

## Isolation, Purification and Characterization of Glutamate Dehydrogenase from Porcine Brain

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**Abstract:** To obtain pure glutamate dehydrogenase from porcine brain and explore its enzymological properties, the glutamate dehydrogenase which showed a single band on SDS-PAGE had been purified from porcine brain by frozen, homogenization, ammonium sulfate precipitation, DEAE-Sepharose chromatography and Superdex-200 chromatography. Its multiple of purification was 70.07 and its specific activity was 13.63 U/mg. 24.61% of the glutamate dehydrogenase activity was recovered. The enzyme had a relative molecular mass of 330.08 kDa. The relative molecular mass of the subunit was about 56.04 kDa. The optimum temperature and pH of this enzyme were 55°C and 8.2 respectively. The glutamate dehydrogenase displayed excellent stability at temperature below 40°C and pH 6-8. Its apparent  $K_m$  towards NADH was 0.084 mmol/L. The enzyme activity could be strongly inhibited when interacting with methanol, ethanol, isopropanol, SDS, oxalic acid, ascorbic acid,  $Cu^{2+}$ ,  $Co^{2+}$ , or  $Zn^{2+}$  and activated when interacting with EDTA.

Glutamate dehydrogenase (GDH) is one of key enzymes of the glutamate metabolism<sup>[1]</sup>. GDH catalyzes the glutamate to deaminate reversibly to be  $\alpha$ -ketoglutaric acid and ammonia when couples with the coenzyme NAD/NADH, which would take part in the synthesis and decomposition of ammonia. As dehydrogenase, GDH exists widely in the animals, plants and microorganism<sup>[2-4]</sup>. The enzyme exists in the form of tetramer or hexamer<sup>[5]</sup>, and plays a very important role in the metabolism of carbon and ammonia<sup>[6-7]</sup>. Because of the extensive application of the enzyme in the area of medicine and chemical analysis<sup>[8]</sup>, there are lots of researches about GDH from different organism except porcine brain so far<sup>[9-15]</sup>. We have isolated and purified the GDH from porcine brain and researched on the partial properties of the enzyme. Our works establish a basis for researching on the metabolism of carbon and ammonia in living organisms, provide the data for the deeply investigation and application of GDH, and found a new pathway to obtain GDH.

### 1. Materials and methods

#### 1.1 Materials and reagents

DEAE-sepharose, superdex-200 and standard substance of protein SDS-PAGE were purchased from GE Healthcare (USA). Molecular mass standard substance in gel filtration chromatography (GFC) was purchased from Pharmacia (USA). Trihydroxymethyl aminomethane was purchased from Farco (Hong Kong). Acrylamide, methylene bisacrylamide were purchased from Fluka (Switzerland); NADH, ADP, BSA were obtained from Sigma (USA). The other agents are analytical pure and made in China.

#### 1.2 Methods

##### 1.2.1 Preparation of crude enzyme solution from porcine brain

Wash the fresh porcine brain with tap water and double-distilled water, cut it in small piece, and store it in refrigerator -20°C before using it. Add it in the precooled buffer A (10 mmol/L pH 8.0

Tris-HCl buffer with 0.5mmol/L EDTA, 0.1mmol/L PMFS) according to the ration 1:3(m/V), extract it 2h after homogenizing. All purification steps are done at 4°C. Then the homogenate is centrifuged at 12000r/min for 30min .Desert the precipitation and add the solid ammonium sulfate to the supernatant until 20% saturation. Two hours latter, the 20% (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub> supernatant is centrifuged at 12000r/min for 30 min. Then desert the precipitation, repeat the previous step except the degree of saturation is 45%. The 45% (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub> supernatant is centrifuged at 6000r/min for 30 min after salting out it 2h. The precipitation is dissolved with buffer A and dialyzed overnight against distilled water, and then centrifuged (6000r/min, 20min).Collect the supernatant which is the crude GDH solution.

