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RESEARCH ARTICLE

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Improvement of megaprimer method for site-directed mutagenesis and its application to phytase

Abstract Site-directed mutagenesis is used extensively for probing gene function. In this paper we describe an improved megaprimer method to the site-directed mutagenesis of phytase from *Aspergillus niger*, which allowed the mutations to be performed more efficiently in less time than other traditional methods. Three rounds of PCR and two pairs of primers were required in this method, and additionally, the restriction enzyme *Dpn* I was used for the elimination of template instead of the gel purification in this process. The entire procedure was performed in one tube. Moreover, this method was easier for obtaining large mutant genes than other methods. We successfully carried out the site-directed mutagenesis of phytase by adopting this method.

Keywords site-directed mutagenesis, phytase, the single-stranded product, PCR

1 Introduction

In recent years, there has been widespread enthusiasm for 'site-directed mutagenesis' as a tool to understand the relationship between protein structure and function. Mutagenesis is now achieved by either PCR-based or non-PCR methods. Due to their characteristics of being rapid, reliable and effective, numerous PCR-based methods are now available (Kuipers et al., 1991), such as the megaprimer method, inverse PCR method, overlap extension polymerase chain reaction (OE-PCR), and quick-changeTM site-directed mutagenesis method.

Scientists have adopted some mutagenesis methods to study phytase (Ling and Robinson, 1997; Tomschy et al., 2000; Nabavi and Nazar, 2005). Among them, the megaprimer method and the quickchangeTM site-directed mutagenesis method are the main ones. The megaprimer method introduced by Kammann et al. and later modified by others is the most effective one among the PCR-based methods (Barettino et al., 1994; Shenoy et al 2003). This method requires one mutagenic primer and two universal primers. The megaprimer from the first PCR can be purified and subsequently used as a primer to amplify the gene. However, a drawback of this method is the low yields of mutated genes due to the inefficient extension by the megaprimer; another drawback is that it needs a step for gel purification, which is cumbersome and time-consuming. The quickchangeTM site-directed mutagenesis only requires two primers, which are two complementary oligonucleotide primers, and the primer-dimer formation becomes more favorable than the primer-template, especially when the primer pairs possess multiple mismatches. In this paper, we describe an improved site-directed mutagenesis method (Fig. 1). This method consisted of three rounds of PCR without the intermediate gel purification. In the first PCR, we added unequal molar amounts of the primers and operated the numbers of the cycles to generate the megaprimer with the desired mutation. In the second PCR, the megaprimer formed a heteroduplex with the template sequence, which was extended by pyrobest DNA polymerase. Subsequently, *Dpn* I was added directly into the tube and the wild template was digested. Only the sequences containing the mutation base could be amplified exponentially with primers U and D in the subsequent cycles. Adding both the Primer U and the Primer D to the mixture, the third PCR produced the final mutant DNA. We successfully applied this methodology to phytase, which provided a simple, highly effective and general application of mutagenesis.

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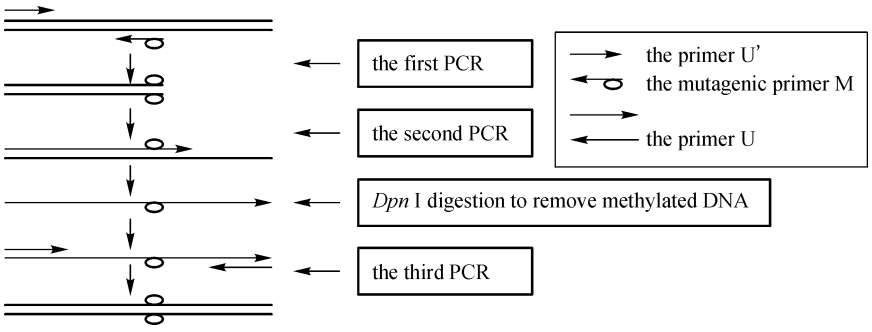

Fig. 1 Schematic diagram of the improved site-directed mutagenesis method

2 Materials and methods

2.1 Materials

Polymerase chain reactions (PCR) were performed on the Tgradient Thermocycler, and pyrobest DNA polymerase was purchased from Takara Biotechnology Co., Ltd., Dalian, China. The *Dpn* I and dNTPs were purchased from Shanghai Sangong Biological Engineering Technology & Services Co. Ltd. Oligonucleotides were synthesized by Sangong Biological Engineering Technology & Services Co. Ltd., *E. coli* JM109 and pMD18-T-phyA.

