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Research Article
Purification of Homogeneous Glutamate Dehydrogenase from Wheat Seedlings Using Ion Exchange Chromatography

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Abstract

Background and Objective: Glutamate dehydrogenase (GDH) is an enzyme that catalyzes the reversible conversion of glutamate to α-ketoglutarate and ammonia. This reaction plays a crucial role in cellular metabolism, particularly in the metabolism of amino acids and nitrogen. This study was twofold: First, to devise a five-stage procedure for the extraction of homogeneous GDH from wheat seedlings and second, to explore the kinetic properties of the enzyme in both amination and deamination reactions. **Materials and Methods:** The purified GDH had a yield of 3.7% and a specific activity of 377 U/mg protein before it was put through the purification process, the enzymes are typically purified by adhering to a certain protocol and using ammonium sulfate precipitation method to separate the enzyme protein from the rest of the mixture using gel filtration, which is performed with Sephadex G-25, then DEAE-Sephacel system is used to carry out the ion exchange chromatography method of separation. The technique for purification was finished by utilizing gel chromatography with Sephadex G-200 as the stationary phase. **Results:** The GDH produced by reductive amination and oxidative deamination processes was shown to function well at a pH value of 8.5, according to the current research and analyzing the kinetic properties of glutamate dehydrogenase allowed researchers to determine the enzyme's affinity for a variety of substrates. The Michaelis constant for 2-oxoglutarate was 2.75, while for glutamate it was 14.9. **Conclusion:** It is possible that the -subunits are most likely the major components that make up the structure of the GDH oligomer and it becomes clear that the enzyme under investigation possesses a substantially greater affinity for 2-oxoglutarate than was first thought.

Key words: Michaelis constant, glutamate dehydrogenase, wheat seedlings, ion exchange chromatography, enzyme kinetics, protein purification

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Glutamate dehydrogenase (GDH) is an enzyme (L-glutamate: NAD (P) H-oxidoreductase 1.4.1.3) that facilitates the transfer of nitrogen-containing compounds and activates the GABA bypass in the tricarboxylic acid cycle. The enzyme facilitates the conversion of reductive 2-oxoglutarate into glutamate by amination, as well as the opposite process of oxidative glutamate deamination[1].

In higher plants, this enzyme is present in every single tissue and organ. Structurally, GDH in most cases represents a homopolymer with a different number of subunits (from 2 to 6) and a molecular weight of 40-60 kDa[2]. The most common and best-known form is the hexamer. The glutamate dehydrogenase properties specific to different organisms have been investigated for a long time. Divalent metal ions, various amino acids and nucleoside phosphates in most cases act as GDH inhibitors[3]. They can also act as activators, along with some amino acids such as aspartate, leucine and asparagine[4] depending on the organism, the Michaelis constant relative to glutamate normally does not exceed 4.6 mM. However, there can be certain exceptions. For example, the K_m value of glutamate for soya-extracted GDH was 7.2 mM at 8.0 pH and 15.8 mM at 9.3 pH[5].

Glutamate dehydrogenase features good thermal stability depending on the organism, the temperature optimum for the enzyme activity is about 20-45°C. According to the literature sources, the temperature optimum of glutamate dehydrogenase for thermophilic bacteria can reach 90°C[6]. The pH optimum values for forward and reverse reactions are different in most cases. Normally, the pH optimum value for forward reactions is lower compared to reverse reactions. For higher plants, the optimum value is around 7-9 pH[7]. For example, the optimum enzyme activity for soya-extracted GDH is observed at 8.0 and 9.3 pH in amination and deamination reactions, respectively[8]. It should be noted that properties directly depend on the subunit composition. It has been established that the α- and the β-subunits can trigger the reactions of 2-oxoglutarate synthesis and glutamate formation, respectively[9].

Therefore, this study aimed to develop the five-stage procedure for obtaining homogenous GDH from wheat seedlings and investigate the kinetic characteristics of the enzyme in amination and deamination reactions.

MATERIALS AND METHODS

Study area: The current research was carried out at the laboratories of the College of Science, Diala University from October, 2023 till January, 2024.

Study design: Hydroponically grown wheat seedlings were the subject of the study. They were subjected to a 10 hrs light cycle and 25 watts per square meter of light intensity grown at a comfortable 25°C.

