

Article

Cloning, Prokaryotic Expression, and Functional Verification of Whole-Cell GABA Synthesis by the MoGAD from *Moringa oleifera*

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Abstract

Moringa oleifera is rich in γ -aminobutyric acid (GABA), a functional non-protein amino acid with significant antihypertensive and neuroprotective activities. However, the key enzymes responsible for catalyzing the conversion of *L*-glutamate (*L*-Glu) to GABA—glutamate decarboxylases (GADs)—have not been functionally characterized in *M. oleifera*, which limits its metabolic engineering applications. In this study, the previously obtained MoGAD1 (PZ458702) and MoGAD2 (PZ458703) genes were heterologously expressed in *Escherichia coli* Rosetta (DE3) to produce recombinant proteins. SDS-PAGE and Western blot analyses showed that both MoGAD1 and MoGAD2 were solubly expressed at 20 °C and 37 °C. Their catalytic functions were verified via whole-cell biocatalysis, and high-performance liquid chromatography (HPLC) analysis confirmed that both MoGAD1 and MoGAD2 could convert *L*-Glu to GABA. The GABA yields of the engineered strains harboring MoGAD1 and MoGAD2 reached 3.67 ± 0.1833 g/L and 0.648 ± 0.002 g/L, with conversion rates of 61.2% and 10.8%, respectively. Both MoGAD1 and MoGAD2 exhibited favorable docking with PLP, with binding energies of -5.489 kcal/mol and -5.297 kcal/mol, respectively; they also showed good docking with *L*-Glu, with binding energies of -4.207 kcal/mol and -4.49 kcal/mol, respectively. This study provides the first experimental evidence for the activity of the MoGAD protein encoded by the GAD gene from *M. oleifera*, elucidates the molecular mechanism underlying GABA accumulation, and offers candidate genes for biotechnological production of GABA.



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Keywords: *Moringa oleifera*; glutamate decarboxylase; γ -aminobutyric acid; heterologous expression; whole-cell biocatalysis

1. Introduction

Moringa oleifera, a perennial tropical deciduous tree of the *Moringaceae* family native to India, has recently been introduced and cultivated in Chinese provinces such as Yunnan

and Guangdong. Owing to its unique geographic and climatic conditions, the *Moringa oleifera* planting area in Yunnan Province accounts for approximately 70% of the national total in China [1]. Renowned as the “diamond of plants,” *M. oleifera* possesses immense nutritional value and unique medicinal properties, leading to its broad application in the food and pharmaceutical industries. In 2012, China’s Ministry of Health officially approved *M. oleifera* leaves as a Novel Food Resource (Announcement No. 19) [2,3]. Modern pharmacological studies indicate that *M. oleifera* exerts antihypertensive and sedative effects, with γ -aminobutyric acid (GABA) identified as a primary bioactive component responsible for blood pressure regulation [4,5]. As a non-protein amino acid ubiquitous in plants, animals, and microorganisms, GABA holds exceptional application potential in the food, pharmaceutical, and cosmetic sectors due to its physiological functions, which include enhancing brain vitality, stimulating growth hormone secretion, and preventing metabolic syndrome [2,6].

Although *M. oleifera* is naturally abundant in GABA (2.07 ± 0.07 mg/g) and its precursor *L*-Glu (3.65 ± 0.11 mg/g) [7], traditional plant extraction methods are constrained by low yields, complex purification processes, and high costs, rendering them unsuitable for industrial-scale demand. Furthermore, the chemical synthesis of GABA poses potential risks regarding biosafety and stereoisomer impurities [8]. In contrast, synthetic biology strategies—specifically utilizing key enzyme genes to construct microbial cell factories for biomanufacturing—have emerged as an efficient and sustainable approach for producing high-value natural products [9–11].

Currently, the biosynthetic pathway of GABA and its key enzyme genes in *M. oleifera* remain unexplored. Glutamate decarboxylase (GAD) is the rate-limiting enzyme that catalyzes the decarboxylation of *L*-Glu to generate GABA. To elucidate the molecular mechanism of GABA synthesis in *M. oleifera* and to develop efficient biocatalysts, this study, based on our previous work, performed the prokaryotic expression of the successfully cloned *MoGAD* genes [12]. The coding sequences of *MoGAD*s were constructed into expression vectors to generate recombinant plasmids, which were then transformed into *E. coli* Rosetta (DE3) competent cells for cultivation. After induced expression, the successfully expressed *E. coli* cells were harvested, and the proteins were purified. Their molecular weights were determined by SDS-PAGE, followed by validation using Western blot with mouse anti-His tag as the primary antibody and goat anti-mouse as the secondary antibody. After obtaining the successfully expressed bacterial cells, synthetic biology approaches were employed to perform whole-cell fermentation of the recombinant *E. coli* for the catalytic conversion of *L*-Glu to γ -aminobutyric acid, aiming to verify the gene function and achieve higher GABA yields. This study aims to provide a theoretical basis and promising genetic resources for dissecting the GABA biosynthetic pathway in *M. oleifera* and facilitating subsequent metabolic engineering.

2. Materials and Methods

2.1. Main Strains and Plasmids

E. coli Rosetta (DE3) competent cells were used as the prokaryotic expression host in this study. The recombinant plasmids *MoGAD1*-pET-28a and *MoGAD2*-pET-28a were previously constructed and preserved by our laboratory [12]. The GenBank accession numbers for *MoGAD1* and *MoGAD2* are PZ458702 and PZ458703, respectively. *MoGAD1* has a protein length of 551 amino acids and a molecular weight of 62,224.4 Da, while *MoGAD2* consists of 367 amino acids with a molecular weight of 40,443.8 Da.

