Orginal Article

J Babol Univ Med Sci
Vol 18, Issu 6; Jun 2016. P:66-72

Cloning and Expression Vector Construction of Glutamate Decarboxylase Gene from Lactobacillus Plantarum

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J Babol Univ Med Sci; 18(6); Jun 2016; PP: 66-72
Received: Mar 2th 2016, Revised: Nov 6th 2015, Accepted: Jan 6th 2016.

ABSTRACT

BACKGROUND AND OBJECTIVE: Gamma-aminobutyric acid (GABA) is a four-carbon non-protein amino acid used in the treatment of hypertension, diabetes, inflammation, and depression. GABA is synthesized by glutamic acid decarboxylase (GAD) enzyme in many organisms, including bacteria. Therefore, cloning of this enzyme is essential to the optimization of GABA production. This study aimed to clone and construct the expression vector of GAD gene from *Lactobacillus plantarum* PTCC 1058 bacterium.

METHODS: In this experimental study, we investigated the morphological, biochemical, genetic and 16s rDNA sequencing of *L. plantarum* PTCC 1058 strain. Genomic DNA of the bacterium was isolated and amplified using the GAD gene via polymerase chain reaction (PCR). Afterwards, the gene was inserted into the pJET1.2/blunt cloning vector and subcloned in vector pET32a. Plasmid pET32a-gad expression vector was transformed in Escherichia coli BL21 strain, and protein expression was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

FINDINGS: Morphological, biochemical and genetic analyses of 16s rDNA sequencing indicated that the studied substrain was of the L. plantarum strain. In addition, results of nucleotide sequencing of the fragmented segment via PCR showed the presence of GAD gene. Results of colony PCR and SDS-PAGE analysis confirmed the accuracy of the cloning and gene expression of the recombinant Escherichia coli BL21 strain.

CONCLUSION: According to the results of this study, cloning of GAD gene from *L. plantarum* PTCC 1058 was successful. These cloned genes could grow rapidly in prokaryotic and eukaryotic systems and be used in cost-effective culture media and even non-recyclable waste.

KEY WORDS: *Cloning, Gamma-aminobutyric acid, Glutamate acid decarboxylase, Lactobacillus plantarum.*

Please cite this article as follows:

Arabpour B, Esmaeili A, Rabbani M. Cloning and Expression Vector Construction of Glutamate Decarboxylase Gene from Lactobacillus Plantarum. J Babol Univ Med Sci. 2016;18(6):66-72.

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[DOR: 20.1001.1.15614107.1395.18.6.10.4]
[DOI: 10.22088/jbums.18.6.66]

Introduction

Gamma-aminobutyric acid (GABA) is a four-carbon amino acid, which is not found in the protein structure (1). GABA is considered an inhibitory neurotransmitter in the central nervous system and peripheral tissues of a large number of organisms (2-8). Critical functions of GABA include blood pressure reduction (6, 8-10), diuretic activities (1, 11), and sedation. Furthermore, it is essential to the treatment of insomnia and depression (12), autoimmune diseases (13), chronic diseases associated with alcohol consumption (14), and inflammation (15, 16). Other studies have denoted the role of GABA in the stimulation of immune cells (17) and diabetes control (18, 19), anti-tumor activities (20), release of free amino acids, improvement of mental health (21), and treatment of neurological disorders, such as epilepsy, Parkinson's disease, schizophrenia, spasticity and Alzheimer's disease (22).

As such, therapeutic functions of GABA have been highlighted in the medicine and food industry (23). In the nature, GABA could be found in a wide range of microorganisms (e.g., bacteria, fungi, and yeasts), plants, and animals. Glutamic acid decarboxylase (GAD) is the catalyzing enzyme in the irreversible reaction of L-glutamate decarboxylation for the production of GABA, which is dependent on pyridoxal phosphate (1). GAD is an intracellular enzyme in lactic acid bacteria (4, 24, 25), which is induced in response to acid stress (26). Differences in the primary structure of this enzyme might affect its ability to produce GABA (24). One of the main purposes of GAD gene cloning is to prepare or access the recombinant bacteria, which could be used in cost-effective culture media and are able to enhance GABA production.

To date, full-length GAD gene has been cloned and sequenced from *Lactobacillus paracasei* NFRI 7415 (24), *Lactobacillus brevis* OPK-3 (27), *Lactobacillus brevis* IFO-12005 (9), and *Lactococcus lactis* 01-7 (28). Moreover, the main components of gadB gene have been cloned and sequenced from *Lactobacillus paracasei* PF6 and *Lactobacillus delbrueckii* subspecies *bulgaricus PR1*, *Lactobacillus lactis* PU1, and *Lactobacillus plantarum* C48 (11). In addition, GAD gene of *Lactobacillus brevis* OPK-3 (27) has been successfully expressed in *Escherichia coli* and *Bacillus subtilis* bacteria (29).