2.2 Primer designing

This method utilized two pairs of primers, a pair of primers were Primer U and Primer D for amplification of the full-length gene in the third PCR; the other pair were Primer M and Primer U' for generation of the megaprimer containing the mutation base in the first PCR. The mutagenic Primer M was designed in such a way that the mutation base was located at the middle of the primer and satisfied the synonymous codons used in *Pichia pastoris* (Zhao et al., 2000)

Table 1 Primers used in the experiments

name of primer	sequence of primers (5'-3')
Primer U	GTGAATTCCTGGCAGTCCCCGCTTC
Primer D	TCGCGGCCTAAGCAAAACACTCCG
Primer U'	TGCCTGCAGGTCGACGATTGTGAATTCC
Primer M	CATGACCGTAGTACTT**CTC**CAGGGACTGGAGGTA

Note: The changed nucleotides causing the desired mutation are in boldface and are underlined.

2.3 Determination of the amount of the primers and the number of PCR cycles

The first PCR was carried out in a total volume of 50 μL reaction mixture containing 1.25 U Pyrobest DNA Polymerase (5 U·μL^{-1}), 5 μL 10×Pyrobest Buffer II (Mg^{2+} Plus), 2 mmol·L^{-1} dNTP Mixture, Primer U (0.05, 0.025 pmol·L^{-1}), and Primer M (0.5 pmol·L^{-1}). The PCR conditions were: preheating at 94°C for 4 min followed by five up to 45 cycles at 94°C, for 1 min; 67°C, for 30 sec and 72°C, for 1 min. Based on the product from the first PCR, the second PCR was performed for 10 cycles at 94°C, for 1 min and at 72°C, for 1 min and then *Dpn* I (10 U) was added into these reaction tubes and digested for 2 h. By adding 1.0 pmol·L^{-1} Primer U and 1.0 pmol·L^{-1} Primer D to the tubes, the third PCR was initially taken at 94°C for 4 min, and then for 30 cycles at 94°C, for 1 min; at 64°C, for 30 sec; at 72°C, for 2 min. At the end of this step, the tubes were held at 72°C for 10 min.

2.4 Mutation frequency analysis

The PCR products (1347 bp) were isolated from an agarose gel, cloned into the pMD18-T vector, and transformed into *E. coli* JM109 competent cells. We screened 5 colonies for the presence of the mutation to calculate the mutation frequency.

3 Results

3.1 Establishment of PCR parameters

The proper concentrations of Primer U' and the number of PCR cycles were also important in this method. We adopted adding unequal molar amounts of primers and different amplification cycles in the first PCR. The results are shown in Fig. 2. When the Primer U' concentration was 0.05 pmol·L^{-1} and 0.025 pmol·L^{-1}, and the number of cycles was from 5 to 45, a megaprimer fragment of about 900-bp was presented on the agarose gel for both concentrations (Figs. 2 (a) and (c)). Subsequently, we chose 8 different circulation numbers (from 5 to 40 cycles) to finish the following steps.