2.2. Main Reagents and Instruments

Isopropyl- β -D-thiogalactopyranoside (IPTG) and o-phthalaldehyde (OPA) were purchased from Beijing Puxitang Biotechnology Co., Ltd. (Beijing, China). The GABA standard, pyridoxal 5'-phosphate (PLP), unstained and pre-stained protein markers, and Western lot antibodies were obtained from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). L-Glu was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Chromatographic-grade acetonitrile, triethylamine, and glacial acetic acid were supplied by Sigma-Aldrich (St. Louis, MO, USA). The buffer compositions were as follows: Buffer A consisted of 1 $\times$ PBS (pH 7.4). Buffer B contained 8 M urea, 50 mM Tris-HCl, and 300 mM NaCl (pH 8.0). Buffer C contained 8 M urea, 50 mM Tris-HCl, 300 mM NaCl, and 0.1% Triton X-100 (pH 8.0). For Ni-NTA affinity chromatography, the binding buffer was composed of 8 M urea, 50 mM Tris-HCl, and 300 mM NaCl (pH 8.0); the washing buffer contained 8 M urea, 50 mM Tris-HCl, 300 mM NaCl, and 20/50 mM imidazole (pH 8.0); and the elution buffer consisted of 8 M urea, 50 mM Tris-HCl, 300 mM NaCl, and 500 mM imidazole (pH 8.0). Luria Bertani (LB) medium was prepared by dissolving 1 g of peptone, 2 g of NaCl, and 2 g of yeast extract in 200 mL of ultrapure water. For LB agar, 3 g of agar powder was supplemented to 200 mL of LB medium. The medium was then autoclaved at 121 °C for 20 min before use. Terrific Broth (TB) medium was prepared by dissolving 10 g of peptone, 24 g of yeast extract, 16.4 g of dipotassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$), 2.31 g of potassium dihydrogen phosphate (KH_2PO_4), and 5 g of glycerol in 1000 mL of ultrapure water, followed by sterilization at 121 °C for 20 min. To prepare a 1 mol/L monosodium glutamate (MSG) solution, 147.13 g of L-Glu was added to approximately 800 mL of ultrapure water and partially dissolved using a magnetic stirrer. Subsequently, 40.00 g of solid NaOH was gradually added under continuous stirring until the solution became completely clear, and the final volume was adjusted to 1000 mL with ultrapure water for storage. For the GABA standard solution (1 mg/mL), 10 mg of GABA powder was accurately weighed, completely dissolved in ultrapure water via sonication, and adjusted to a final volume of 10 mL. A series of working standard solutions (100–1000 μ g/mL) was prepared through serial dilution and stored at room temperature. The 100 mmol/L PLP solution was prepared by dissolving 0.249 g of PLP (Pyridoxal phosphate) powder in ultrapure water with sonication, adjusted to a final volume of 10 mL, and stored at -20 °C. The primary equipment used in this study included an ultrasonic cell disruptor (Tianjin Jinli Instrument Equipment Technology Development Co., Ltd. (Tianjin, China)), a full-temperature shaking incubator (Changzhou Ruipin Precision Instrument Co., Ltd. (Changzhou, China)), a high-speed refrigerated centrifuge (Hunan Xiangyi Laboratory Instrument Development Co., Ltd. (Changsha, China)), an electronic balance (Sartorius, Göttingen, Germany), a clean bench (Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd. (Shanghai, China)), a thermostatic water bath (Guohua Electric Co., Ltd., Jiangsu, China), a low-temperature high-speed centrifuge (Eppendorf, Hamburg, Germany), an ultra-low temperature freezer (Thermo Fisher Scientific, Waltham, MA, USA), a high-pressure steam autoclave (ALP, Tokyo, Japan), and a High-Performance Liquid Chromatograph (HPLC, Agilent Technologies, Santa Clara, CA, USA).

2.3. Methods

2.3.1. Plasmid Transformation and Positive Clone Identification

The previously constructed and preserved recombinant plasmids *MoGADs*-pET-28a were transformed into *E. coli* Rosetta (DE3) competent cells. Positive clones were screened on agar plates containing kanamycin (50 μ g/mL) and chloramphenicol (34 μ g/mL). Single colonies were selected for colony PCR identification and further verified by double restriction enzyme digestion using *Sal* I and *Hind* III.

2.3.2. Induced Expression and Purification of Recombinant Protein

The recombinant plasmid *MoGADs*-pET-28a was transformed into *E. coli* Rosetta (DE3) competent cells via heat shock at 42 °C for 90 s. The cells were then plated on agar containing 30 µg/mL kanamycin and 34 µg/mL chloramphenicol and incubated at 37 °C. A single colony was inoculated into a liquid medium containing the corresponding antibiotics and cultured at 37 °C. When the OD₆₀₀ reached 0.6–0.8, the inducer IPTG was added to a final concentration of 0.5 mM. To optimize the expression, the cultures were incubated separately under two conditions: at 20 °C overnight, or at 37 °C for 6 h. Finally, the cells were harvested by centrifugation at 4000 rpm for 10 min, and the supernatant was discarded.

The harvested cell pellets were resuspended in Buffer A and fully lysed using an ultrasonic cell disruptor. Following centrifugation, the supernatant and pellet were separated. The pellet was redissolved in Buffer B. Both the supernatant and pellet fractions were processed and prepared for SDS-PAGE analysis to evaluate protein solubility. For large-scale expression, the bacterial culture was grown in a medium with appropriate antibiotics until the OD₆₀₀ reached 0.6–0.8. IPTG (0.5 mM) was added, and the culture was incubated overnight at 20 °C. The cell biomass was subsequently harvested by centrifugation.

For affinity purification, the entire protein purification process was carried out at 4 °C. The large-scale harvested cells were resuspended in Buffer C and disrupted by sonication. The lysate was centrifuged to collect the supernatant containing the crude protein. Purification was performed using a Ni-NTA agarose affinity chromatography column. Briefly, the Ni-NTA resin was packed into the column and washed with Binding buffer for equilibration. The crude protein extract was loaded onto the column and incubated with the equilibrated resin, followed by the collection of the flow-through. The column was washed sequentially with Binding buffer and Washing buffer, and the wash fractions were collected. The target protein was then eluted using Elution buffer, and the eluate was collected. Finally, the crude protein extract and all collected fractions were prepared for SDS-PAGE analysis to verify the purification efficiency.

