDING L-GLUTAMATE DECARBOXYLASE BY AFFINITY CHROMATOGRAPHY

	Protein (mg)	Specific activity (μmol/min per mg × 10^3)	Yield (%)	Purification grade from brain homogenate
Nerve ending extract	10.0	5	100.0	6 ×
Eluate without NaCl	4.0	44	40.0	53 ×
Eluate with 0.2 M NaCl	2.4	166	24.0	200 ×
Eluate with 0.5 M NaCl	0.86	412	8.6	493 ×

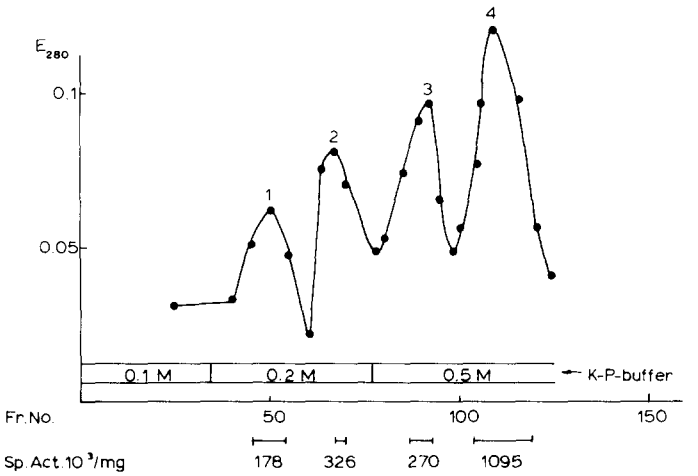

Fig. 3. DEAE-Sephadex A 50 column cheomatography of rat brain L-glutamate decarboxylase purified by affinity chromatography. The eluate with 0.5 M NaCl in Fig. 2 was subjected to DEAE-Sephadex A 50 column chromatography. A 2.5 X 40 cm column was used. The eluting solution contained 15 mg dithiothreitol per l of potassium phosphate buffer, pH 7.2. 3 ml in each fraction was collected. Specific activity indicated was μmol CO_2 evolved/min per mg protein $X10^3$.

phoresis coincided with that of the mouse L-glutamate decarboxylase of molecular weight 85 000 [2]. pH optimum of rat brain L-glutamate decarboxylase was found to be 7.2. In our preparation the highest specific activity of the enzyme was 1.124 μmol/min per mg protein which was half the maximum purity obtained by others [2], but as a purification technique, the application of affinity chromatography as a single step offers a much higher recovery from the starting material.

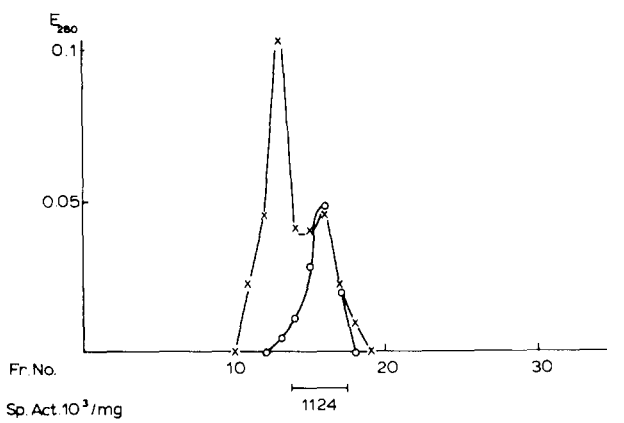

Fig. 4. Sephadex G-200 column chromatography of rat brain L-glutamate decarboxylase. Combined eluates of Nos. 1—3 from the DEAE-Sephadex A 50 column chromatography (X———X), and fraction number 4 (○———○) were chromatographed on Sephadex G 200. A 1.5 X 30 cm column was used. Standard buffer with isothiuronium bromide and pyridoxal phosphate, as described in the text, was used for elution. 3-ml fractions were collected. Specific activity is indicated in the same units in the previous figures.

TABLE II

SUBSTRATE SPECIFICITY OF NERVE ENDING L-GLUTAMATE DECARBOXYLASE FROM RAT BRAIN

Mean of three experiments. * Indicates significant differences from the control. $P < 0.01$.

Effector	L-Glutamate	L-Glutamate (μmol/min per enz. $\times 10^3$)	L-Aspartate (μmol/min per enz. $\times 10^3$)
None		10.3	0.117
Phenylalanine	10^{-6} M	8.74	0.197 *
	10^{-5} M	9.29	0.168 *
6-Azauracil	10^{-6} M	9.81	0.221 *
	10^{-5} M	8.93	0.208 *
	10^{-4} M	8.14	0.155 *
Actinomycin D	10^{-6} M	10.5	0.181 *
	10^{-5} M	10.4	0.174 *

Substrate specificity of decarboxylases is rather broad and L-glutamate decarboxylase is known to have some affinity towards L-aspartate to yield β-alanine [2]. Table II summarizes some effects on L-glutamate decarboxylase which might shift the specificity of the enzyme to other substrates. 6-Azauracil is known to be an inhibitor on orotidylate decarboxylase but is diverse in its actions on pyrimidine metabolism resulting in β-alanine accumulation in rat liver [3] as well as in guinea pig brain [10]. On a molecular basis, the effect of 6-azauracil should be in favour of β-alanine increase from aspartate by L-glutamate decarboxylase as is shown in the Table II. Phenylalanine is a competitive transport inhibitor towards tyrosine which is a precursor substance of neurotransmitters at nerve endings in rat brain slices [11], again shown to increase affinity of the enzyme to L-aspartate. These findings should be confirmed more precisely by a more purified enzyme to suggest L-glutamate decarboxylase as a regulator molecule of ω-amino acids in the brain.

In conclusion, for advancement of affinity chromatography in the field of purification techniques, a specific elution method has to be introduced, different from nonspecific increase of ionic strength in the eluting solution of the substance which is specifically attached to the matrix.

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