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Original Article

Mutating Asn-666 to Glu in the O-helix region of the *taq* DNA polymerase gene

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Abstract

Taq DNA polymerase is widely used in laboratories and for this reason many investigators have focused their attention on understanding the role of various regions and amino acids in this enzyme. O-helix is a part of *taq* polymerase suggested to play an important role in the enzyme fidelity. The influence of Asn666 in this helix on the enzyme function has never been investigated, and therefore by using nested PCR, a portion of *taq* DNA polymerase gene containing Asn666Glu mutation was amplified. This DNA was digested with *Eco* RI restriction enzyme to confirm the presence of Asn666Glu mutation. After digesting this product and the wild type *taq*-pET-15b plasmid with *Nhe*I and *Bam*HI restriction enzymes, they were ligated and used for the transformation of *E. coli* DH5α competent cells. The obtained colonies were screened for the presence of the mutated *taq* polymerase gene using *Eco*RI, *Nde*I and *Bam*HI restriction enzymes. In conclusion, with the use of the obtained recombinant plasmid it is possible to study the role of this amino acid on *taq* DNA polymerase function.

Keywords: Taq DNA polymerase; Mutation; Recombinant plasmid

INTRODUCTION

Polymerase chain reaction is a widely used technique for the amplification of DNA for the cloning, identification and other laboratory purposes. In this reaction, *taq* DNA polymerase plays an important role, and its activity and fidelity can significantly influence the obtained results. Structurally, this enzyme can be divided into three segments: the N terminus which is stretched from amino acids 1-290, the inverting segment from amino acids 291-419 and the C-terminus which is from amino acids 420-832 (1). The active site is suggested to face the O-helix region of the enzyme (Fig. 1) and Asp-785, Glu-786, and Asp-610 are present in the catalytic site of all DNA polymerase I family (2). It is suggested that Arg-659 and Lys-663 in the O-helix region are crucial in enzyme activity and mutations of these amino acids have dramatically decreased the *taq* polymerase enzyme activity (1). It is also shown that mutations of Phe-667 (1), Tyr-671 (2), and Ile-
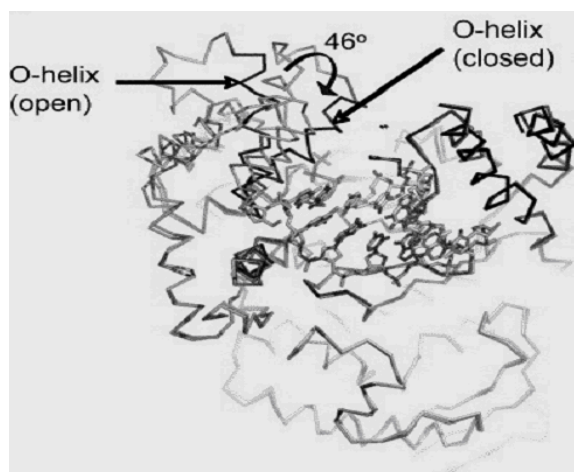

Fig. 1. Schematic representation of the O-helix present in the C-terminal of the *taq* DNA polymerase (4).

665 (3) in this region have decreased the fidelity of the enzyme. Therefore, it seems that due to the important function of the O-helix region of the *taq* polymerase enzyme in its activity and fidelity, by mutating amino acids in this region and altering hydrophobicity and polarity of these amino acids important

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information regarding the function of the enzyme can be obtained. Since no data are available regarding the role of Asn-666 in the enzyme activity, we designed the present study to produce an expression vector containing mutated Asn666Glu *taq* polymerase gene, in order to investigate the effect of this mutation on the enzyme function.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *E. coli* DH5α strain used in this study was purchased from Cinnagen (Tehran, Iran). Oligonucleotide primers were synthesized by Faza biotech (Tehran, Iran). *Taq* polymerase was purchased from BioRon (Tehran, Iran). DNA ligase, DNA molecular size marker, *Hind*III Digested λDNA, and all restriction enzymes were from Fermentas (Tehran, Iran). High Pure PCR Template Preparation Kit and QIA quick Gel Extraction Kit were obtained from Roche and Qiagen, respectively. All other chemicals were from Merck (Tehran, Iran).

