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Original Research

Cloning and expression of rice glutamate decarboxylase (GAD) in *Escherichia coli*

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Abstract: Glutamate decarboxylase (GAD) converts L-glutamate to *g*-aminobutyric acid (GABA), which is a non-protein amino acid present in all organisms with some activities including improvement of neurve and cytoskeltal functions. Therefore, GAD is considered as a key molecule to use in molecular therapy of genetical human diseases. Accordingly, cloning of GADs from various plants is an important aim of researchers. The aim of this study was to clone rice (Oryza sativa L.) GADs in *Escherichia coli* (*E.coli*) MC 1061 bacterium.In this study, rice GADs was cloned in *E.coli* in both 37°C and 28°C. Two concentrations of Isopropyl-β-D-thiogalactoside (IPTG) (0.5mM and 1mM) were investigated in TB medium. Purification was done with Ni^{2+}-nitrilotriacetic acid (NTA) resin in various concentration of imidasol. According to SDS-PAGE analysis, rice GADs was cloned and expressed successfully in *E.coli* MC 1061 bacterium and the most expression was done in 37°C and 0.5mM IPTG and the best concentration of imidasol was 100mM for purification step. Based on the results, rice GADs can be expressed in *E.coli* MC 1061 bacterium and, hence, it is a suitable way to produce the plant enzyme in industrial scales.

Key words: *Escherichia coli*; Glutamate decarboxylase; Overexpression.

Introduction

Glutamate decarboxylase (GAD) is an enzyme that catalyses the conversion of L-glutamate to alpha-aminobutyric acid (GABA), a four-carbon nonprotein amino acid, in the presence of the cofactor pyridoxal 5'-phosphate (1). The pathway responsible for converting glutamate to succinate via GABA named the GABA shunt (2). GAD decarboxylates glutamate directly and irreversibly which is the first step of this shunt. The second step is the reversible conversion of GABA to succinic semialdehyde by GABA transaminase. Finally succinic semialdehyde dehydrogenase, irreversibly, oxidizes succinic semialdehyde to succinate (3, 4). GABA is ubiquitously present in bacteria, animals and plants. Although, the role of GABA in plants remains controversial (5, 6), in vertebrates, GABA is known to be a major inhibitory neurotransmitter. In humans, GABA is also directly responsible for the regulation of muscle tone (7). GABA reduces the blood pressure level of experimental animals and humans, recovers alcohol-related symptoms (8), improves memory and learning ability of mouse (9, 10) and prevents diabetes (11). Therefore, GAD is considered as an important molecule involvs in the methabolisms of human cells and can be usded as molecular therapy to overcome human genetical diseases. Brown rice (Oryza sativa L.) extracts with high levels of GABA had an inhibitory function on leukemia cell proliferation and a stimulatory action on the cancer cell apoptosis (12). Thus, rice has been introduced as a main source of GADs (12). Several hosts have been suggested to be used for cloning of rice GADs (13, 14). Based on the fact that bacteria are considered as a suitable hosts for cloning of plant genes (14), the main purpose of this study was to optimize recombinant GAD production in *Escherichia coli* (*E.coli*) MC 1061 bacterium (T7 express strain of *E. coli*).

Materials and Methods

Vector

pET-32a(+) carrying OsGAD1 gene was used for this study (Gift from Dr.Akama university of Shimane, Japan).

Transformation

The *E. coli* MC 1061 bacterium was used to prepare competent cells for transferring the recombinant pET-32a(+). Ten microliters of gift plasmid were added to 150 microliters of competent bacterial cells. Finally, the prepared cells were placed in the LB culture medium containing ampicillin (100mg/mL) for 12 hours at 37°C. Selection step of recombinant bacterium, was done in direct PCR technique.