Spectrophotometric analysis was performed on a solution with a pH of 8.0 that included 2.5 mM 2-oxoglutarate, 0.25 mM NADH, 50 mM ammonium chloride and 100 mM HEPES buffer[10]. The purpose of this experiment was to determine the function that GDH plays in amination processes. The enzymatic process could not have begun without the enzyme's participation. The deamination-related GDH activity of a solution that included 100 mM HEPES buffer (pH 8.5), 3 mM NAD, 1.0 mM calcium chloride and 50 mM sodium glutamate was evaluated by optical density measurements[11]. The enzymatic process could not have begun without the enzyme's participation.

A modified five-step procedure was used to purify GDH at a constant temperature of 4°C[12]. The wheat stems and leaves were combined in the separator. Ammonium sulfate fractionation was carried out in two stages, during which the solution was saturated from 0 to 35% and again from 35 to 70%. A 50 mM Tris-HCl buffer at pH 7.8 was used to suspend the resultant residue in a 2 cm^3 container. To remove the ammonium salts, a Sephadex G-25 column was utilized for gel filtration. Separation of the proteins was carried out in a 50 mM Tris-HCl solution at pH 8.0. Using a DEAE Sephacel column from American supplier Sigma-Aldrich, an ion exchange chromatography method was carried out. A linear gradient of NaCl concentration from 0.15 to 0.3 m was used for the protein desorption process as prescribed by Kujo and Ohshima[13]. At 254 mM NaCl, the enzyme was precipitated from the surface. What followed was a purge and performed using gel filtration on Sephadex G-200.

Electrophoretic investigations of proteins were conducted in 7.5% polyacrylamide gel and universal dying of proteins in the gels was carried out using $AgNO_3$. Specific GDH staining was based on the tetrazolium method[14]. Proteins were estimated using the Lowry method[15].

Molecular GDH forms were produced and their enzymatic reaction rates were measured at varying pH levels to establish the effect of pH on those rates. Using 2-oxoglutarate (forward reaction) and glutamate (reverse reaction), the Michaelis constants of the purified enzymes were determined. Lineweaver-Burk plots based on double-inverse coordinate measurement were used to calculate K_m.

Statistical analysis: Each sample was analyzed three times and the experiments were repeated three times. The distribution pattern was first assessed using asymmetry and excess (Excel, Microsoft Office) and using the

Kolmogorov-Smirnov test. According to the obtained values, the pattern of distribution was evaluated as normal. The Student's test was used with correction for multiple comparisons (Bonferroni correction)[16]. The one-way ANOVA test (the impact of the factor is significant at $p<0.05$) was applied as supplementary.

RESULTS AND DISCUSSION

Electrophoretically homogenous GDH was separated and purified based on the five-stage procedure of purification from green wheat seedlings, which had been modified by our Department (Biology Department, College of Science, Diala University) (Table 1). The ion exchange chromatography on DEAE-Sephacel represents the most significant stage, which allowed purifying GDH by more than 300 times. The obtaining of homogenous glutamate dehydrogenase provided the possibility to explore the important catalytic and kinetic characteristics of the enzymatic reaction.

Ammonium sulphate-based fractionation (up to 70% saturation) and gel filtration on Sephadex G-25 allowed obtaining the enzyme with a specific activity of 16.1 E/mg protein. Ion exchange chromatography performed on a column of DEAE Sephacel identified the peak activity level for GDH subjected to desorption with sodium chloride (254 mM). The obtained enzyme with a specific activity of 163 E/mg protein was subsequently subjected to gel chromatography on Sephadex G-200 (Table 1).

The conducted activities resulted in GDH purification to the specific activity of 377 E /mg protein. At the same time, the degree of purification was 319.5 times and the yield was 3.7%. The implemented PAGE electrophoresis involving universal protein dying with silver nitrate discovered that GDH was obtained in a homogenous condition (Fig. 1a). The modified tetrazolium method of dying discovered that the purified protein represented glutamate dehydrogenase (Fig. 1b).