Primer design

To produce Asn666Glu mutation, the Asn codon (aac) had to be changed to Glu codon (gaa). This replacement would create an *Eco* RI restriction site and thus making it easier to screen for the DNA sequences containing this mutation. Therefore, two complimentary primers containing this mutation were designed using WDNASIS program (Hitachi. Software Engineering Co., Ltd., Japan):

FM: 5ʹ-CAAGACCATCGAATTCGGGGTCC-3ʹ
RM: 5-GGACCCCGAATTCGATGGTCTTG-3ʹ

Two primers were also designed against DNA regions in the pET-15b plasmid near the multiple cloning site:

FpET: 5ʹ– ATAGGGGAATTGTGAGCGG-3ʹ
RpET: 5ʹ– GGGGTTATGCTAGTTATTGC-3ʹ

For the nested primers, two sequences inside the *taq* polymerase gene were selected:

Ftaq: 5ʹ-CCCAACCTCCAGAACATCC-3ʹ
Rtaq: 5ʹ– GGACGGGCATGTTGAAGG-3ʹ

Nested PCR

The method of nested PCR was used for the introduction of the desired mutation (5) in the *taq* DNA polymerase gene. For the first step PCR, in one tube FM and RpET primers (2.5 M each) and in another tube FpET and RM primers (2.5 M each) were used. Into each tube 50 ng of the wild type recombinant *taq*-pET-15b vector (6) was added as the template followed by 0.5 mM (each) deoxyribo-nucleotide triphosphate and 10X-PCR buffer (containing 2 mM $MgCl_2$) in a total volume of 50 μl reaction mixture. The thermocycling profile was as follows: initial denaturation at 94 ^oC for 5 min; 35 repeated cycles of 94 ^oC for 1 min, 55 ^oC for 2 min, and 72 ^oC for 3 min; and a final extension of 72 ^oC for 20 min. The PCR product was analyzed using 0.8% agarose gel electrophoresis. The DNA bands were then extracted from the gel using QIA quick Gel Extraction Kit (Qiagen, Germany) and the obtained DNA fragments were mixed and 50 ng of this mixture was used for the second step PCR (nested PCR). For this step, the reaction mix and PCR conditions were similar to the first step except for the primers in that Ftaq and Rtaq (2.5 M each) were used instead. Afterwards, to confirm the presence of mutation, digestion of the PCR product was performed with *Eco*RI restriction enzyme (1 h, 37 ^oC).

Ligation and transformation

The product of the nested PCR and the wild type recombinant *taq*-pET-15b vector were digested with *Nhe*I and *Bam*HI restriction enzymes (1 h, 37 ^oC). The digested insert and vector were isolated from agarose gel and then ligation was performed with T4 DNA Ligase (Fermentas, Germany) at 16 ^oC overnight in a water bath. Before ligation, the vector was dephosphorylated by treatment with calf intestinal alkaline phosphatase (Roche, Germany) to remove 5ʹ-terminal phosphate and to prevent recircularization of the plasmid. The ligated DNA was used for the trans-formation of *E. coli* DH5α competent cells using heat shock method (6).

Plasmid preparation was performed on the obtained colonies using High Pure Plasmid Isolation Kit (Roche, Germany) and the

recombinant plasmids containing the Asn666Glu were screened by digestion with *Eco*RI, *Bam* HI, and *Nhe*I restriction enzymes (1 h, 37 °C) (7).

RESULTS

Nested PCR

To produce a segment of the *taq* polymerase gene containing the desired mutation, a two-step nested PCR was performed. In the first step, in one tube the forward primer recognizing a sequence in the pET-15b and the reverse primer containing the Asn666Glu mutation were used. In another tube the forward primer containing the mutation and the reverse primer designed against a region of pET-15b were utilized. As shown in Fig. 2A, the PCR product of the first tube was about 2000 bp which is similar to the expected size of 2134 bp. The PCR product obtained in the second tube was about 600 bp which matches the expected size of 584 bp (Fig. 2B).

In the second step the obtained PCR products were gel purified and mixed to form the template for the nested PCR. Forward and reverse primers were designed to recognize sequences inside the amplified PCR products obtained in the first PCR reactions. The DNA band of the nested PCR had the expected size of 526 bp (Fig. 2). Since mutation of the *taq* polymerase gene should have created an *Eco*RI restriction site, therefore, digestion with this enzyme should result in two bands of about 250 bps which can be seen in Fig. 3. as expected.