Review of domestic studies shows that GABA is not produced in Iran and is mainly imported from other countries. Gabapentin is an analog therapeutic material of GABA, which was initially used for the treatment of epilepsy and neuropathic pain relief. In 1994, gabapentin was approved by the Food and Drug Administration (FDA) as an effective agent to control epilepsy (30). However, use of gabapentin is associated with several side effects (e.g., dizziness, fatigue, weight gain, and drowsiness), which have been reported at higher doses of this agent in elderly patients. Therefore, long-term consumption of gabapentin is not recommended (31).

Given the importance of GABA in the medical, healthcare and food industry, this study aimed to clone and construct the expression vector containing the GAD gene from *L. plantarum* PTCC 1058 bacterium.

Methods

In this experimental study, *L. plantarum* PTCC 1058 bacterial strain was obtained from the Department of Microbiology at the University of Isfahan (Iran), and BL21 and TOP10 *E. coli* strains were provided from the National Organization for Scientific and Industrial Research of Iran.

Bacterial culture and verification of L. plantarum PTCC 1058: In order to isolate the GAD gene, L. plantarum PTCC 1058 strain was cultured in Man, Rogosa and Sharpe (MRS) medium (Merck, Germany). In addition to morphological and biochemical tests to verify the strain, polymerase chain reaction (PCR) was carried out using 16s rDNA gene primer sequences (AGGAGGTGATCCACCCGCA DG47F and RW01RAACTGGAGGAAGGTGGGGAT) (Takapouzist, Iran).

Initially, genomic DNA was extracted using the CinnaGen Plasmid Extraction Kit. PCR reaction consisted of template genomic DNA (50 ng), buffer PCR 10x (2.5 ml), 0.5 ml of each of the forward and reverse primers, dNTP (1 µl), Mgcl2 (1 µl), Taq DNA polymerase enzyme (0.5 µl), and injection of distilled water (25 µl) in 30 cycles (94°C for 45 seconds, 58°C for 45 seconds, 72°C for 60 seconds). Final incubation in PCR was performed for 10 minutes at the temperature of 72°C. Purified PCR product was sent to Takapouzist (Tehran, Iran) for single-read gene sequencing. Results of 16s rRNA sequencing were analyzed using the BLASTn software in the database of NCBI BLAST Search tool (http: www.ncbi.nlm.nih.gov/BLAST).

Amplification of GAD gene: GDA gene primers were designed and developed using the Oligo 7 software

[DOR: 20.1001.1.15614107.1395.18.6.10.4]
[DOI: 10.22088/jbums.18.6.66]

with restriction enzyme sites (CGCGGATCCATGGCAATGTTATACGGTAAA PLANre-F and CCGGAATTCTCAGTGTGTGAATCCGTATTTCTT PLANre-R). To amplify the GDA gene, PCR was performed as described above. To detect the PCR results, electrophoresis was carried out on 1% agarose gel and observed using the Gel Doc system. After purifying the PCR product, the purified segment was sent to Takapouzist for paired-end sequencing.

The extracted purified segment was attached to pJET1.2/blunt cloning vector (Fermentas, U.S.A) or T/A vector with a flat end using CloneJET™ PCR Cloning Kit. Based on the standard protocols, the fragmented segment was amplified in the vector. For the transformation of the recombinant pJET1.2 vector, TOP10 E. coli strain was used as the cloning host.

Subcloning of GDA gene in plasmid expression vectors: Plasmid was extracted from the bacteria containing pET-32a (+) and pJET1.2 gad (+).

For double enzyme digestion, we used the restriction enzymes BamHI and EcoRI (Fermentas, U.S.A) separately. The recombinant pET-32a (gad^+) expression vector became susceptible in the expression strain of BL21 bacteria and inserted via thermal shock. After growing in antibiotic medium, colony PCR was performed randomly in some colonies.

Analysis of GDA gene expression via SDS-PAGE: GDA gene expression was performed by adding 0.5 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a colony of the recombinant BL21 strain cultured in **lysogeny broth** (LB) (antibiotic medium) via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Results

According to the results of morphological and biochemical analyses after comparison with Bergey's Manual of Systematic Bacteriology (http: www.bergeys.org/outlines.html, data not shown) and 16s rRNA gene sequencing (Figure 1.A, bond at 370 bp) after the blast and evaluation, the strains in question were determined as gram-positive bacillus and catalase-negative Lactobacillus plantarum.