2.3.3. Detection of Target Protein

SDS-PAGE Analysis: Protein samples were prepared and subjected to electrophoresis using a 5% stacking gel and a 12% resolving gel to evaluate their molecular weights.

Western Blot Verification: Protein samples were similarly prepared and resolved on a 5% stacking gel and a 12% resolving gel by electrophoresis. For immunodetection, a mouse anti-His tag antibody was used as the primary antibody, followed by a goat anti-mouse secondary antibody. To further confirm the identity of the purified target protein, signal visualization was performed using a TMB colorimetric detection kit in accordance with standard Western blot procedures.

2.3.4. High-Efficiency Biosynthesis of GABA Using Recombinant *E. coli*

The *E. coli* Rosetta (DE3) cells harboring the *MoGADs*-pET-28a recombinant plasmid, prepared and cultured as described in Section 2.3.2, were utilized for the following experiments.

Detailed Experimental Procedures

The whole-cell catalytic procedure was performed based on the method described by Li et al. [13–15] with slight modifications. The previously harvested bacterial cells were inoculated at a ratio of 2% (*v/v*) into a shaking flask containing 10 mL of LB medium. Kanamycin (stock solution 50 mg/mL) and chloramphenicol (stock solution 50 mg/mL) were added to the medium at a 1:1000 (*v/v*) ratio. The culture was incubated at 37 °C and 220 rpm for 12 h to prepare the seed culture. Subsequently, the seed culture was transferred

into a shaking flask containing 50 mL of TB medium at a 2% (*v/v*) inoculation ratio, with the same antibiotics added (1:1000, *v/v*). The culture was grown at 37 °C and 220 rpm until the OD₆₀₀ reached 0.6–0.8. At this point, IPTG (final concentration: 0.5 mM), the substrate MSG (final concentration: 6 g/L), and pyridoxal 5'-phosphate (PLP) (final concentration: 0.5 mM) were simultaneously supplemented into the broth. The bacteria were first induced at 16 °C and 200 rpm for 20 h, followed by a whole-cell catalytic conversion phase at 36 °C and 200 rpm for 24 h. For the blank control group, the substrate MSG was omitted, while all other conditions remained identical. After the bioconversion, a 100 µL aliquot of the reaction mixture was collected for GABA detection by HPLC.

Detection of the Product GABA by OPA-HPLC Method

The pre-column derivatization method using o-phthalaldehyde (OPA) for HPLC analysis to determine GABA concentration was adapted from Gu et al. [16–18] with minor modifications. Briefly, a 600 µL aliquot of 0.4 mol/L borate buffer (prepared by accurately weighing 2.47 g of boric acid, dissolving it in 100 mL of ultrapure water, and adjusting the pH to 9.5 with NaOH) was blended with 120 µL of the standard or sample solution. Then, 120 µL of OPA derivatization reagent was added. For the OPA reagent: 20 mg of OPA powder was accurately weighed, dissolved in 5 mL of acetonitrile via sonication, and homogeneously mixed with 40 µL of β-mercaptoethanol. Due to the susceptibility of OPA to degradation, all manipulation steps for this reagent were strictly protected from light. The mixture was vortexed and incubated for 2 min at room temperature in the dark. Finally, 10 µL of the derivative was injected into the HPLC system.

Chromatographic conditions: Separation was performed on a Merck RP C18 column (4.6 mm × 250 mm, 5 µm). The mobile phase consisted of acetonitrile and a 20 mmol/L sodium acetate buffer at a volume ratio of 23:77. The sodium acetate buffer was prepared by dissolving 1.64 g of anhydrous sodium acetate in 1000 mL of ultrapure water, adding 200 µL of triethylamine, and adjusting the pH to 7.3 with glacial acetic acid. The detection wavelength was set at 333 nm, the flow rate was 0.9 mL/min, the column temperature was maintained at 30 °C, and the run time per injection was 20 min.

Preparation of GABA Standard Curve

To construct the standard curve, a series of GABA working standard solutions with concentrations of 100, 200, 500, 800, and 1000 µg/mL were prepared by diluting the 1 mg/mL GABA stock solution. These standards were derivatized according to the procedure described in Section 2.3.4 Detection of the Product GABA by OPA-HPLC Method and subsequently analyzed by HPLC under the specified chromatographic conditions.

2.3.5. Bioinformatics Analysis of MoGADs Gene

Phylogenetic Tree Analysis of MoGADs Genes

Ten plant-derived GAD gene sequences were screened from the NCBI BLAST website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 25 May 2026), and a phylogenetic tree of *MoGAD1* and *MoGAD2* genes was constructed using MEGA 6.0 with the Neighbor-Joining method.

Molecular Docking Analysis of MoGADs Protein

The molecular docking program AutoDock Vina (Version 1.2.3) [19,20] was used. This program employs the “Iterated Local Search” algorithm, which continuously performs local searches and repeated iterations to find the optimal molecular docking conformation, selecting the solution with the lowest binding energy as the final result. First, AutoDock Tools software (Version 1.5.7) was used to preprocess the compounds and proteins, select appropriate docking sites, and export configuration files for docking with AutoDock Vina.

Next, AutoDock Vina was used to continuously perform local searches and repeated iterations to identify the best molecular docking conformation. Finally, PyMOL software (Version 3.0.3) [21] was used for visualization and analysis of the three-dimensional complex structures, while LigPlot⁺ was used to identify and display two-dimensional interactions [22]. PyMOL primarily identifies and visualizes hydrogen bonds and conformations in three-dimensional space based on geometric algorithms, whereas LigPlot⁺ generates two-dimensional interaction diagrams using built-in geometric and statistical rules. The two methods complement each other in terms of presentation. The two-dimensional structure of the ligand was drawn using ChemDraw 20.0 and then converted to a three-dimensional structure using Chem3D 20.0.

All experiments were performed in triplicate, and the results are expressed as the mean $\pm$ standard deviation (SD). The figures display representative data from these replicates. The GABA content in the samples was calculated using the regression equation derived from the standard curve.