Obtaining the mutated recombinant plasmids

The PCR product containing the mutation and the wild type *taq*-pET-15b vector was then digested by two restriction enzymes, *Bam*HI and *Nhe* I. The digested DNA fragments were then ligated and used for the transformation of the *E. coli* DH5α competent cells. Subsequently, the plasmids extracted from the obtained colonies were digested with *Eco*RI (Fig. 4). One of the plasmids having the correct orientation was selected and double digestion with *Bam*HI and *Nde* I was performed. This digestion should have resulted in the production of a 1767 bp DNA fragment if the orientation of the insert was correct. As can be seen in Fig. 5, this digestion produced the expected fragment.
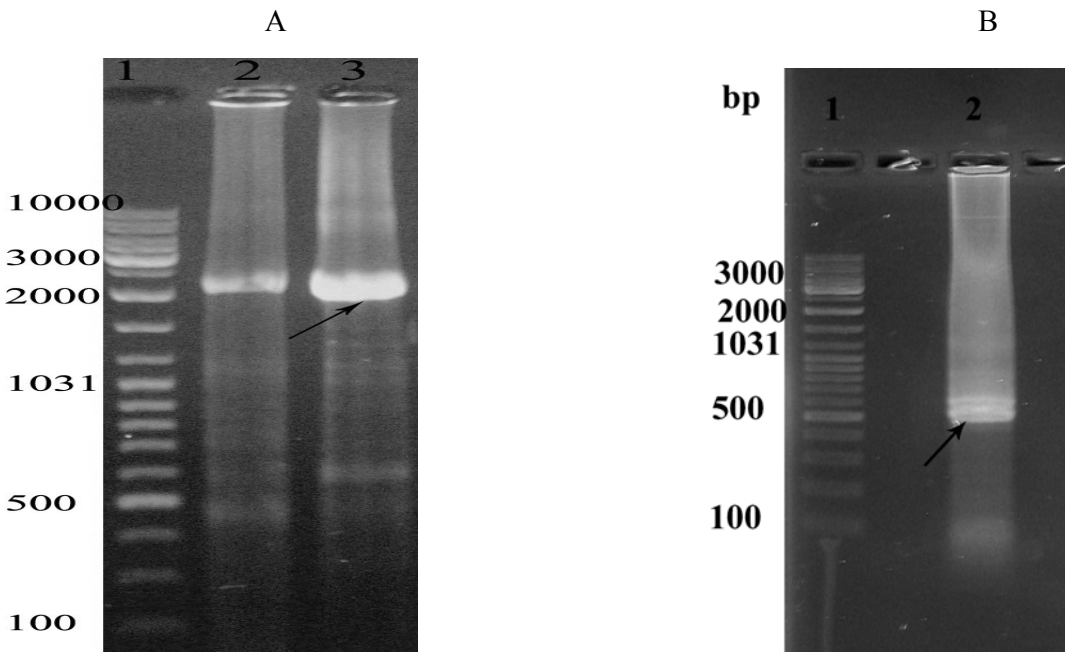