From Fig. 2 (b) and (d), we found that the final results from different Primer U' concentrations were greatly different. Under the condition of 0.05 pmol·L^{-1} Primer U', the bright bands (900 bp) were found (Fig. 2 (a)), but the target bands were not found (1347 bp) in the third PCR

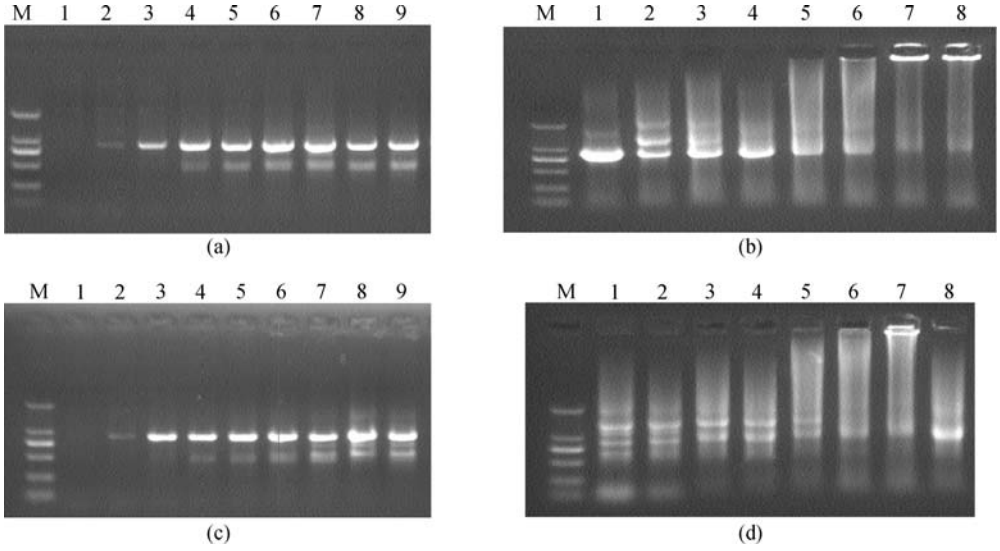

Fig. 2 Optimization of key parameters and conditions for maximum efficiency of mutagenesis
Note: (a) represents the products generated from the different circulation numbers of the first PCR with 0.05 pmol·L^{-1} of Primer U'. M represents DNA Marker-D (100–2000 bp). From Lane 1 to Lane 9, the number of cycles is 5, 10, 15, 20, 25, 30, 35, 40 and 45 respectively. (b) represents the effects of different circulation numbers in the first PCR with 0.05 pmol·L^{-1} of Primer U'. M is DNA Marker-D (100–2000 bp). From Lane 1 to Lane 8, the number of cycles is 5, 10, 15, 20, 25, 30, 35 and 40. (c) represents the products generated from the different circulation numbers of the first PCR with 0.025 pmol·L^{-1} of Primer U'. M is DNA Marker-D (100–2000 bp). From Lane 1 to lane 9, the number of cycles is 5, 10, 15, 20, 25, 30, 35, 40 and 45. (d) represents the effects of the different circulation numbers of the first PCR with 0.025 pmol·L^{-1} Primer U'. M is DNA Marker-D (100-2000 bp). From Lane 1 to Lane 8, the number of cycles is 5, 10, 15, 20, 25, 30, 35 and 40, respectively.

product except in lane 2 (Fig. 2 (b)). On the contrary, we found that the band (1347 bp) in the product of 0.025 pmol·L^{-1} (U') presented from Lane 1 to Lane 5 (Fig. 2 (d)), and the amplification product was more specific than the product from the 0.05 pmol·L^{-1} Primer U'. This indicated that the concentrations of Primer U' influenced the final amplification efficiency. Meanwhile, we found that the circulation numbers also influenced the result greatly. Based on the results obtained, the optimum concentration for Primer U' and cycle number were determined to be 0.025 pmol·L^{-1} and 20 cycles in the first PCR.