3. Results and Analysis

3.1. Plasmid Transformation and Identification

The agarose gel electrophoresis results of the intact *MoGAD1*-pET-28a and *MoGAD2*-pET-28a recombinant plasmids are shown in Figure 1a and 1b, respectively. Following double restriction enzyme digestion with *Sal I* and *Hind III*, the *MoGAD1*-pET-28a and *MoGAD2*-pET-28a plasmids yielded the expected target fragments of 1505 bp (*MoGAD1*) and 953 bp (*MoGAD2*), respectively, along with the 5369 bp backbone fragment of the pET-28a vector. These results confirmed the successful integration of the target genes into the pET-28a expression vector, laying the foundation for subsequent heterologous expression.

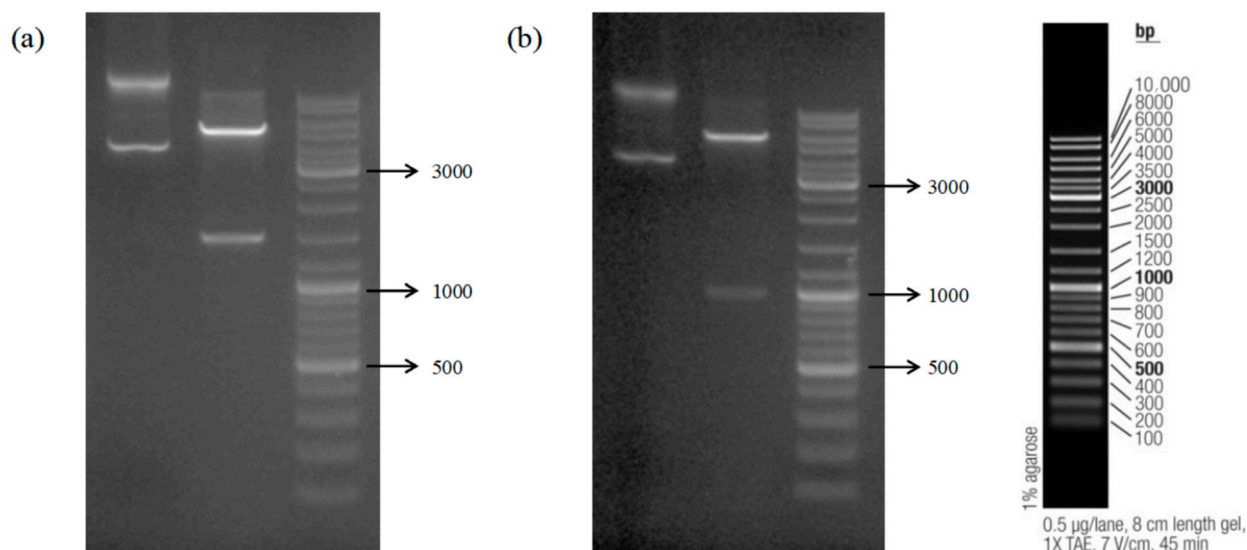


Figure 1. (a) Agarose gel electrophoresis of the *MoGAD1*-pET-28a recombinant plasmid. Lane 1: Undigested plasmid; Lane 2: Double digestion with *Sal I* and *Hind III* (Expected sizes: 1505 + 5369 bp); Lane 3: DNA marker. (b) Agarose gel electrophoresis of the *MoGAD2*-pET-28a recombinant plasmid. Lane 1: Undigested plasmid; Lane 2: Double digestion with *Sal I* and *Hind III* (Expected sizes: 953 + 5369 bp); Lane 3: DNA marker.

3.2. Protein Expression

The SDS-PAGE analysis results of *MoGAD1* and *MoGAD2* protein expression are shown in Figure 2. Both *MoGAD1* and *MoGAD2* were successfully heterologously expressed in *E. coli*, but differences in solubility were observed between the two. SDS-PAGE

analysis revealed that, compared with the total protein before induction (lane 1), the induced samples (lanes 2 and 5) showed significantly intensified specific bands at the expected molecular weights, indicating that both genes were effectively expressed in *E. coli* Rosetta (DE3). Both MoGAD1 and MoGAD2 exhibited soluble expression at both induction temperatures (20 °C and 37 °C). However, compared with MoGAD1, MoGAD2 showed relatively more soluble protein in the supernatant fraction under low-temperature (20 °C) induction conditions, displaying a certain trend toward improved solubility. This finding provides a basis for subsequent protein purification strategies.

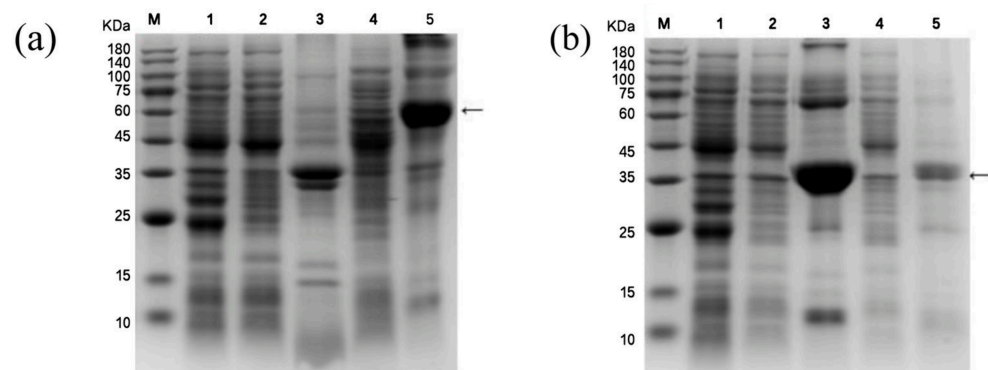


Figure 2. SDS-PAGE analysis of the expression of (a) MoGAD1 and (b) MoGAD2 fusion proteins. Lane M: Protein marker; Lane 1: Pre-induction total protein; Lane 2: Supernatant fraction (induced at 20 °C); Lane 3: Pellet fraction (induced at 20 °C); Lane 4: Supernatant fraction (induced at 37 °C); Lane 5: Pellet fraction (induced at 37 °C). The arrows indicate the positions of the target proteins.