Quality and quantity of the genomic DNA, which were extracted from Lactobacillus plantarum using CinnaGen DNA Extraction Kit, were assessed via electrophoresis and spectrophotometry. Accordingly, concentration of 1693.8 µg/ml and absorbance ratio at 260/280 (1.82) were determined for the extracted sample, and electrophoresis was indicative of the high quality of this sample.

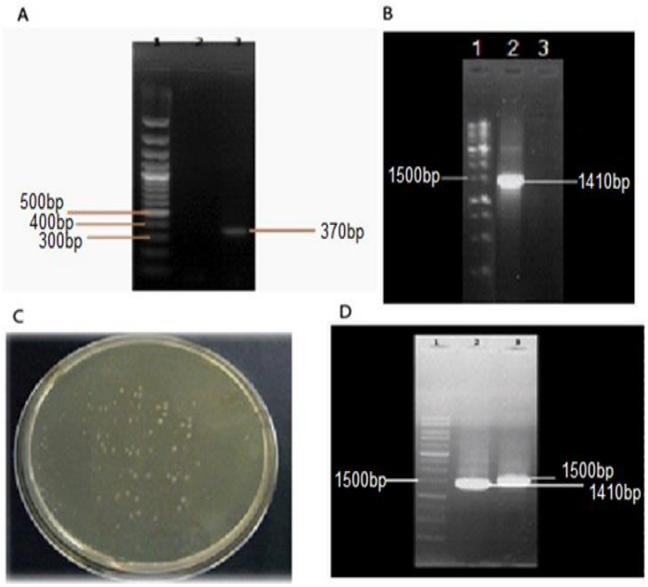


Figure 1. (A) Column 1: DNA ladder 1kb; **Column 2:** bond of approximately 370 bp (results of Lactobacillus plantarum PTCC 1058 genome PCR with primers DG47F and RW01R)
(B) Column 1: DNA ladder 1kb; **Column 2:** extracted genome; **Column 3:** negative control (results of agarose gel for extracted DNA from Lactobacillus plantarum PTCC 1058 with primers PLANreF and PLANreR)
(C) TOP10 recombinant bacteria containing T/A (gad+) vector grown on LB plates with ampicillin
(D) Column 1: DNA ladder 1kb; **Column 2:** colony PCR using GAD primers; **Column 3:** colony PCR using vector primers (colony PCR results of a random colony using specific primers of GAD gene and vector pJET1.2)

Moreover, results of PCR on the extracted genome with the designed primers showed the bond at 1410 bp (Figure 1.B), which was indicative of the presence of full-length GAD gene. As depicted in figures 1.C and 1.D, GAD gene accession to T/A cloning vector in TOP10 cloning strain was successful. In addition, plasmid extraction, purification, and sequencing of the segment confirmed the purified fragment to be a substrain of Lactobacillus plantarum. Results of plasmid colony PCR confirmed the presence of GAD gene in both the recombinant TOP10 strain and BL21 expression strain (figures 2.A & 2.B).

In this study, SDS-PAGE results verified the cloning and enzyme expression of the recombinant expression strain (figures 2C & 2D). However, western blot or other tests are required for the final confirmation.

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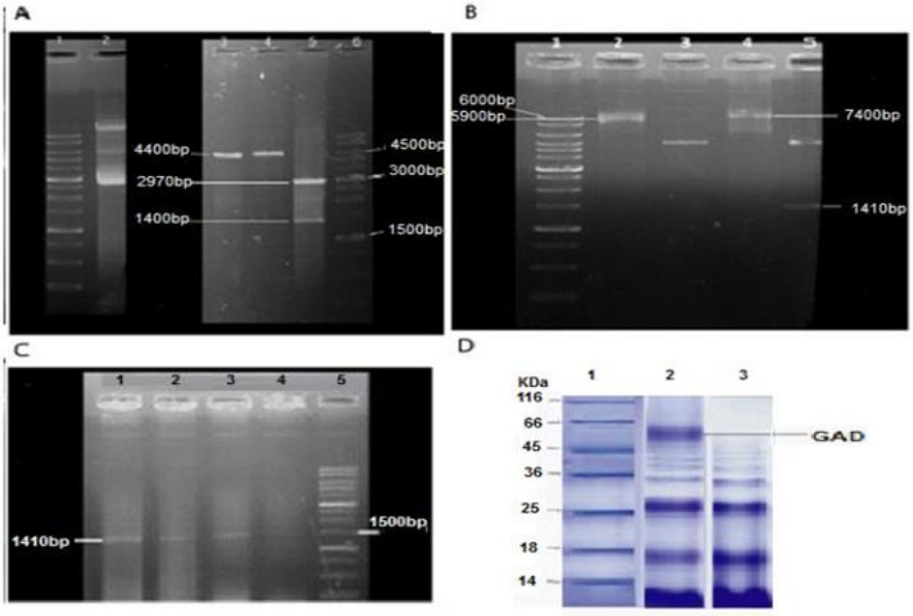


