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Cloning, expression, and functional analysis of lysine decarboxylase in mulberry (*Morus alba* L.)

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ABSTRACT

1-Deoxynojirimycin (DNJ) is the main bioactive compound of *Morus alba* L.. DNJ has pharmacological effects, including blood sugar level regulation and antiviral activity. In this study, the mulberry lysine decarboxylase gene (*MaLDC*), which is involved in the biosynthesis of DNJ alkaloids, was cloned, expressed, and functionally verified. *MaLDC* was induced and expressed in *Escherichia coli* BL21 (DE3). The recombinant soluble MaLDC protein had a relative molecular mass of 24.0 kDa. The protein was purified by Ni-NTA separation. The results showed that MaLDC protein could catalyze lysine decarboxylation to produce cadaverine. The K_m and V_{max} values were 19.2 μM and 3.31 μM/min, respectively. Quantitative real-time reverse transcription polymerase chain reaction revealed that *MaLDC* expression was positively correlated with DNJ content ($P < 0.001$), indicating that the *MaLDC* could encode a functional protein involved in the biosynthesis of DNJ alkaloid in mulberry. Our results provided a foundation for further studies of the enzymatic properties of LDC and established a basis for the analysis of key enzymes involved in the biosynthetic pathway of mulberry DNJ alkaloid.

1. Introduction

Dried leaves of the mulberry plant (*Morus alba* L.) are commonly used as a traditional Chinese medicine for the treatment of various diseases [1]. Mulberries are also an important food source [2]. Mulberry leaves were first recorded as a medicine in the book “Shen Nong's Herbal Classic, the Compendium of Materia Medica” (Ming Dynasty, 16 t h century) for the treatment of diabetes. The Pharmacopoeia of the People's Republic of China states that the collecting requirement of mulberry leaves was after frost, and that mulberry is sweet and bitter in flavor and cold in property. Furthermore, it states that mulberry has several beneficial functions, including effect on the lung and liver channels, expelling pathogenic wind-heat, clearing away lung heat and moisturization, and clearing up heat from the liver to improve acuity vision [3]. Multiple active ingredients were found in mulberry leaves, such as alkaloids, flavonoids and terpenoids. Some studies have shown that mulberry leaves contain polyhydroxylated alkaloid 1-deoxynojirimycin (DNJ), the major compound facilitating the reduction of blood glucose [4].

DNJ has inhibitory effects on intestinal α-glucosidase, blocks the absorption of postprandial intestinal glucose, regulates hepatic glucose metabolism enzyme activity, and promote glucose utilization, moreover, DNJ can enhance insulin sensitivity and it plays a significant role in reducing blood glucose [5]. DNJ reduced fat accumulation by altering the activity of fatty acid metabolizing enzymes and stimulates glucose transporter expressions in the rat liver [6]. DNJ also reduced the risk factors of atherosclerosis through enhancing cholesterol efflux and attenuating high glucose-accelerated senescence in human umbilical vein endothelial cells [7,8]. DNJ exhibits antiviral effects against human immunodeficiency virus by inhibiting glycoprotein processing [9]. Owing to its extensive pharmacological effects, DNJ has attracted a great deal of attention from scholars worldwide. However, the content of DNJ in mulberry leaves is low, and it is difficult to obtain DNJ in large quantities using traditional separation methods. Researchers use a combination of chemical synthesis and microbial transformation to obtain DNJ alkaloids [10], however, some isomers formed during the preparation process make the separation difficult, and the resultant pharmacological activities are different from those of natural DNJ. Synthetic biology techniques have been applied to the development of natural products and can maximize the yield of the target product, this approach has been used in artemisinin [11], paclitaxel [12], and tanshinone [13]. This technology provides a novel method for obtaining large amounts of natural active ingredients. Therefore, synthetic biology techniques may have applications in the production of DNJ.

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DNJ alkaloids are lysine-derived polyhydroxy piperidine alkaloids [14], natural sugar analogs formed by replacing the oxygen atom on the pyran ring by an NH group. Its synthesis in plants begins with lysine decarboxylation by lysine decarboxylase (LDC) to form cadaverine. Subsequently, cadaverine generates Δ^1-piperideine, which is catalyzed by primary amine oxidase, and then a series of enzymatic reactions result in the generation of DNJ alkaloids [15]. LDC plays an important role as the first key enzyme in alkaloid biosynthesis [16]. Some scholars have cloned and obtained *LDC* genes from many types of plants, allowing them to analyze the activity and function of LDC enzymes. Moreover, in a previous study, two LDC genes from Huperzia serrata were cloned, and function verification results showed that the enzyme could catalyze the generation of cadaverine from lysine [17]. Additionally, Bunsupaet al. [18] transferred the *LDC* into the model plants *Arabidopsis* and tobacco, and the accumulation of cadaverine was significantly altered. Yang et al. [19] studied on *LDC* expression and matrine and oxymatrine contentsin *Sophora alopecuroides* and found that changes in *LDC* expression and in the contents of the two alkaloids were consistent. However, to our knowledge, no studies have examined *LDC* in mulberry leaves.