The Rf value (retention factor) for the obtained enzyme was 0.12 Premnath[17]. It should be noted that the relative electrophoretic mobility of glutamate dehydrogenase extracted from different objects represents a conservative value. For example, Rf for GDH extracted from 12-day wheat seedlings is 0.13, this value depends on the number of subunits and the relationship between them.

The measured optimum pH value for the purified glutamate dehydrogenase was 8.5 both for forward and reverse reactions (Fig. 2 and 3) and (Table 2). It was demonstrated earlier that the optimum H-value for GDH (amination reaction) extracted from corn and soya leaves was 8.5 and 8.0, respectively. In the case of the deamination reaction, the optimum pH value for corn-extracted GDH was 9.0.

The obtained glutamate dehydrogenase enzyme extracted from wheat seedlings had different K_m values for 2-oxoglutarate (K_m = 2.75 (Fig. 4a)) and glutamate (14.9) (Fig. 4b, Table 2). Higher affinity was discovered between the GDH extracted from corn leaves and 2-oxoglutarate, which could be associated with the molecular structure of protein and the specific nature of metabolism for C3 and C4 plants.

It has been established that the α-subunits act as the enzyme catalyst in deamination reactions and the β-subunits can trigger the amination of keto acids. The obtained enzyme

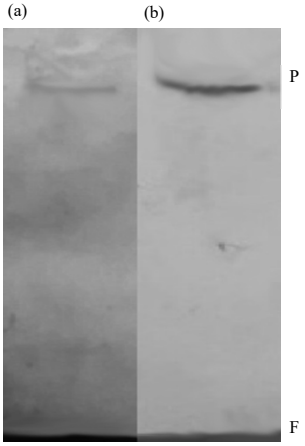

Fig. 1(a-b): Pure GDH electrophoresis, (a) Silver nitrate and (b) Specific GDH expression
P: Protein band and F: Dye front

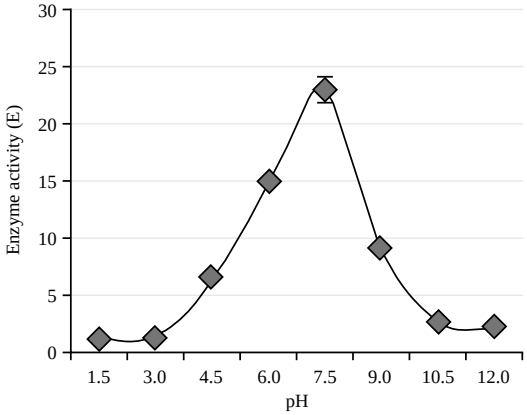

Fig. 2: Glutamate dehydrogenase activity achieved by reductive 2-oxoglutarate amination is pH-dependent

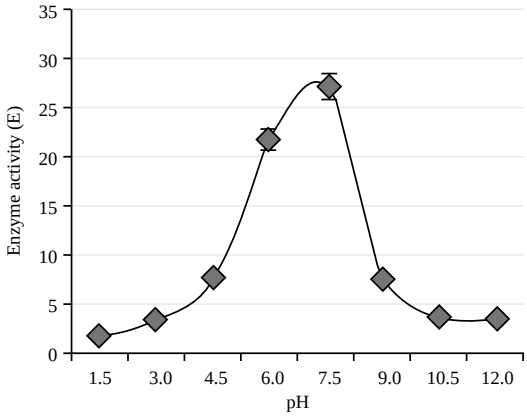

Fig. 3: Dependence of the obtained glutamate dehydrogenase activity on the pH value for glutamate deamination

