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Important sequence for overexpression of NADH oxidase gene from *Thermus thermophilus* HB8 in *Escherichia coli*

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Abstract

Enzymes fixed on the electrode of biosensor are gradually inactivated and the electrode is discarded after using several times. In order to prepare the stable biosensor, we try to use a stable enzyme from extreme thermophilic bacteria, *Thermus thermophilus* HB8. It is very important that a stable enzyme from *T. thermophilus* HB8 is overproduced in *Escherichia coli*, for the purpose of enough supply of enzyme. Thereby, we determined the important sequence for overexpression of NADH oxidase (*nox*) gene from *T. thermophilus* HB8 in *E. coli*. As a result, it is revealed that ten nucleotides sequence, GAAATTAACT, in the upstream of start codon of *nox* gene was important for its overexpression in *E. coli*.

Key words: biosensor; stable enzyme; extreme thermophilic bacteria; overexpression

Introduction

Nowadays, biosensor is widely used to measure concentration of various substances. For example, glucose sensor is used by patients of diabetes mellitus to measure the concentration of blood glucose. However, the electrode fixed with enzyme of biosensor is discarded for the instability of enzyme.

While, *Thermus thermophilus* HB8 is an extreme thermophilic bacteria, and can live in hot water at 85°C. Thereby, we think glucose dehydrogenase (GlcDH) from *T. thermophilus* HB8 is proved to be particularly useful for glucose sensor because of its stability. Since large quantity of GlcDH is required for use to biosensor, the overexpression of *GlcDH* gene from *T. thermophilus* HB8 in *Escherichia coli* is a prerequisite. Park and Kreutzer (1994) reported that the expression of *nox* gene from *T. thermophilus* HB8 in *E. coli* was extremely increased by the insertion of twelve nucleotides into the upstream of start codon, ATG, of the gene and the change of the ten nucleotides sequence upstream of the insertion. However, in our study, *GlcDH* gene was not expressed in *E. coli* by the same modification as *nox* gene. It is necessary for overexpression of the *GlcDH* gene in *E. coli* to clarify a mechanism of overexpression by modification of upstream sequence of *nox* gene. Therefore, we determined the important sequence in the modification of *nox* gene for overexpression in *E. coli*.

1 Materials and methods

1.1 Bacterial strains and plasmids

E. coli JM109 was the host strain for all plasmids. Cells were grown at 37°C in LB medium supplemented with 0.1 mg/mL ampicillin. Plasmid pKK223-3 was used for cloning and expression vector. For the overexpression of the genes, *E. coli* JM109 harboring the appropriate plasmids were grown as reported previously (Kreutzer *et al*., 1992).

1.2 DNA manipulation procedures

Genomic DNA of *T. thermophilus* HB8, restriction enzymes, PCR amplification kit, and *E. coli* JM109 competent cell were purchased from TaKaRa, Japan. T4 DNA ligase was purchased from Promega, USA.

1.3 Plasmid constructions

The *nox* and *GlcDH* genes from *T. thermophilus* HB8 was amplified by the polymerase chain reaction (PCR) as previously reported (Park *et al*., 1992b), and using primers listed in Table 1 were purchased from Sigma-Aldlich, Japan. PCR product was digested with *Eco*RI, and then with *Hin*dIII and eluted from an agarose gel. This was ligated with the expression vector pKK223-3 linearized by *Eco*RI, and *Hin*dIII. *E. coli* JM109 was transformed with the ligation mixture, the resulting clones were analyzed by restriction map and sequencing.

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Table 1 Nucleotide sequence of primers used in PCR for the amplification of NADH oxidase (*nox*) and Glucose dehydrogenase (*GlcDH*) genes

Primer 1/Primer 2	Amplified gene	Expression vector
5'-TTCGAATTCTTTAACTTTAGGAGAAATTAACTATGAGAGGATC-CATGGAGGC/5'-TCCCAAGCTTCTAGCGCCAGAGGACCACCCGCT	*nox*	P12
5'-TTCGAATTCTTTAACTTTAGGAAACACAATTCATGAGAGGATC-CATGGAGGC/5'-TCCCAAGCTTCTAGCGCCAGAGGACCACCCGCT	*nox*	N12
5'-TTCGAATTCTTTAACTTTAGGAGAAATTAACTATGGAGGCGAC-CTTCCCGT/5'-TCCCAAGCTTCTAGCGCCAGAGGACCACCCGCT	*nox*	P
5'-TTTAGAATTCTTTAGGAGAAATTAACTATGAGAGGATCCATGGAC-CGGAGGCGCTTTCT/5'-TTTAAAGCTTCTAAAGGAGGCGTAGCACCC	*GlcDH*	

1.4 Enzyme activities

NADH oxidase activity was determined by measurement of absorbance at 340 nm of NADH according to Park *et al*. (1992a) with some modification. A suitable diluted enzyme solution was added to the solution containing 50 mmol/L potassium phosphate (pH 7.0), 0.067 mmol/L NADH and 0.1 mmol/L FAD (coenzyme of Nox), then absorbance at 340 nm was measured by the spectrophotometer (Hitachi U-2000, Japan). The specific activity of the Nox in soluble proteins from extracts of *E. coli* was calculated by measuring the initial decrease in absorbance of NADH in large excess of NADH according to the zero-order kinetics.

2 Results

2.1 Expression of *GlcDH* gene from *T. thermophilus* HB8 in *E. coli*

GlcDH gene from *T. thermophilus* HB8 genome was cloned into the expression vector pKK223-3, using PCR with modification of sequence upstream of start codon of *GlcDH* gene, according to Park and Kreutzer (1994) on the overexpression of *nox* gene from *T. thermophilus* HB8 in *E. coli*.