3.3. Protein Purification

The SDS-PAGE analysis of the MoGAD1 and MoGAD2 proteins following nickel-affinity purification is presented in Figure 3a,b. As observed in the gels, the flow-through fraction (Lane 2) contained almost no target protein band, indicating that the His-tagged fusion proteins bound tightly to the Ni-NTA resin with high adsorption efficiency. The 20 mM (Lane 3) and 50 mM (Lane 4) imidazole wash fractions removed some impurity proteins, while the target protein bands were extremely faint or nearly invisible. This indicated that the washing gradient was reasonably established, effectively eliminating non-specifically bound impurities while minimizing the loss of the target protein. The 500 mM imidazole elution fraction (Lane 5) displayed a single, distinct target protein band, demonstrating that impurities were essentially removed. Based on the estimation by gel image analysis software, the purities of both purified MoGAD1 and MoGAD2 exceeded 90%, and approximately 3 mg of recombinant protein was finally obtained from a 400 mL culture system. Western blot analysis was performed using a mouse anti-His primary antibody and a goat anti-mouse secondary antibody, combined with TMB colorimetric detection. As shown in Figure 3e,f, specific positive signals were observed at the expected molecular weights, further confirming that the purified products were the target His-tagged fusion proteins.

3.4. Detection of the Product GABA

Through HPLC analysis, whole-cell biocatalysis experiments confirmed for the first time at the functional level that both MoGAD1 and MoGAD2 possess glutamate decarboxylase activity, as they are capable of catalyzing the decarboxylation of MSG to generate GABA. The GABA standard curve is presented in Figure 4. Under the established o-phthalaldehyde (OPA) pre-column derivatization chromatographic conditions, the retention time of the GABA standard was approximately 8.41 min (Figure 5a). In the catalytic reaction mixtures

of recombinant *E. coli* expressing MoGAD1 (Figure 5b) and MoGAD2 (Figure 5c), distinct chromatographic peaks were detected at the exact retention time of the GABA standard, confirming the generation of GABA in the reaction system. Conversely, the blank control group lacking the substrate MSG (Figure 5d) did not exhibit the characteristic GABA peak at the corresponding retention time. This excluded the possibility of interference from the endogenous GABA metabolism of the host strain, thereby verifying that the detected GABA was indeed the product of *L*-Glu decarboxylation catalyzed by the MoGADs.

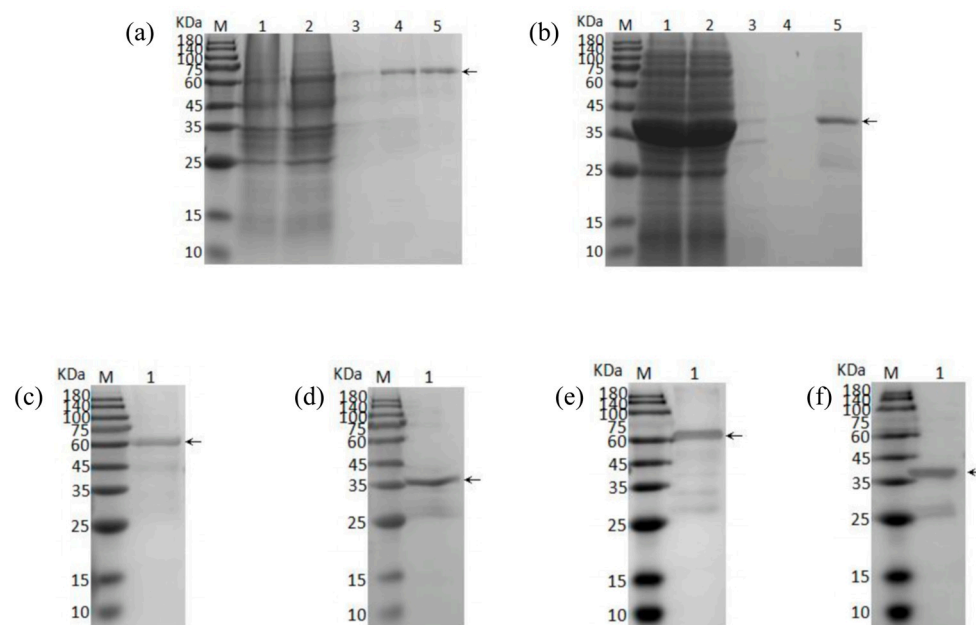


Figure 3. SDS-PAGE analysis of the purification of (a) MoGAD1 and (b) MoGAD2 fusion proteins by nickel-agarose affinity chromatography: Lane M: Protein marker; Lane 1: Load; Lane 2: Flow-through; Lane 3: 20 mM imidazole fraction; Lane 4: 50 mM imidazole fraction; Lane 5: 500 mM imidazole fraction. SDS-PAGE analysis of the final purified (c) MoGAD1 and (d) MoGAD2 protein: Lane M: Protein marker; Lane 1: the target fusion protein. Western blot analysis of the final purified (e) MoGAD1 and (f) MoGAD2 protein. Lane M: protein marker; Lane 1: purified His-tagged MoGAD1 detected with anti-His antibody. The arrows indicate the positions of the target proteins.

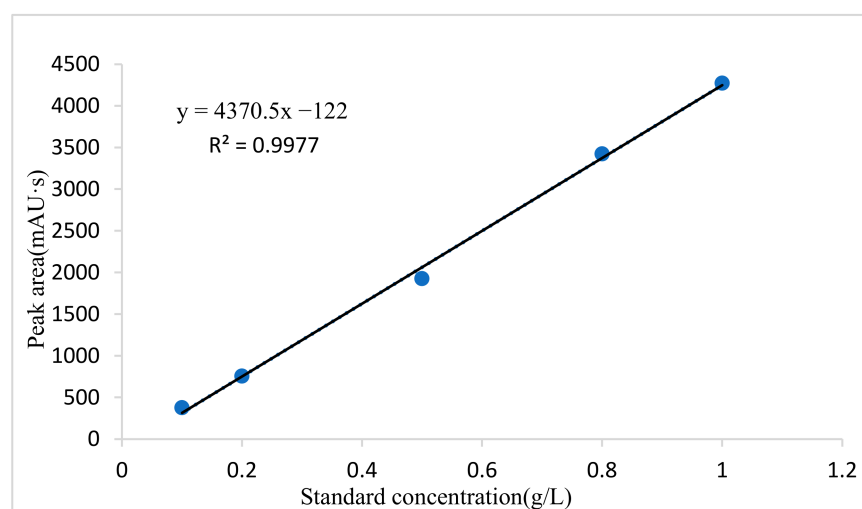


Figure 4. GABA standard curve.