3.2 Establishment of the improved site-directed mutagenesis method

The improved megaprimer was performed as a one-tube PCR, with 3 rounds of PCR. In the first PCR, the reaction was started with the addition of 1.25 U Pyrobest DNA Polymerase (5 U·μL^{-1}), 5 μL 10 × Pyrobest Buffer II (Mg^{2+} Plus), 2.5 mmol·L^{-1} dNTP Mixture, 0.5 pmol·L^{-1} of internal mutagenic primer M and 0.025 pmol·L^{-1} of Primer U' in a total volume of 50 μL, following the conditions of pre-denaturation at 94°C for 4 min, and then denaturation for 20 cycles at 94°C for 1 min, 67°C for 30 s, and 72°C for 1 min, followed by the second PCR amplification at 94°C for 1 min, and at 72°C for 2 min for 10 cycles, without adding anything into the tube during this step. After the second PCR, *Dpn* I (10 U) and TangoTM buffer (2 μL) were added directly into the reaction tubes. Both methylated and hemimethylated DNA were digested after incubation with *Dpn* I (10 U) for 2 h at 37°C. In the third PCR, 1.0 pmol·L^{-1} Primer U and 1.0 pmol·L^{-1} Primer D were directly added into the tubes without any purification step. After preheating at 94°C for 4 min, 30 cycles of amplification were performed at 94°C, for 1min, 64°C, for 1 min and 72°C, for 2 min. The target product (1347 bp) with the desired mutation was purified and cloned into the cloning vector.

3.3 Comparative analysis of the two methods

Compared with the standard megaprimer PCR method, the improved one produced a more satisfactory result, but the resulting fragment (1347 bp) was not found with the standard method (Fig. 3).

We also observed other bands in addition to the target band (1347 bp), the reason being that Primer M was competing with Primer D in the third PCR, thereby, non-specific bands were presented. The resulting fragment (1347 bp) was purified and cloned into the cloning vector pMD18-T. We picked 5 transformed colonies for sequencing (Table 2). The results showed that the mutation frequency was 100%. Therefore,

Table 2 Frequency of the improved megaprimer method mutation

gene name	mutation sequence	mutation frequency
K300E	ACCTCCAGTCCCTG**GAG**AAGTACTACGG	100%(5/5)
Q297LK300E	ACCTC**TTG**TCCCTG**GAG**AAGTACTACGG	100%(5/5)
KK	ACCTCCAGTCCCTGAAAAAGTACTA CGG	

Note: KK is the part of the wild template; K300E and Q297LK300E are the result of the improved megaprimer method.

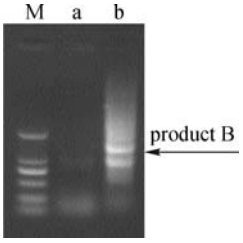

Fig. 3 Comparison of the two methods
Note: Lane a shows the results of the stranded method; Lane b shows the results of the improved method; Lane M shows marker DL2000.

the improved site-directed mutagenesis had an obvious advantage over the standard method for its simplification and efficiency.

4 Discussion

In this study, we used the improved site-directed mutagenesis method to create base substitutions of the phytase gene. Compared with the standard megaprimer PCR method, one of the novel features of the improved method is that it overcomes the problem of intermediate gel purification by adding unequal molar amounts of primers and operating the number of cycles. The standard method requires intermediate gel purification, which can decrease the influence of the residual primer and wild template, but can increase the influence of ultraviolet rays and other factors. Moreover, it is laborious and circumvented. In this improved method we adopted a new strategy to overcome the problem of gel purification. Through a series of experiments, the Primer U' concentration of 0.025 pmol·L^{-1} is somewhat lower than the normally used concentration and only 20 cycles of amplification in the first PCR were found to be suitable, which more or less could not achieve the ideal effect. The second novel feature of the improved method was that mutant DNA could be synthesized more readily using the single-stranded DNA containing the mutation base as template than a double-stranded template, since two denatured complementary template strands of DNA may be quickly reannealed at a low temperature, interfering with the primer annealing with the template strand (Gyllensten and Erlich, 1988). It is possible to generate millions or more copies of the DNA with a few copies of DNA as template. Finally, the third feature of the improved method appears to be that two outside primers in the third PCR could amplify the single-stranded DNA, but only a little. The difference between the standard method and the novel method was that the former used the large fragment from the first PCR as the primer. However, it is difficult to complete the megaprimer extension due to the limitation from the sizes and the annealing of the megaprimer.

In conclusion, we have successfully applied this strategy to site-directed mutagenesis of phytase. This approach is efficient and simple for site-directed mutagenesis. The mutagenesis frequency approaches 100% under optimal conditions.

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