E. coli JM109 was transformed by the expression vector including *GlcDH* gene, and cultured in the LB medium of 30 mL under the presence of several concentration of IPTG (isopropyl-β-D-thiogalactopyranoside) that was the inducer of *GlcDH* gene. After the heat treatment (at 70°C for 1 h) of the extracts of the *E. coli* in order to remove the thermolabile proteins from *E. coli* by centrifugation, soluble proteins were analyzed by SDS-PAGE (data not shown). However, *GlcDH* was not detected by SDS-PAGE. The modification of upstream sequence of start codon on the *nox* gene was not effected for expression of *GlcDH* gene. Therefore, we determined the sequence of the modification in *nox* gene required for overexpression in *E. coli*.

2.2 Determination of the important sequence for overexpression of *nox* gene in *E. coli*

In order to determine which modification of the upstream sequence of *nox* gene reported by Park and Kreutzer (1994), is important for overexpression of *nox* gene in *E. coli*, we constructed the three kinds of expression vectors changed the upstream sequences of *nox* gene using PCR. The first vector, called P12, had the same modification in the report of Park and Kreutzer (1994), namely the twelve nucleotides insertion, ATGAGAGGATCC, into the upstream of start codon and the change to the ten nucleotides sequence, GAAATTAACT, we called "Park sequence". The second vector, called N12, was changed only to the twelve nucleotides insertion. The last vector, called P, was changed only to the Park sequence (Fig. 1).

E. coli JM109 was transformed by three kinds of expression vectors, and cultured similarly in the case of expression vector of *GlcDH* gene. After the heat treatment, soluble proteins of extracts of the each *E. coli* were analyzed with SDS-PAGE (Fig. 2). The quantity of *nox* about 23 kDa was most in the *E. coli* transformed by the vector P. The expression quantity of *nox* gene of vector P12 was less than that of vector P. *nox* gene in the vector N12 was hardly expressed in *E. coli*. Moreover, when the specific activity of Nox in soluble proteins was measured by decrease of the absorbance at 340 nm, λ_{max} of NADH,

Expression vector P12:
- GAAATTAACT ATGAGAGGATCC ATG -
Park sequence 12 ntd insertion

Expression vector N12:
- AACACAATTC ATGAGAGGATCC ATG -
Wild-type sequence 12 ntd insertion

Expression vector P:
- GAAATTAACT ATG -
Park sequence No insertion

Fig. 1 Modification in the upstream *nox* gene in the expression vectors. ntd: nucleotides.

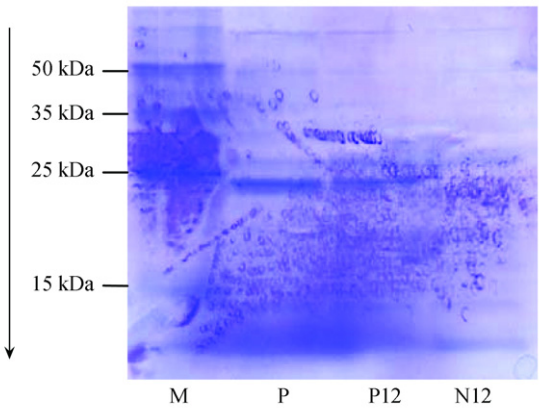

Fig. 2 SDS-PAGE of soluble proteins extracted from *E. coli* transformed by each vector. M: size marker.

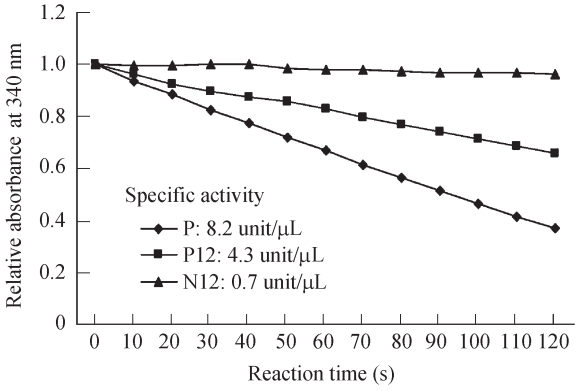

Fig. 3 Measurement of activity of Nox in soluble proteins.

the specific activities were the same order with the amount of expression quantities estimated by SDS-PAGE (Fig. 3).

3 Discussion

Park and Kreutzer (1994) reported that the expression efficiency of *nox* gene and malate dehydrogenase (*mdh*) gene from *T. thermophilus* HB8 in *E. coli* increased by the insertion of twelve nucleotides into the upstream of start codon, ATG, of the gene and by the change of the ten nucleotides sequence upstream of the insertion. Park described that the cause in the improvement of expression efficiency is to make base pairing between 16S rRNA and twelve nucleotides insertion, and the ten nucleotides modification to Park sequence upstream was not referred. To confirm the reason of improvement of expression efficiency, we investigated that which modification of twelve nucleotides insertion and Park sequence was more effective for improvement of expression efficiency of *nox* gene expression. As our results, the modification to Park sequence is more effective in improving expression efficiency than the twelve nucleotides insertion. However, why the modification to Park sequence of the array improves expression efficiency has not been elucidated.

To clarify the reason of improvement of expression efficiency, *nox* gene is modified by only first half of the ten nucleotides to the Park sequence, and modified the latter half to the Park sequence independently. The expression of the modified genes in *E. coli* is in progress.

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