Polymerase chain reaction

The PCR were performed using a thermal cycler (GeneAmp 9600; Perkin-Elmer Cetus) in a final 15 µl of a reaction mixture. Accordingly, the following materials have been added to the PCR reaction: H2O ultrapure 10.4 µl, PCR Buffer 1.5 µl plus MgCl2 (50 Mm) 0.5 µl (Promega®), DNTPs (10 Mm) 0.3 µl, forward and reverse primers (20 pmole/µl) 1 µl, taq DNA polymerase (5 U/µl) 0.3 µl and DNA template (1 µl). Negative control was used in each run which was free of template DNA. PCR profile consisted 35 cycles including

denaturing temprature for 30 s, annealing for 30 s and extension for 90 s, at 94, 60 and 72°C, respectively. The primers sequences which have been used for the amplifications were 5´TAA TAC GAC TCA CTA TAG GG3´ for T7 promotor, as forward, and 5´GCT AGT TAT TGC TCA GCG G3` for T7 terminator primer, as reverse, to amplify a 2243 bp product. The PCR products were evaluated by electrophoresis on 1.0% agarose gel and visulaized under UV light transilluminator (UV Star, Biometra, Germany).

The plasmids of selected clone were extracted and transferred to T7 express competent *E.coli* bacterium.

Recombinant Glutamate decarboxylase expression

For this step, selected clone of recombinant MC 1061 bacterium were first cultured in the liquid LB culture medium containing the antibiotic ampicillin (100 mg/mL) for 12 hours at 37℃, with the culture medium rotated at 150 revolutions per minute (rpm). 200 microliters of the cultured bacteria were then added to 200 milliliters of the liquid TB culture medium containing ampicillin with 180rpm (at the 28 and 37 degrees centigrade). Induction was done by two final concentrations of IPTG (0.5mM and 1mM) at OD600 of 0.6; the bacteria were cultured for four more hours. The culture medium containing the bacteria was then centrifuged at 6000 revolutions per minute (4629 g) for 10 minutes at four degrees centigrade and the supernatant liquid was then discarded. Precipitation kept at –20℃ until the next steps were carried out. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to observe the expression of recombinant plasmids (15). Recombinant enzyme was purified with Ni^{2+}-nitrilotriacetic acid (NTA) resin (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Results

Transformation

In this research, the pET-32a (+) expression vector, which is one of the strongest known expression vectors, was employed. The PCR method was used to identify recombinant bacteria containing the fragment. These reactions were done by universal T7 forward and reverse primers (Fig. 1).

Expression of the recombinant glutamate decarboxylase enzyme

The T7 express strain of *E. coli* was used to transfer and express fragments. This strain has great ability in expressing recombinant genes. TB culture medium that contained greater quantities of various nutrients including carbon sources was used for the abundant expression of the recombinant proteins. IPTG was used for the expression of the recombinant protein, after the light absorption coefficient had reached 0.6. The 12 percent SDS-PAGE was used to study the expression of the recombinant proteins. Figure 2 shows the protein profiles of the electrophoresed samples. Control group consisted of recombinant bacteria to which IPTG had not been added. This group of bacteria grew as the non-induced sample beside the induced ones. The presence of a protein band in induced samples, and its absence in control ones, indicated the expression of the recombinant protein. Protein purification is important for study of the function of desired protein. The purification process separates the protein of interest from all other proteins. The purification was done with Ni^{2+}-nitrilotriacetic acid (NTA) resin. This step was successful and the most purified protein has been shown in presence of 100mM imidasol.

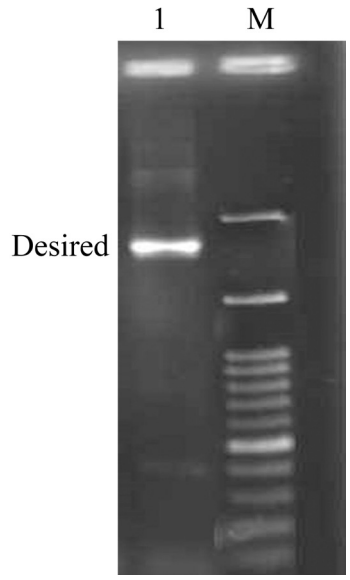

Figure 1. Isolation of 2243bp fragment by polymerized chain reaction. The results demonstrated that the bacteria have been transfected successfully. 1: polymerized fragment, M: 100bp plus ladder.

