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Heterologous expression of glutamine transaminase in Escherichia coli

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Abstract: Transglutaminase (TGase) has been widely used in food, textile, leather, medicine and other industries. It is currently a research hotspot of industrial production of mTG enzyme to express the gene sequence encoding mTG enzyme in engineering strains by means of genetic engineering. This paper successfully expresses the gene sequence of mTG enzyme in Escherichia coli, providing a reference for industrial production of mTG enzyme.

Keywords: Transglutaminase; E. coli; Heterologous expression.

1. Introduction

Transglutaminase (TGase)was first found in the liver of guinea pigs by Clarke et al.It is a kind of transfer enzyme, which widely exists in human body, animals, plants and microorganisms. TG enzyme from microorganisms is also called mTG enzyme. It can catalyze the formation of glutamine residues in protein peptide chain γ- Carboxylamide groups as acyl donors react with different acyl receptors to produce amide transfer reactions, resulting in intermolecular and intramolecular cross-linking of proteins. At present, TG enzymes are widely used in food, pharmaceutical, textile and other industrial fields, mTG enzyme has become the main source of commercial TG enzyme due to its excellent physical and chemical properties and catalytic ability. However, the production of mTG enzyme by conventional fermentation has a long fermentation cycle, complex composition of fermentation broth, difficulty in downstream purification and low yield. Therefore, using genetic engineering means to express the gene fragment encoding mTG enzyme in engineering strains is another important way for industrial production of TG enzyme.

Compared with other expression systems, E. coli expression system has the advantages of clear genetic background, simple culture conditions, short growth cycle, and can express different exogenous gene products economically and efficiently. Therefore, E. coli is the first-choice tool for foreign gene expression. In this study, PET plasmid was used as the vector to express the gene fragment of mTG enzyme in Escherichia coli host, providing a reference for the expression of mTG enzyme in engineering strains.

2. Material and method

2.1. Materials used in the experiment

The expression plasmid pET-duet and the host Escherichia coli BL21 (DE3) were stored in the laboratory; DNA Marker and Protein Marker were purchased from Shanghai Biotech; BamHIand HindIII restriction endonucleases were purchased from TaKaRa Bio; 2 × Phanta high-fidelity enzyme was purchased from Vazyme; All reagents are purchased from Chinese medicine; Ligase was purchased from AGbio. Ampicillin, IPTG and other chemical reagents are domestic analytical reagent. Primer

P1: CGCGGATCCGTCGACGCGGGCCGGGAGGG, P2: CTCAAGCTTCGCTCACATCACGGCCAGCC

synthesized by Shanghai Biotech.

2.2. Experimental method

The mTG enzyme gene fragment was amplified from the genome of Streptomyces mobaraensis CICC 11019, and connected to the pET-duet expression vector to construct a recombinant plasmid. The recombinant plasmid was transformed into the BL21 receptive state of Escherichia coli. The enzyme activity of the fermentation broth was measured after IPTG induction and expression.

2.2.1. S. mobaraensis CICC11019 genome extraction

(1) Take 0.5-1 mL of cultivated S. mobaraensis bacterial solution, add it into a 1.5 mL centrifuge tube, centrifuge at room temperature of 8000r for 1min, discard the supernatant and collect the bacterial body. Add 100μL Buffer Digestion and 80μL lysozyme solution (add the corresponding lysozyme to the Enzymatic lysisbuffer before use, and prepare 20 mg/mL lysozyme solution) and resuspension, and water bath at 37℃ for 30 minutes. During the water bath process, mix it upside down every 10min to promote sample cracking;

(2) Add 20μL Proteinase K solution, shake and mix well. 56℃ water baths for 30 min to complete cell lysis;

(3) Add 200μL Buffer BD, fully invert and mix, and take a water bath at 70℃ for 10 min;

Note: After adding Buffer BD, white precipitates may be produced, which generally disappear after water bath at 70℃, and will not affect subsequent experiments. If the solution does not become clear, it means that the cell lysis is not complete, which may lead to less extracted DNA and impure extracted DNA.

(4) Add 200 μL of anhydrous ethanol, fully invert and mix. The addition of anhydrous ethanol may produce translucent fibrous suspension, which does not affect the extraction and application of DNA.

(5) Put the adsorption column into the collection tube, use a pipette to add all the solution and translucent fibrous suspended solids into the adsorption column, leave it for 2min, and then centrifuge at 12000r room temperature for 1min, and then pour out the waste liquid in the collection tube.

(6) Put the adsorption column into the recovery header, add 500 μL PW Solution, and pour out the filtrate after

centrifugation for 30s.

(7) Put the adsorption column into the recovery header, add 500 μL Wash Solution, and pour out the filtrate after 10000r centrifugation for 30s.