### **1.2.2 DEAE-Sepharose ion exchange chromatography**

The DEAE-Sepharose ion exchange chromatographic column is balanced with buffer B (25mmol/L PH8.0 Tris-HCl with 0.5mmol/L EDTA and 1mmolβ-mercapto- ethanol). The protein is eluted from the column with 0-0.6mol/L NaCl(prepared by buffer B) in linear gradient at the speed of 0.5ml/min. Measure the enzymatic activity and protein concentration in every tube, collect the tubes with high specific activity which are used for the gel filtration chromatography.

### **1.2.3 Superdex-200 gel filtration chromatography**

Superdex-200 gel chromatographic column is balanced with buffer C(25mmol/L PH8.0 Tris-HCl with 0.5mmol/L EDTA and 1mmolβ-mercaptoethanol), The protein is eluted from the column with buffer C 200ml at the speed of 0.3ml/min. Measure the GDH activity and protein concentration in every tube; collect the tubes with high specific activity. Fractions containing GDH activity are dialyzed overnight against distilled water (with 1mmol/L EDTA) at 4°C, and then lyophilized for use.

### **1.2.4 Identification of enzymatic purity and molecular mass**

Measure the purity and the molecular mass of the subunit by using SDS-polyacrylamide gel electrophoresis<sup>[16]</sup>. The concentration of the gel is 12%.

### **1.2.5 Measurement of the protein concentration**

We use the ultraviolet spectrophotometry method and the Bradford's method respectively to measure the concentration of the enzyme<sup>[17]</sup>.

### **1.2.6 Measurement of GDH activity**

The method we used to measure the activity of GDH which is according to reference [1] with some modified. The system contains 1mol/L (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub> ( or 2mol/L NH<sub>4</sub>Cl) 150μL, 0.1mol/L α-oxoglutarate 150μ, 10mmol/L NADH 50μL, 18mmol/L ADP 20μL,50mmol/L PH8.2 Tris-HCl buffer 2.53mL and enzyme solution 100μL, react the system at 55°C after mixing sufficiently. Measure the reduction value of the absorbance in 340nm after reacting 1min. One unit of enzyme was defined as the amount of the GDH which is required to oxidize 1μmol of NADH per 1 min.

### **1.2.7 Measurement of the properties of GDH**

The method to measure the enzyme is according to the reference [18, 19] with some modified.

## **2. Results and Analysis**

### **2.1 Separation and purification of GDH in porcine brain**

The GDH crude solution that had been extracted from porcine brain was placed over DEAE-Sepharose, the result has been showed as figure.1. The peak of enzymatic activity mainly concentrated in 23~29 tubes, especially the twenty-sixth tube showed the max enzymatic activity. Fractions containing high specific activity were placed over Superdex-200. The elution profile was showed in figure.2. The enzymatic activity peak concentrated between 27th~31th tubes. SDS-PAGE showed that the enzyme had been purified (figure.3). The purification fold of the GDH

was 70.07, recovery rate was 24.61% and specific activity came up to 13.63U/mg.