Fig. 2. The PCR products obtained after the first PCR. These products were electrophoresed on an 0.8% agarose gel. A) Lane 1: DNA molecular weight marker. Lanes 2-3: PCR amplified DNA using FpET and RM primers. B) Lane 1: DNA molecular weight marker. Lane 2: PCR amplified DNA using FM and RpET primers.
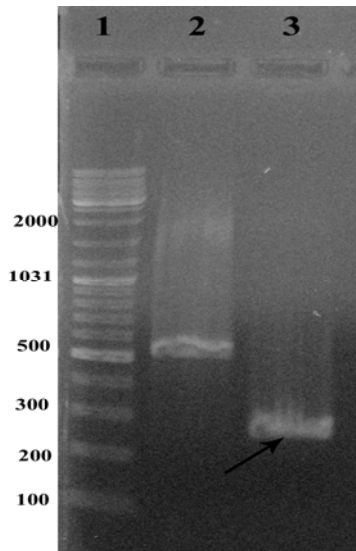
Fig. 3. The PCR products obtained after the second PCR (nested PCR). These products were electrophoresed on an 0.8% agarose gel. Lane 1: DNA molecular weight marker. Lanes 2: nested PCR product. Lane 3: Digestion of the nested PCR product with *Eco*RI restriction enzyme.
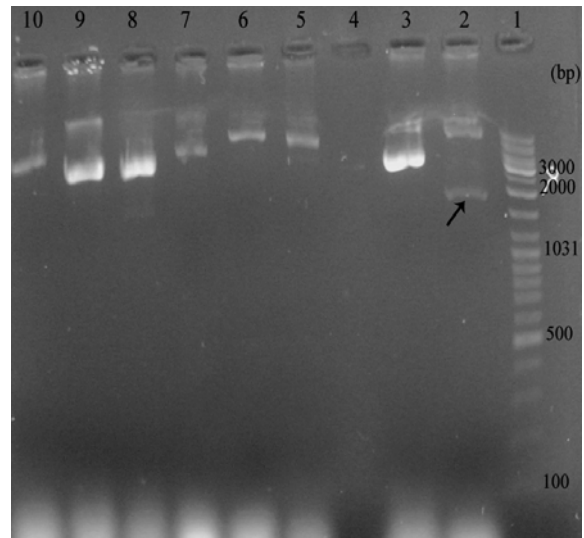

Fig. 4. Digestion of the obtained plasmids with *Eco* RI restriction enzyme. These plasmids were then electrophoresed on an 0.8% agarose gel. Lane 1: DNA molecular weight marker. Lanes 2-10: digested plasmids.
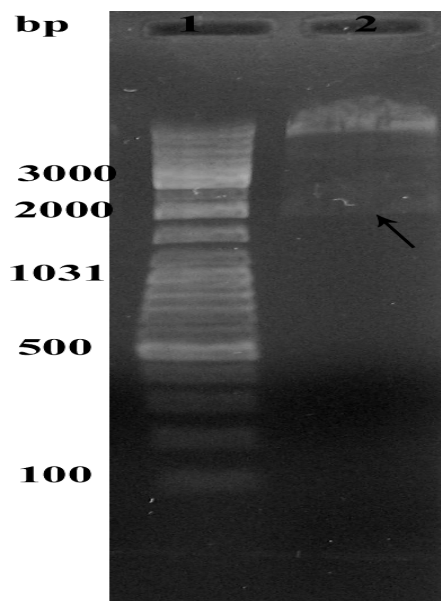

Fig. 5. Digestion of the recombinant plasmid with *Nhe* I and *Bam* HI restriction enzymes. This plasmid was then electrophoresed on an 0.8% agarose gel. Lane 1: DNA molecular weight marker. Lane 2: Digested plasmid.

DISCUSSION

The O-helix region of the *taq* polymerase enzyme has been of interest to many investigators. Tosaka et al. has shown that T664P mutation results in a decreased enzyme fidelity but not activity (8). Mutating other amino acids in this region such as Ile-614 (8-9) and Ala-661 (10) also have resulted in the attenuation of the enzyme fidelity. These data suggest that amino acids located in the O-helix play an important role in the replication of

DNA by the *taq* polymerase enzyme and that they influence the proper placement of nucleotides rather than the rate of DNA replication.

Understanding the structure-function relationship of the *taq* polymerase enzyme can help the investigators to create mutated enzyme with higher or lower fidelity. The latter can be of great importance since this property can be used for the production of random mutations in an amplified gene. These mutations, if screened properly, can lead to the production of industrially more favorable proteins.

In the present study we decided to mutate Asn666 to Glu. The reasons for selecting this mutation were as follows: first, no one has studied the Asn666 mutations before and therefore this would be the first time that the role of this amino acid on the function of *taq* DNA polymerase can be studied. Second, change in the charge and polarity of the amino acid occupying position 666 may influence the activity and fidelity of *taq* DNA polymerase. While Asn is a polar hydrophilic amino acid, Glu is acidic.

Therefore, mutating Asn666 to Glu in the O-helix of Taq DNA polymerase and investigating its effect on the function of this enzyme is an important step towards understanding the role of O-helix in the enzyme fidelity and activity.

CONCLUSION

This is the first report in which a recombinant plasmid having mutated Asn666Glu *Taq* DNA polymerase gene has been produced. This plasmid can be used in the future experiments for the investigation of the role of Asn666 on the enzyme's function.

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