(8) Put the adsorption column back into the recovery header, centrifuge at 12000r room temperature for 2min, leave the residual Wash Solution, open the cover of the adsorption column and place it at room temperature for several minutes.

(9) Take out the adsorption column, put it into a new 1.5mL centrifuge tube, add 50-100μL CE Buffer, and let it stand for 3min, 12000r room temperature centrifugation for 2min, and collect DNA solution. The extracted DNA can be further tested or stored at - 20℃.

2.2.2. Amplification of target fragment

The primers P1 and P2 were designed according to the mTG gene fragment obtained from NCBI. P1 had BamHIrestriction site and P2 had HindIII restriction site. To extract the S. mobaraensis genome is the template, P1 and P2 are primers for PCR amplification of mTG gene fragment reaction system and amplification conditions are shown in the following table.

Table.1 The PCR amplification reaction system

component	volume(μL)
2×Phanta Max Master Mix	25
P1	2
P2	2
Formwork (*S. mobaraensis* genome)	2
DMSO	1
ddH_2O	18

PCR amplification conditions: 95℃ pre-denaturation for 3 min, 95℃ denaturation for 15s, 70℃ annealing for 15s, 72℃ extension for 2min, 72℃ complete extension for 5min, a total of 30cycles. After PCR, prepare 1% agarose gel for electrophoresis. After electrophoresis, the gel recovers and purifies the target fragment.

2.2.3. PCR gel recovery and purification

(1) Cut the gel block containing the target segment from the agarose gel, place it in a clean and sterile 1.5mL centrifuge tube, and weigh it. Each tube of agarose gel block should not exceed 400mg;

(2) Add solution Buffer 2, and take a water bath at 50℃ for 5-10 min until the rubber block is completely dissolved. During the water bath, pay attention to turning the centrifuge tube to ensure that the rubber block is completely dissolved;

(3) Use a pipette gun to transfer the dissolved solution to the adsorption column, centrifuge the membrane, and pour out the waste liquid. If the volume of the solution is more than 800 μL, Multiple centrifugation;

(4) Add Buffer 2, centrifuge the membrane, and pour out the waste liquid;

(5) Add the Wash Solution, centrifuge the membrane, and pour out the waste liquid;

(6) Repeat the previous step;

(7) Open the cover and centrifuge the adsorption column for 60s, and dry the residual ethanol;

(8) Add 30μL sterile ddH2O to the adsorption membrane in the middle of the adsorption column, stand for 2min, centrifuge the membrane, and ddH2O can be preheated at 60℃;

(9) Repeat the previous step, and the eluted target fragment is stored at - 20℃。

2.2.4. PET-dute plasmid extraction

(1) Overnight culture of target strains;

(2) Take 1-5mL of bacterial solution into a 1.5 mL clean centrifuge tube, centrifuge at 12000 r for 1 min, and leave the bacterial body to precipitate;

(3) Add 250 μL solution P1 resuspended slime;

(4) Add 250 μL solution P2, gently turn it over for 6-8 times to fully mix, at this time, the solution in the centrifuge tube should become clear;

(5) Add 350 μL solution P3, immediately turn it gently for 6-8 times, fully mix it, and white flocs will appear at this time, and centrifuge for 10min at 12000r;

(6) Add 500μL to the balance column The balance liquid BL of is centrifuged to pass the balance liquid through the membrane and balance the column;

(7) Use a pipette gun to transfer the supernatant in (5) to the treated column and centrifuge the waste liquid;

(8) Add solution PW (containing anhydrous ethanol), and centrifuge the waste liquid;

(9) Repeat the previous step;

(10) Centrifuge the empty adsorption column for 2min, then open the cover and dry for 3min, and dry the residual anhydrous ethanol;

(11) Place the adsorption column in a clean centrifuge tube, and add 70μL ddH2O to the middle adsorption membrane of the adsorption column, placed at room temperature for 2min, centrifuged for 2min, ddH2O can be preheated at 65℃;

(12) The collected plasmid DNA should be stored at -20℃。

The extracted pET-dute plasmid needs to be verified by agarose gel electrophoresis to ensure successful extraction.

2.2.5. Construction of recombinant plasmid

The mTG enzyme gene fragment recovered and purified by gel and the pET-dute plasmid extracted were digested with BamHIand HindIII at 37℃ for 3.5-4h. The products of the double digestion were purified by agarose gel electrophoresis and stored at -20℃.

After digestion, two fragments with the same digestion site were obtained. The mTG and the linearized pET-dute plasmid were connected by DNA Ligation Kit ligase, and the connection condition was 16℃ overnight to construct the pET-mTG recombinant plasmid. See the table below for the two-enzyme digestion system and linking system.