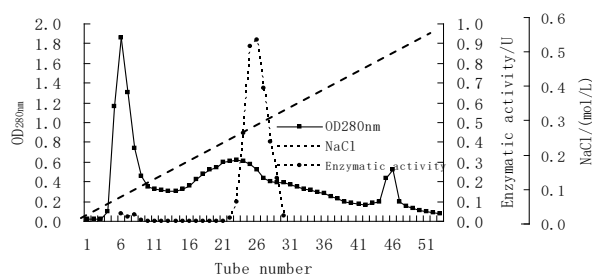


Fig.1 DEAE-Sepharose chromatography of GDH

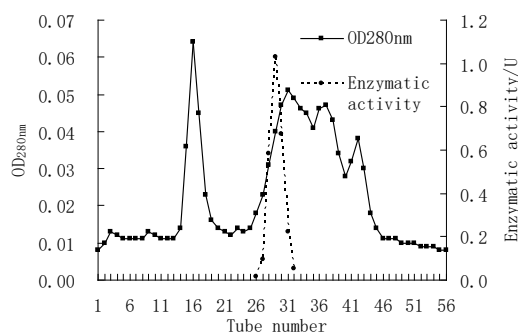


Fig.2 Superdex-200 chromatography of GDH

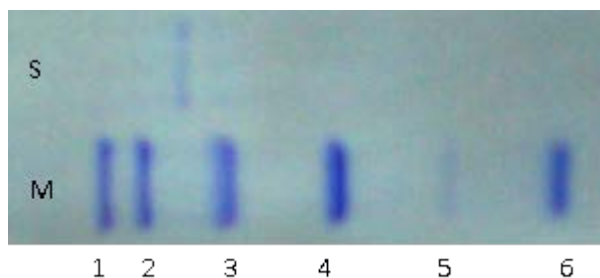


Fig.3 SDS-PAGE of GDH from porcine brain

M. Molecular mass standard substance; 1. Rabbit muscle phosphorylase B 97.0kD; 2. Bovine serum albumin 66.0kD; 3. Chicken egg white ovalbumin 45.0kD; 4. Bovine erythrocyte carbonic anhydrase 30.0kD; 5. Soybean trypsin inhibitor 20.1kD; 6. Bovine milks  $\alpha$ -lactalbumin 14.4kD; S. Porcine brain glutamate dehydrogenase 56.04kD.

## 2.2 Measurement of molecular mass of GDH

We found the relative molecular mass of the subunit of GDH is 56.04KD (figure.3) by the way of SDS-PAGE. The relative molecular mass of the holoenzyme is 330.08KD, so the porcine brain GDH may have six same subunits.

## 2.3 Measurement of the properties of GDH

### 2.3.1 The optimum temperature and thermal stability of GDH

The optimum temperature of GDH was about 55°C (Figure.4). The relative enzymatic activity remained 70% after staying the enzyme at 40°C 5h. There was no activity lost when the temperature was lower than 30°C, which indicates the enzyme has excellent thermal stability below 40°C. However, the enzymatic activity reduced rapidly when the temperature was up to 50°C, and there was no activity at 60°C (Figure.5). We also found there would be no enzymatic activity lost when the pure GDH stays at 4°C three months or 30°C half a month.

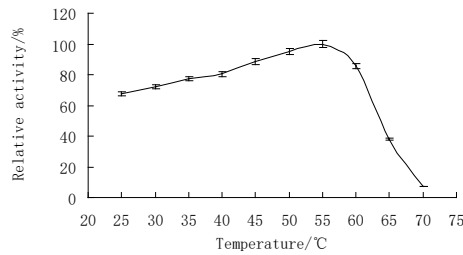


Fig.4 Effect of temperature on activity of GDH from porcine brain

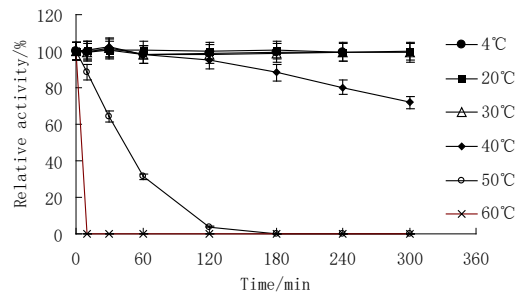


Fig.5 Thermal stability of GDH from porcine brain

### 2.3.2 The optimum pH and pH stability of GDH

We found the optimum pH of GDH is about 8.2(Figure.6). GDH had excellent tolerance to pH. The enzymatic activity had almost no change after staying in 4°C 6 days when the pH value was between 6~8. There would almost be no enzymatic activity after the enzyme stayed 2h lower than pH4. When the pH value was 9, the enzyme would have no activity after 6 days(Figure.7)..