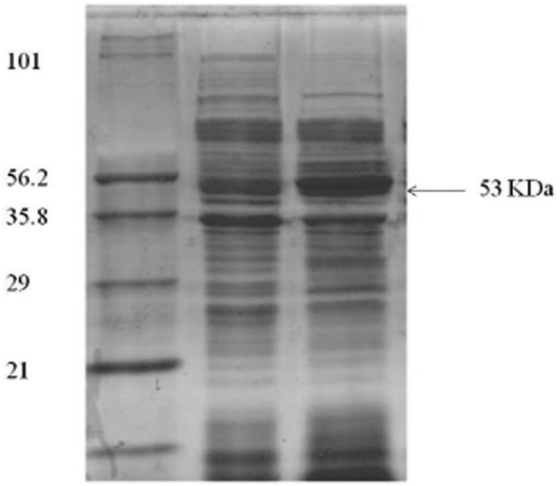

Figure 2. SDS-PAGE analysis. The figure illustrates that rice GADs were successfully cloned and expressed in MC 1061 *E.coli* bacterium. L: Protein ladder, 1: Control, 2: Recombinant protein.

Discussion

Because of the importance of GABA in various aspects in organisms, and due to the low-level expression of this enzyme in various organisms (16), we expressed this enzyme in MC 1061 *E.coli*. pET-32a(+) was used as vector harboring rice GAD gene. This is one of the strongest known expression vectors and can secrete the recombinant protein to the periplasmic space (17). The periplasmic expression causes some posttranslational modification events, which cannot be done in cytoplasmic expression. In addition, secretion of recombinant

proteins to the periplasm of *E. coli* has several advantages over intracellular production among which are separation from cytoplasmic proteins, enhanced biological activities, enhanced product solubility and easy in protein purification. We have successfully transferred and expressed rice GADs gene in *E. coli* MC 1061 bacterium. Based on the results it seems that the bacterium is a suitable host for cloning of rice GADs, as a reach source of GADs. A study by Oh et al., revealed that rice GADs can be cloned and expressed in another plant entitled *Scutellaria baicalensis* (13). Based on the fact that cloning and expression of rice GADs in another plants and also the processes of plant growing and purification of rice GADs from the plants is more difficult than badteria, hence, it appears that the currecnt investigation introduce a more suitable method to cloning and production of rice GADs with less costs and more speed. Acccordingly, in parallele with our results, Akama and colleagues reported that rice GADs can be expressed in *E.coli* strain BL21 (DE3) (14). Collectively, it seems that *E.coli* can be considered as a suitable candidate for cloning of rice GADs in inductrial scales. Additionally, our results demonstrated that the best condition for producing of purified recombinant protein was 37℃ and 0.5mM IPTG for expression procedure and 100mM imidasol for purification step which can be usefull to considered fro practical production of rice GADs. However, Park and colleagues have cloned the rice GADs gene in another bacterium entitled Bifidobacterium longum (18).

Based on the fact that rice is reach of several isoforms of GADs, hence, investigations are performing to find new kind of the enzyme isoforms in the rice. For example, Kato et al., found new GADs in the rice bran (19). Akama et al., also reported a novel glutamate decarboxylase isoform which is defected in C-terminus calmodulin-binding domain (20). Due to the fact that rice has several kinds of GADs, and based on our results which demonstrated that *E.coli* can be considered as a good carrier for cloning of GADs, it seems that more investigations need to be performed to determine the best rice GADs isoform which can be cloned in the bacterium.

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