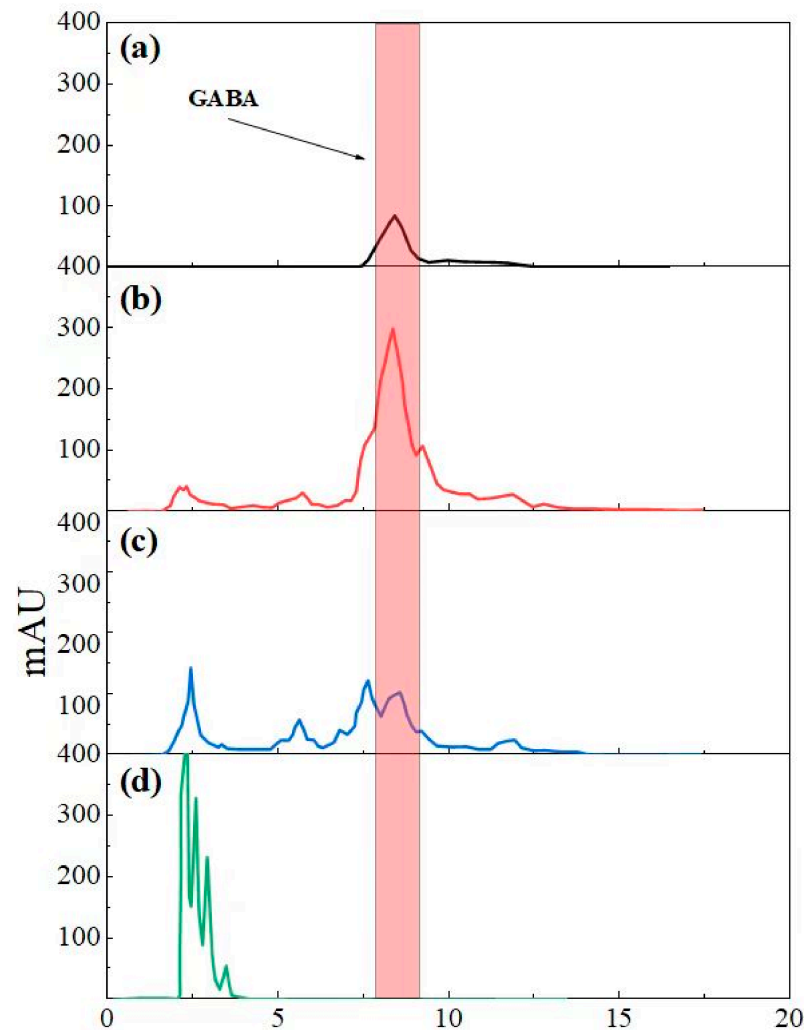


Figure 5. HPLC chromatograms for GABA detection. (a) GABA standard; (b) Reaction sample catalyzed by MoGAD1; (c) Reaction sample catalyzed by MoGAD2; (d) Blank control.

Based on the analysis of the experimental results and the GABA standard curve (Figure 4, linear regression equation: $y = 4370.5x - 122$, $R^2 = 0.9977$, linear range: 100–1000 $\mu\text{g/mL}$), the sample peak areas were substituted into the regression equation. The GABA yield in the MoGAD1 catalytic reaction was calculated to be 3.67 ± 0.1833 g/L, while that of MoGAD2 was 0.648 ± 0.002 g/L. Using the initially supplemented 6 g/L MSG as the substrate, the direct mass conversion rates were 61.2% and 10.8%, respectively, indicating that the GABA yield of MoGAD1 was approximately 5.66 times that of MoGAD2. Notably, although the SDS-PAGE results showed that both proteins were predominantly expressed in the insoluble form (pellet fraction), considerable GABA yields were still detected in the whole-cell catalytic system. This indicates that a certain amount of soluble, fully active protein exists within the cells to participate in the catalytic reaction. The aforementioned yields and conversion rates were obtained under initial, unoptimized conditions. By subsequently optimizing critical parameters—such as substrate concentration, cofactor (PLP) concentration, induction and biotransformation temperatures, pH, and reaction time—both GABA yield and conversion rate are expected to be further enhanced.

3.5. Molecular Informatics Analysis of MoGADs

3.5.1. Phylogenetic Tree Analysis of MoGADs

Through BLAST alignment (Online NCBI BLAST version 2.17.0) and phylogenetic analysis of the two target DNA sequences, it was found that these sequences share high

homology with the GAD gene sequences of *Hevea brasiliensis*, the *Juglans regia* complex population, and *Carya illinoensis*. As shown in Figure 6, The phylogenetic tree shows that the target sequences cluster into the same group as the GAD genes of the aforementioned plants, indicating a close genetic relationship. Therefore, it is speculated that the two target DNA sequences are plant GAD genes or their partially homologous fragments. This gene encodes glutamate decarboxylase, which may be involved in the biosynthesis of GABA in plants.