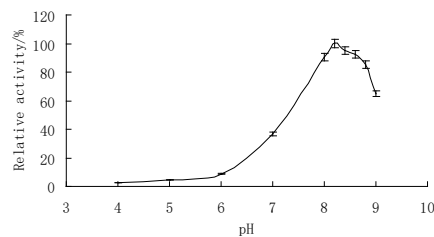


Fig.6 Effect of pH on activity of GDH from porcine brain

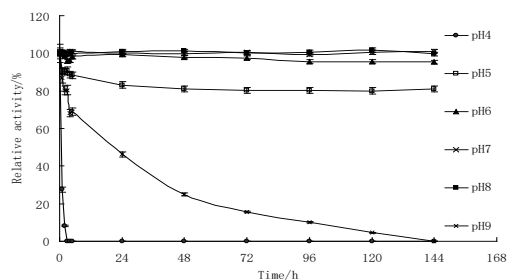


Fig.7 pH Stability of GDH from porcine brain

### 2.3.3 The $K_m$ value of GDH

We obtained the  $K_m$  of GDH to NADH is 0.084mmol/L by using Lineweaver-Burk (Figure.8). The  $K_m$  value from porcine brain was close the GDH from human brain<sup>[1]</sup> and ox brain<sup>[9]</sup>, but was distinct with that came from duck liver<sup>[12]</sup> and corynebacterium glutamicum S<sub>9114</sub><sup>[7]</sup>, which indicated the  $K_m$  of GDH from different source might have some distinctions with each other.

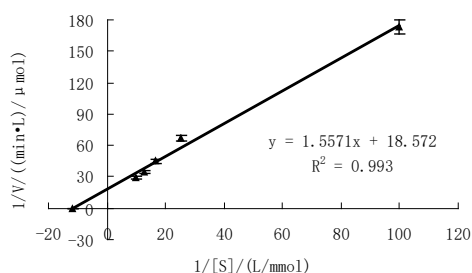


Fig.8 Km Determination of GDH from porcine brain

### 2.3.4 Effect of different chemical compound on the activity of GDH

With the concentration of compounds increased, GDH was inhibited by SDS, oxalic acid, ascorbic acid and  $H_2O_2$ , especially SDS, oxalic acid and ascorbic acid. Our data showed EDTA can activate GDH (Figure.9). It was worth noting that EDTA plays important role in maintaining the activity of GDH. The enzyme solution would absolutely lost activity after dialysis if there was no EDTA in distilled water. Inversely, if we added 1mmol/L EDTA to the water, the enzymatic activity will have almost no change.

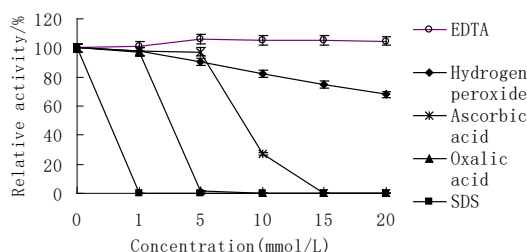


Fig.9 Effect of various compounds on activity of GDH from porcine brain

### 2.3.5 Effect of different organic solvent on the enzymatic activity

Methanol, ethanol and isopropyl alcohol had noTable depressant effect on the activity of GDH. With the volume ratio of the three organic solvents increased, the depressant effect was more and more obvious. When the ratio achieved 40%, the enzymatic activity was almost lost(Figure.10)..

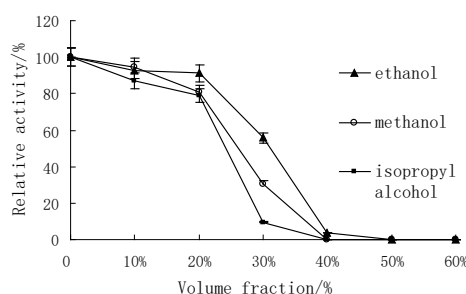


Fig.10.Effect of methanol, ethanol and isopropyl alcohol on activity of GDH from porcine brain

### 2.3.6 Effect of different metal ions on enzymatic activity

Different metal ions or the same metal ions with different concentrations had obvious discrepant effects on the activity of GDH. With the concentration of metal ions increased,  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Ba^{2+}$  partially inhibited the enzymatic activity,  $Cu^{2+}$ ,  $Co^{2+}$ ,  $Zn^{2+}$  showed strong inhibitory action on the enzymatic activity, which deactivated the enzyme at last.  $Na^+$ ,  $Li^+$ ,  $K^+$  had no obvious effect on the activity of GDH(Figure.11).