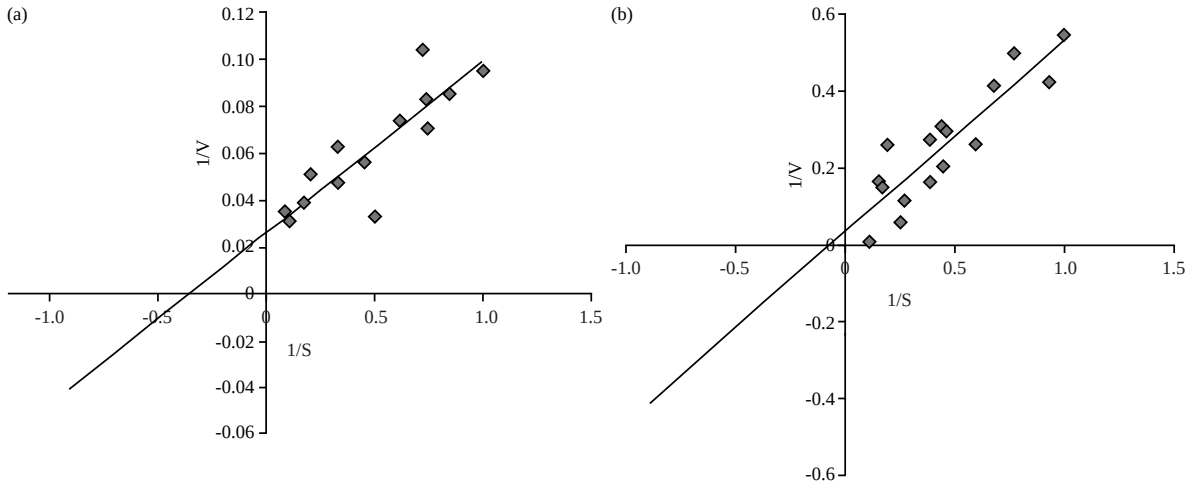

Fig. 4(a-b): Calculating the Michaelis constant of the newly discovered enzyme, (a) Two-oxoglutarate and (b) Glutamate

Table 1: Stages of glutamate dehydrogenase purification from wheat seedlings

Stage	Volume (mL)	Protein (mg)	Total activity (E)	Specific activity (E/mg protein)	Yield (%)	Degree of purification
Homogenate	10	148	175.2	1.18	100.0	1.0
Ammonium sulphate-based fractionation, 70% saturation	7.5	31.3	90.3	2.88	51.5	2.4
Gel filtration through Sephadex G-25	4.0	1.21	19.5	16.1	11.1	13.64
DEAE-Sephacel	2.0	0.1	16.3	163	9.3	138.1
Gel filtration through Sephadex G-200	2.0	0.017	6.55	377	3.7	319.5

Table 2: Constants of Michaelis and the optimum pH range for glutamate dehydrogenase (n = 3, p = 0.05)

Enzyme	K_m for 2-Og (mM)	K_m for Glut (mM)	pH optimum for amination reaction	pH optimum for deamination reaction
GDH	2.75	14.9	8.5	8.5

contains the predominant number of β-subunits. Accurate quantitative analysis of the subunit structure of glutamate dehydrogenase can be explored using mass-spectrometric methods or blotting, which currently represents a challenge[18-20].

This study presents a comprehensive and highly effective methodology for the purification of Glutamate Dehydrogenase (GDH) from wheat seedlings. The meticulous approach outlined in the research offers significant insights into the biochemical properties of GDH and opens up a range

of potential applications in both biochemistry and agricultural biotechnology. By developing a robust and reproducible purification protocol, this study not only enhances our understanding of GDH but also provides a valuable tool for future research in related fields. The detailed methodology and results obtained have the potential to inform various applications, from improving crop yields to developing new biotechnological processes, thereby demonstrating the far-reaching impact of this research.

CONCLUSION

The study developed a five-step purification process for GDH isolated from wheat seedlings. After 319.5 purification cycles, the GDH obtained had a specific activity of 377 E/mg of protein and a yield of 3.7%. Electrophoresis using PAGE and silver nitrate staining confirmed the sample's homogeneity. The purified glutamate dehydrogenase enzyme was tested to determine its characteristics, revealing an optimal pH of 8.5 for both reductive amination and deamination reactions. The Michaelis constants for 2-oxoglutarate and glutamate were found to be 2.75 and 14.9 mM, respectively. This study provides a detailed and effective methodology for the purification of glutamate dehydrogenase (GDH) from wheat seedlings, offering significant insights and potential applications in biochemistry and agricultural biotechnology.

SIGNIFICANCE STATEMENT

The study had 2 main goals: To develop a five-stage method for extracting homogeneous GDH from wheat seedlings and to investigate the enzyme's kinetic properties in amination and deamination reactions. The findings suggest that the-subunits are likely major components of the GDH oligomer structure and the enzyme has a significantly higher affinity for 2-oxoglutarate than initially believed.

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