Figure 2. (A) Columns 1 & 6: DNA ladder 1kb; **Column 2:** T-vector containing GAD gene; **Columns 3 & 4:** enzymatic digestion of T-vector containing GAD gene with EcoRI and BamHI, respectively; **Column 5:** double enzymatic digestion of T-vector containing GAD gene vector

(B) Column 1: DNA ladder 1kb; **Column 2:** expression vector pET-32a (+); **Column 3:** double enzymatic digestion of expression vector pET-32a (+) with EcoR1 and BamHI enzymes; **Column 4:** expression vector pET-32a (+) containing GAD gene; **Column 5:** double enzymatic digestion and pET-32a (gad^+) with EcoRI and BamHI enzymes

(C) Confirmation of fragment presence in expression vector (colony PCR of pET32a [gad^+] colonies grown on LB plates containing ampicillin); **Columns 1, 2, & 3:** colony PCR results of three different colonies of BL21 transformed with recombinant vector pET32-(a)+ containing GAD gene; **Column 4:** negative control; **Column 5:** DNA ladder 1kb

(D) SDS-PAGE results; **Column 1:** protein marker; **Column 2:** enzyme expressed in recombinant strain BL21; **Column 3:** lack of enzyme expression in non-recombinant strain BL21

Discussion

The most important finding of our research was the cloning of GAD gene from the bacterium Lactobacillus plantarum PTCC 1058. In the present study, for the first time, the gene coding the GAD enzyme of the strain Lactobacillus plantarum PTCC 1058 was identified, sequenced, and cloned, along with the construction of the expression vector and investigation of its expression. The obtained recombinant strain was capable of developing and could be used efficiently in the food and pharmaceutical industries. In 1991, human GAD was isolated from chromosome 10 (32). In 1997, GAD gene was isolated from Lactobacillus brevis, and its characteristics were studied (25). Furthermore, in 1999, this gene was isolated from Lactococcus lactis, cloned, and sequenced (28). In 2006, the GAD gene from Lactobacillus brevis OPK-3 was cloned in pLip shuttle vector, where the expressed enzyme activity in the recombinant strains was significantly higher compared to that of Bacillus subtilis (29). In 2007, after the segregation of some lactic acid bacteria from Italian cheese, the central area of GAD gene was isolated from these strains (11). GAD gene was isolated from Lactobacillus brevis BH2 and cloned, and its characteristics were studied as well (33). In 2007, GAD gene was isolated from Lactobacillus brevis OPK-3, and its characteristics were investigated (27). In 2008, the GAD gene was cloned, and the characteristics of GAD enzyme of Lactobacillus paracasei NFRI 7415 were determined (34). In the same year, cloning and gene sequence analysis of GAD gene of Lactobacillus brevis IFO-12005 was performed (35). In 2009, GAD was cloned from Streptococcus thermophilus Y2, and its expression was investigated (10). Moreover, GAD gene has been cloned from Lactobacillus delbrueckii, Lactobacillus reuteri, and Lactobacillus casei (36, 37). In one case, this gene was isolated from L. plantarum ATCC 1497 and cloned in a shuttle expression vector (E. coli-Lactobacillus pTRKH2), and the product

[DOR: 20.1001.1.15614107.1395.18.6.10.4]
[DOI: 10.22088/jbums.18.6.66]

(pTRKH2GAD) was expressed and observed in Lactobacillus sakei B2-16, where the recombinant Lactobacillus sakei B2-16 had higher GABA production (1.35-1.42 times) (38). This enzyme could assist researchers in enzyme extraction and purification, as well as the evaluation of the characteristics and optimization of enzymatic expression. Moreover, enzyme immobilization technique could be used for the direct conversion of glutamate to GABA for the large-scale production of GABA in different industries. Some of the advantages of enzyme compared to whole cell include the specificity of enzymatic reactions, which prevents unwanted byproducts and biodegradability of enzymes, which is associated with less environmental pollution. Furthermore, enzymes could function in conditions at near neutral pH, low temperature, and normal atmospheric pressure saving large amounts of energy. These features are considered to be of paramount importance in industries. The present study was the first to clone GAD gene from Lactobacillus plantarum 1058.

Acknowledgements

Hereby, we extend our gratitude to the Deputy of Research and Technology at the University of Isfahan for the financial support of this research project.

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[DOR: 20.1001.1.15614107.1395.18.6.10.4]
[DOI: 10.22088/jbums.18.6.66]