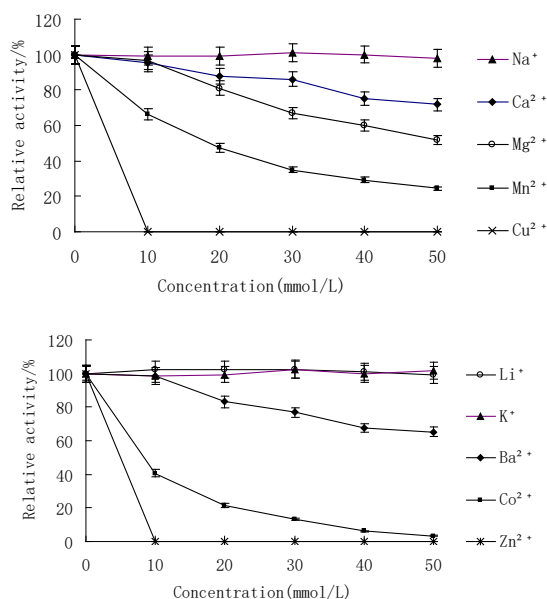


Fig.11 Effect of metal ions on activity of GDH from porcine brain

### 3. Conclusion

We succeed to separate and purify the GDH from porcine brain. Compared with the purification of the GDH from *Pseudomonas pseudoalcaligenes* [4], *corynebacterium glutamicum* S<sub>9114</sub> [7], *Brassica napus* [11] and bovine brain [9], our method is more convenient. Under the similar pretreatment condition, this method reduced the utilization frequency of chromatography. After just only twice chromatography, we gained the electrophoretic pure GDH contrast to three times or more times from other material resources. Compared to the purification proceed from duck liver [12], although our experiment reduced pretreatment steps, we obtained the same results. Furthermore, the recovery rate was also increased as the same as purify fold.

The thermal stability of the GDH in our experiment is good, and its pH tolerance is also wide compared to the GDH from duck liver [12]. There were no enzymatic activities lost during half month when put it in buffer C at 30°C or below 30°C. No enzymatic activity lost when the GDH was placed at 4°C 6 days in the condition of pH 6~8. This means the GDH may have high application value. The optimum temperature of the GDH is 55°C, higher than general GDH from other materials. The optimum pH is 8.2, which is similar to the GDH from *Pseudomonas pseudoalcaligenes* (pH 8.0) [4], lower than the GDH from duck liver (pH 10.0) [12] and higher than the GDH from *corynebacterium glutamicum* S<sub>9114</sub> (pH 7.5) [7] and *Ectomycorrhizal Fungus Laccaria bicolor* (Maire) Orton (pH 7.4) [10]. The molecular mass of the subunit is 56.04KD, which is close to the subunit of GDH human brain [1]. The molecular mass of the holoenzyme is 330.08KD, indicates that the GDH has six same subunits. This also matches to the report that GDH exist mainly in the form of tetramer or hexamer [5].

Different metal ions have different effect on the enzymatic activity. With the concentration of metal ions increased, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ba<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, Zn<sup>2+</sup> showed partial depressant effects on the activity of GDH, but Na<sup>+</sup>, Li<sup>+</sup>, K<sup>+</sup> had no obvious influence on the enzymatic activity. Gamier A et al. [10] had reported that Mg<sup>2+</sup>, Mn<sup>2+</sup>, Cu<sup>2+</sup> inhibited the activity of GDH. Zhu H et al. [12] found that Cu<sup>2+</sup>, Zn<sup>2+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup> depressed the enzymatic activity in their experiment, But Li<sup>+</sup>, K<sup>+</sup> also inhibited the enzymatic activity and the effect of Mn<sup>2+</sup> was not obvious. Wang Y et al. [7] had reported that Na<sup>+</sup> had no obvious influence on the enzymatic activity and K<sup>+</sup> could activate the enzymatic activity. These indicate that the GDH from different genus may be different in properties which may relate to the environment they adapt to.

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