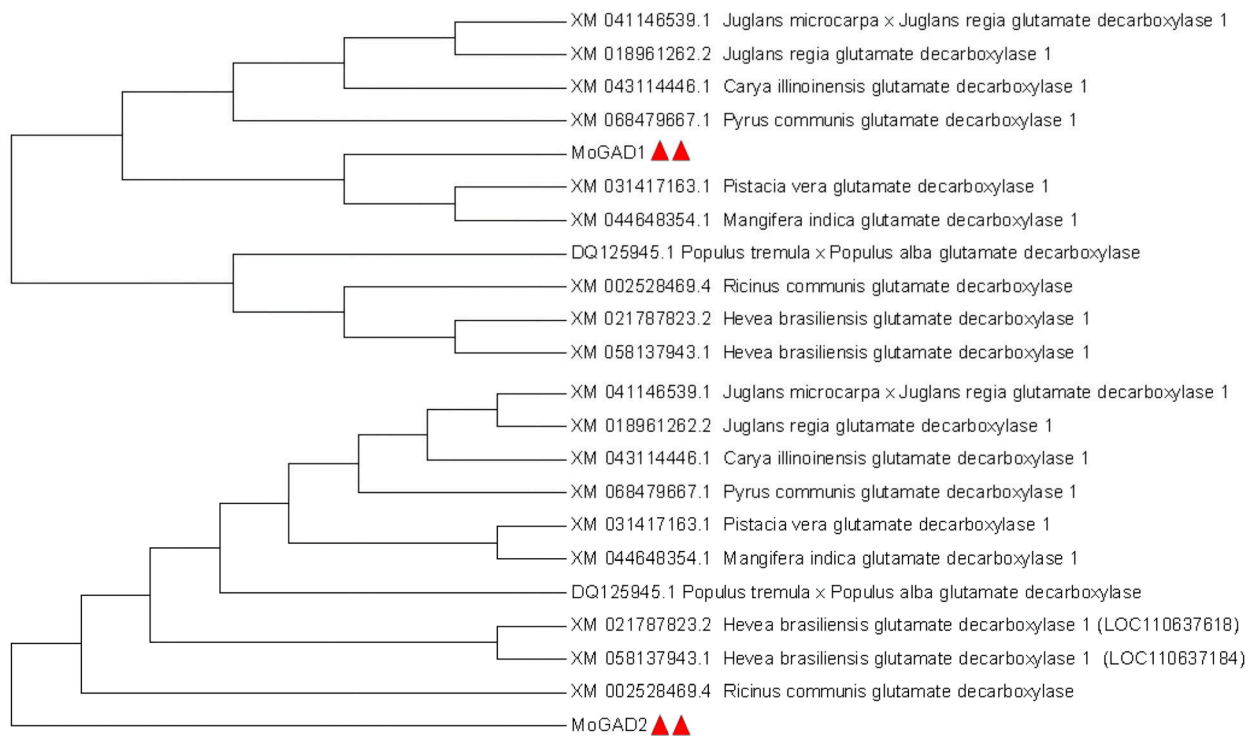


Figure 6. Phylogenetic analysis of MoGAD genes.

3.5.2. Molecular Docking Analysis of MoGADs

The molecular docking results of MoGADs with *L*-Glu and PLP are shown in Figure 7 and Table 1. It is generally accepted [20,23] that a docking energy value less than -4.25 kcal/mol indicates certain binding activity between the two, less than -5.0 kcal/mol indicates good binding activity, and less than -7.0 kcal/mol indicates strong binding activity. Molecular docking results showed that stable conformations could be formed in the binding pockets for MoGAD1 with PLP, MoGAD1 with *L*-Glu, MoGAD2 with PLP, and MoGAD2 with *L*-Glu, although their binding affinities exhibited some differences. For MoGAD1, the optimal docking binding energy with PLP was -5.489 kcal/mol, significantly lower than that with *L*-Glu (-4.207 kcal/mol). Specifically, PLP formed five hydrogen bonds with ASN139, ALA159, GLY161, and PHE355 of MoGAD1, whereas *L*-Glu formed only one hydrogen bond with ASP279. This indicates that MoGAD1 binds more stably and with higher affinity to PLP, while its binding to *L*-Glu is relatively weaker. For MoGAD2, the optimal docking binding energy with PLP was -5.297 kcal/mol, also better than that with *L*-Glu (-4.49 kcal/mol). PLP formed four hydrogen bonds with TYR61, THR110, TRP112, and GLN113 of MoGAD2; *L*-Glu formed four hydrogen bonds with TRP112, GLN113, SER123, and SER125. Although MoGAD2 showed certain binding activity to both ligands, the binding energy with PLP was lower, suggesting that binding to PLP is more favorable. Comprehensive analysis shows that both target proteins have stronger binding ability to PLP than to *L*-Glu. According to common molecular docking evalua-

tion criteria, the binding energies of MoGAD1-PLP and MoGAD2-PLP are both less than -5.0 kcal/mol, indicating good binding activity; the binding energy of MoGAD2-*L*-Glu is -4.49 kcal/mol, suggesting certain binding activity; the binding energy of MoGAD1-*L*-Glu is -4.207 kcal/mol.