Table 2. Double enzyme digestion system

component	volume(μL)
BamHI	2.5
HindIII	2.5
buffer	5
mTG/pET-dute	25
ddH_2O	15

Table 3. The connection system

component	volume(μL)
mTG	6
pMA5	2
Ligation Solution A	16

2.2.6. BL21/pET-mTG recombinant strains construction

The recombinant plasmid pET-mTG was transformed into E. coli BL21 receptive cells were coated with a resistant plate and cultured at 37℃ for 12h. The single colonies grown on the plate were selected and inoculated into LB liquid medium with ampicillin for culture. After the bacterial liquid was turbid, it was diluted 10 times for PCR verification. At the

same time, the plasmid extracted from the bacterial solution was digested and verified by single enzyme digestion, and then sequenced. The correct one was E. coli BL21/pET-mTG positive transformant. After the positive transformant was expanded in the medium supplemented with ampicillin, IPTG was added to induce the recombinant strain to express the target protein.

2.2.7. Determination of glutamine transaminase activity

The standard curve of glutamine transaminase activity was determined according to the enzyme activity determination method, and the absorbance of the measured supernatant was brought into the standard curve, and then the enzyme activity in the supernatant was further calculated.

3. Results and discussion

3.1. BL21/pET-mTG recombinant strains construction

To extract the S. mobaraensis genome was used as the template, p1 and p2 were used as primers to amplify the mTG enzyme gene fragment. The PCR reaction system was subjected to gel electrophoresis. After the correct bands were cut, recovered, purified and verified. It can be seen from the validation results in Figure 1 that the experiment successfully obtained about 1200 bp of mTG target gene fragment, which is in line with the expected length.

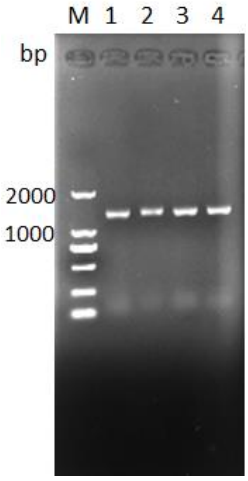

Figure 1. PCR products of mTG M: DNA marker

The mTG enzyme gene fragment and the extracted pET-dute plasmid were digested and linked, and the linked recombinant plasmid pET-mTG was transformed into E. coli BL21 receptive cells were coated with a plate containing ampicillin and cultured overnight. The single colonies were selected for colony PCR to obtain a band of about 1200 bp. The recombinant plasmid was digested and validated to obtain a band of about 8900 bp. The validation results are shown in Fig 2 and 3.

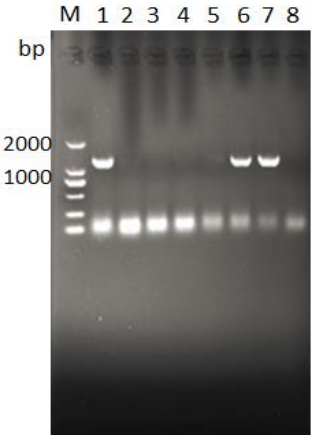

Figure 2. PCR products of E. coli/pET-mTG M: DNA marker

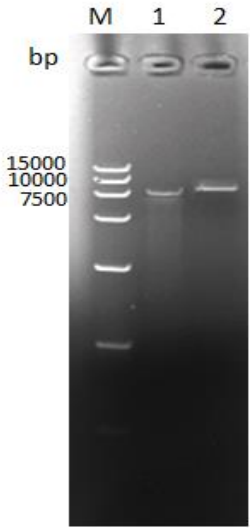

Figure 3. Pet-mTGvalidation of recombinant plasmid by single enzyme digestion M: DNA marker

The correct transformants were sent to the company for sequencing, and the measured results were consistent with the mTG enzyme sequence on NCBI.

3.2. Determination of glutamine transaminase activity

The absorbance of the supernatant was measured according to the enzyme activity measurement method, and the standard curve was brought in to further calculate the enzyme activity of the supernatant.

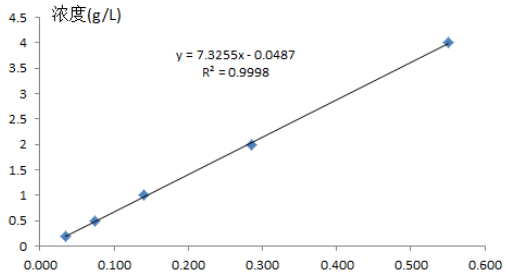

Figure 4. Standard curve of enzyme activity

After calculation, the enzyme activity of the recombinant strain BL21/pET-mTG is 0.1593U/mL.

4. Conclusion

In this paper, pET-mTG recombinant plasmid was successfully constructed and transformed into E. coli BL21, successfully constructed BL21/pET-mTG recombinant strain, and realized the gene fragment encoding mTG enzyme in E. coli BL21 was successfully expressed and the enzyme activity of fermentation broth was 0.1593U/mL, which provided a new idea and method for the industrial production of mTG enzyme.

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