Therefore, in this study, the mulberry lysine decarboxylase gene (*MaLDC*) from mulberry leaves was cloned, expressed, and functionally analyzed by reverse transcription polymerase chain reaction (RT-PCR). The relationships between *MaLDC* expression levels and mulberry leaf DNJ content were also studied. Our results provide a foundation for further studies on the enzymatic properties of LDC and establish a basis for the analysis of key enzymes involved in the biosynthetic pathway of mulberry DNJ alkaloids.

2. Materials and methods

2.1. Materials

Mulberry top leaves were collected from mulberry plantations in Jiangsu University (Zhenjiang, China), immediately placed into liquid nitrogen, and then transferred to −80 °C freezers. Mulberry leaves were collected from the same location for DNJ content determination. 1, 5-Diaminopentane was purchased from Sigma (St. Louis, MO, USA).

2.2. Total RNA extraction and cDNA synthesis

Mulberry leaves were quickly ground into powder in liquid nitrogen. Total RNA was extracted from mulberry leaves using TRIzol reagent (Sangon Biotech, Shanghai, China). Total RNA was detected with 1% agarose gel electrophoresis, and the purity and concentration were detected using a Biospec-mini nucleic acid protein detector (Shimadzu, Japan). A Revert Aid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to generate cDNA for subsequent experiments.

2.3. MaLDC cloning and sequencing

The mulberry transcriptome data (SRA accession: SRP127713) was obtained by our previous work, one *LDC* sequence was used as a template. Primer Premier 5.0 software was used to design primers LDC-F and LDC-R (Table 1) for the PCR amplification. The PCR system (Sangon Biotech, shanghai, China) comprised the following reaction mixture (25 μL total volume): 2.5 μL GreenBuffer, 0.25 μL of 10 μM dNTPs, 0.5 μL of each primers (LDC-F and LDC-R), and nuclease-free water to 25 μL. The reaction conditions were pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 49 °C for 30 s, extension at 72 °C for 90 s, and extension at 72 °C for 10 min for 35 cycles.

A gel recovery kit (Sangon Biotech) was used to recover the PCR products, and a Biospec-mini nucleic acid protein detector was used to measure the concentration. The purified DNA was ligated with the cloning vector pMD18-T and transformed into *Escherichia coli* DH5-α competent cells (TiangenBiotech Co., Ltd.) using ampicillin resistance and blue-white screening. The universal primer M13 (Table 1) was used for bacterial PCR validation, and qualified bacteria were sent to Sangon Biotech for sequencing.

2.4. Bioinformatics analysis of MaLDC

Conserved regions of the sequences were analyzed using specialized searches (CDD-search) in BLAST on the NCBI website (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree was constructed using MEGA 6.0. The neighbor joining method was used to map the confidence of each branch with the bootstrap method. The number of cycles was set at 1000. ProtParam online software (http://web.expasy.org/protparam/) was used to analyze the physicochemical properties of the protein, and three-dimensionalstructure prediction was carried out using SWISS-MODEL (http://swissmodel.expasy.org/).

2.5. MaLDC prokaryotic expression and purification

A pair of primers (LDC-eF and LDC-eR) containing restriction enzyme sites were designed according to the *MaLDC* sequence (Table 1). BamH I and Sal I restriction enzyme recognition sites (TaKaRa, Japan) were introduced into the 5′ ends of the primers. The purified PCR products and pET28a (+) vector were double-digested with BamH I and Sal I restriction enzymes. The double digested products were ligated overnight at 4 °C using T4 DNA ligase (TaKaRa, Japan) to construct the recombinant plasmid pET-28a (+)/*MaLDC*. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) by the heat shock method. The common primers T7 (Table 1) were used for bacterial PCR verification. Successfully identified strains were inoculated in Luria-Bertani medium, treated with isopropyl β-D-1-thiogalactopyranoside (IPTG, concentration of 1 mM), and cultured at 30 °C for 12 h. Prior to sonicating, phenylmethylsulfonyl fluoride was added to a final concentration of 100 μg/mL, and the cells were then sonicated as follows: 400 W ultrasonic power for 5 s and stop for 5 s. Analysis of proteins was the performed via sodium dodecyl sulfate polyacrylamide gel electrophoresis on 12% gels. A chelated high-flow agarose preparative column (Ni-NTA) was used to purify the protein, the particle size of the purification column was 50–100 μL, the yield of this purified material was 40 mg per gram of wet weight cell pellet, and the MaLDC protein was then eluted with 50 mM imidazole buffer. The eluate containing the MaLDC protein was transferred to a dialysis bag (molecular weight cutoff: 17 kDa) containing 10 mM phosphate-buffered saline as a

Table 1
Primers used in this study.

Primer name	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
MaLDC	TGCAGGTCGACGATTCGG	ATTGTGAGCGGATAACAA
MaLDC-e	CGCGGATCCATGGAGATAGAGAGTGAAATG	GCGTCGACGCTTCATTTCACTCTCTATCTCC
MaLDC-rt	AAGTTCGTGATGGTGGTTGC	AAGCTGAGACCACGTGATCA
β-actin	CATTGTCTTGTCTGGTT	TCATCATACTCCGACTTTGC
M13	CGCCAGGGTTTTCCCAGTCACGAC	CACACAGGAAACAGCTATGAC
T7	TAATACGACTCACTATAGGG	TGCTAGTTATTGCTCAGCGG