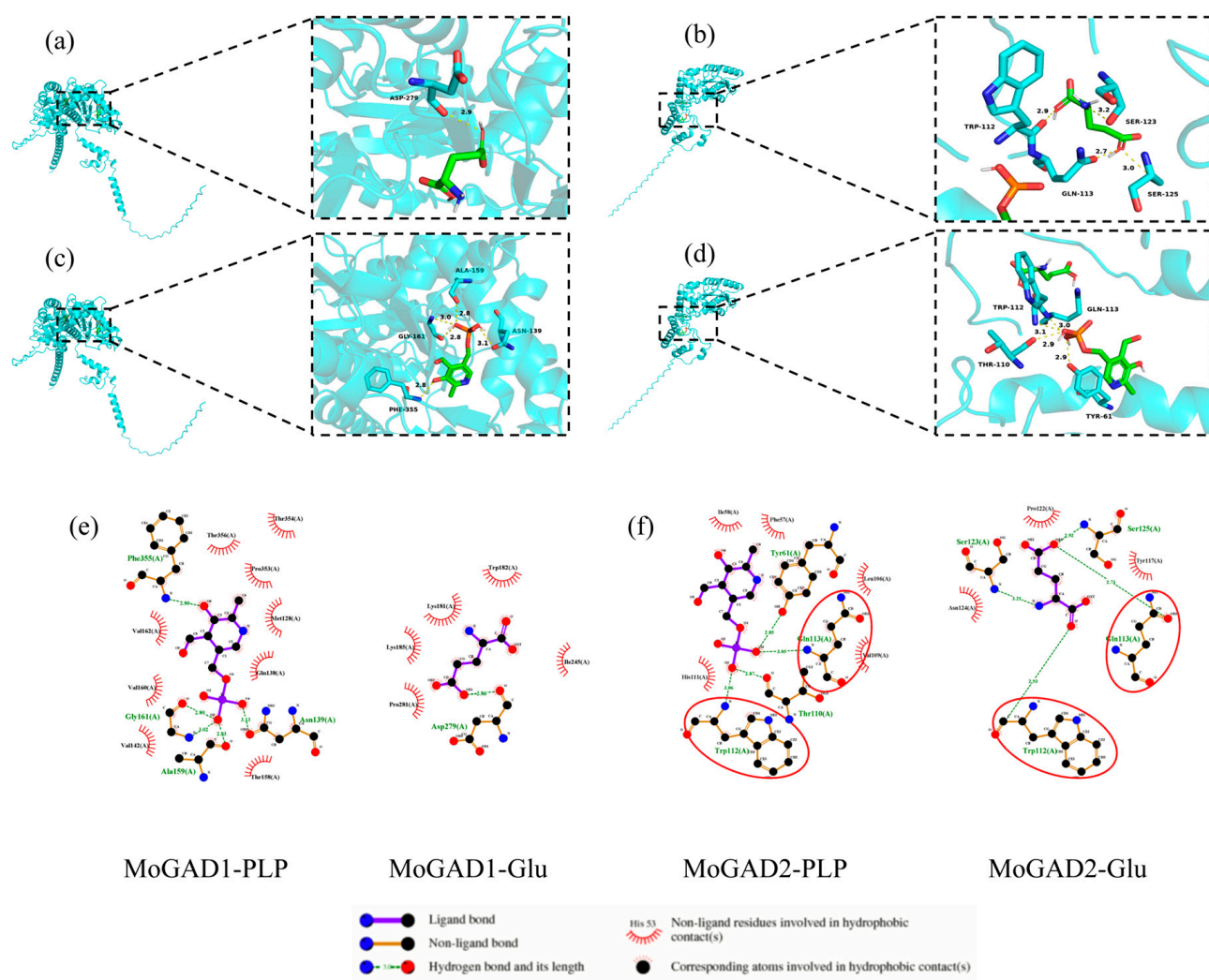


Figure 7. (a) Molecular docking of MoGAD1 with *L*-Glu; (b) Molecular docking of MoGAD2 with *L*-Glu; (c) Molecular docking of MoGAD1 with PLP; (d) Molecular docking of MoGAD2 with PLP; (e) Two-dimensional LigPlot interaction diagram of MoGAD1 with *L*-Glu and PLP; (f) Two-dimensional LigPlot interaction diagram of MoGAD2 with *L*-Glu and PLP.

Table 1. MoGAD protein molecular docking analysis.

Ligand	Receptor Protein	Binding Affinity (kcal/mol)	Hydrogen Bond Number	Binding Site
<i>L</i> -Glu	MoGAD1	-4.207	1	ASP279
<i>L</i> -Glu	MoGAD2	-4.49	4	TRP112, GLN113, SER123, SER125
PLP	MoGAD1	-5.489	5	ASN139, ALA159, GLY161, PHE355
PLP	MoGAD2	-5.297	4	TYR61, THR110, TRP112, GLN113

4. Discussion

MoGAD1 and MoGAD2 proteins exhibited detectable soluble fractions when expressed under induction at both 20 °C and 37 °C. In the future, the soluble expression of MoGADs could be improved, and the yield of active proteins increased, by optimizing

induction conditions (e.g., prolonged induction at low temperatures, co-expression with molecular chaperones) or by selecting more suitable expression hosts (e.g., *Bacillus* or yeast systems).

Compared with previous reports on whole-cell GABA biosynthesis, the yields obtained in this study (0.648–3.67 g/L) are relatively modest. For instance, Sun et al. achieved a high molar conversion rate of 98.6% using a recombinant *E. coli* system expressing a *Bacillus*-derived GAD [24]; Wen and Bao reported a GABA titer of 77.6 g/L through secretory expression in *Corynebacterium glutamicum* [25]; Yang et al. integrated enzyme evolution with metabolic engineering to reach 307.5 g/L GABA with a productivity of 61.49 g/L/h in a 5 L bioreactor [26]; and Chang et al. further increased the productivity to 117.8 g/L/h by enhancing cell permeability and constructing a PLP self-sufficiency system [27]. The lower yields in our study may be attributed to several factors [28–30]: (i) the soluble expression level of MoGAD proteins was relatively low, resulting in insufficient active enzyme available for catalysis; (ii) the transmembrane transport of the substrate MSG may serve as a rate-limiting step in the whole-cell system; and (iii) the reaction conditions (temperature, pH, PLP concentration, and induction time) still require systematic optimization.

5. Conclusions

In summary, this study provides the first direct experimental evidence that glutamate decarboxylases derived from *Moringa oleifera* (MoGAD1 and MoGAD2) catalyze the decarboxylation of MSG to generate GABA. This fills a critical gap in the functional validation of key enzyme genes within the GABA biosynthetic pathway of *Moringa*. It confirms that both MoGAD1 and MoGAD2 are functional genes responsible for GABA accumulation in vivo. Furthermore, this work lays an experimental foundation and provides genetic resources for subsequent in-depth research, including the characterization of MoGAD1 and MoGAD2 enzymatic properties, protein engineering to enhance solubility and catalytic efficiency, systematic optimization of whole-cell catalysis conditions, and the construction of highly efficient GABA-synthesizing engineered strains. Ultimately, it provides a theoretical basis and technical support for the green biomanufacturing and high-value exploitation of GABA, a functional component of *Moringa*.

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Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request. The data are not publicly available due to institutional privacy and institutional data protection regulations.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

GABA (γ -aminobutyric acid); L-Glu (L-glutamate); MSG (monosodium glutamate); SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis); OPA (o-phthalaldehyde); PLP (Pyridoxal phosphate).

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