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Construction of a Thermally Stable Cholesterol Oxidase Mutant by Site-Directed Mutagenesis

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Abstract: Site-directed mutagenesis was used for improvement of thermal stability of cholesterol oxidase from *Rhodococcus* sp. PTCC 1633. Cholesterol oxidase catalyzes the oxidation of cholesterol to cholest-5-en-3-one and its subsequent isomerization into cholest-4-en-3-one. This enzyme is industrially important and is commonly used for the enzymatic transformation of cholesterol. It is also useful for the clinical determination of total or free serum cholesterol by coupling with a related enzyme for the assessment of arteriosclerosis and other lipid disorders. Substitution in glutamine145 was created by means of site-directed mutagenesis using SOE-PCR (Splicing by Overlap Extension-Polymerase Chain Reaction). The presence of directed mutations and adventitious base changes were verified by DNA sequencing. The properties of mutant enzyme were similar to those of the wild type but the thermal stability of enzyme was obviously increased. Also, the range of pH for activity of mutant enzyme differed from that of the wild-type cholesterol oxidase. Substitution of the glutamine (Q) 145 by glutamic acid (E) could create not only a hydrogen bond between glutamic acid (E) 145 and aspartic acid (D) 134, but also a salt bridge between glutamic acid (E) 145 and arginine (R) 147 and subsequently improve the thermal activity of cholesterol oxidase.

Key words: Cholesterol oxidase, site-directed mutagenesis, thermostable enzyme, PCR technique, *Rhodococcus* sp. PTCC 1633

INTRODUCTION

Cholesterol oxidase (EC 1.1.3.6) catalyzes the oxidation of cholesterol (5-cholesten-3-β-ol) to 4-cholesten-3-one with the reduction of oxygen to hydrogen peroxide (Smith and Brooks, 1974). Cholesterol oxidase (Cho) is an enzyme of great commercial value, widely employed by laboratories routinely devoted to the determination of cholesterol concentrations in food, serum and other clinical samples (Richmond, 1976). This enzyme may be used for production of starting materials for chemical synthesis of pharmaceutical steroid (Ahmad *et al*., 1993) and for determination of the optical resolution of non-steroidal compounds, such as allylic alcohols (Dieth *et al*., 1995). Also, cholesterol oxidase exhibits potent insecticidal activity that is very important and vital for pest control strategies in case of transgenic crops (Purcell *et al*., 1993). Furthermore, some pathogenic bacteria require cholesterol oxidase to infect their host macrophage, probably because of the cholesterol oxidase ability in altering the physical structure of the membrane by converting cholesterol to cholesten-4-en-3-one (Brzostek *et al*., 2007). As these enzymes are unique to bacteria, they represent a potential target for a new class of antibiotics. More recently, it has been demonstrated that Alzheimer's disease β-amyloid selectively oxidizes cholesterol at the C-3 hydroxyl group and catalytically produces 4-cholesten-3-one; therefore it mimics the activity of cholesterol oxidase (Puglielli *et al*., 2005). The amino acid sequence of cholesterol oxidases is conserved among Cho-producing bacteria (Nishiya and Hirayama, 1999). The DNA of the cholesterol oxidase of *Rhodococcus* sp. PTCC 1633 (choR) was similar to 99% of *Rhodococcus equi* (*choE*), 98% of *Brevibacterium sterolicum* (*choB*) and 82% of *Streptomyces* sp. (*choA*).

Although ChoAs exhibits high catalytic activity for cholesterol, its thermal stability and stability at alkaline pH are not satisfactory. Thus, some efforts were performed to improve thermal stability of ChoA by random mutation (Nishiya *et al*., 1997). They produced a more thermally stable form of the Cho from a *Streptomyces* sp. The enhancement of thermal stability was achieved by means of *in vitro* random mutagenesis on a pre-targeted segment of gene. The decision as to where the region of

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mutagenesis should occur was based on the previously recorded structure of *choB* determined by Vrielink *et al.* (1991). The region chosen was shown not to contain the FAD binding domain or active site hollow.

The *choR* gene has been previously expressed and characterized. Here, successful enhancement of the thermal stability of ChoR by means of site-directed mutagenesis technique was reported. The mutational effects are discussed using a structural model of ChoR based on the 3-D structure of ChoB.

MATERIALS AND METHODS

Strains, plasmids and culture conditions: *Escherichia coli* Novablue was used as the host for recombinant DNA manipulations and site-directed mutagenesis of *choR* gene. *E.coli* BL21(DE3)pLysS was used for production of cholesterol oxidase. Plasmid pET23 (*choR*) containing the *choR* gene was used for additional plasmid construction and site-directed mutagenesis, respectively. Recombinant strains were grown in LB broth or on LB agar. The antibiotic used was ampicillin (100 mg mL^{-1}).

Manipulation and analysis of DNA: DNA purification, restriction enzyme digestion, ligation, agarose gel electrophoresis and transformation of competent *E. coli* cells were performed according to the procedures proposed by Sambrook *et al.* (2001).

Site-directed mutagenesis and construction of expression plasmids encoding mutant cholesterol oxidases: Substitution in Glutamine145 was created by means of site-directed mutagenesis using SOE-PCR (Splicing by Overlap Extension-Polymerase Chain Reaction). The presence of directed mutations and adventitious base changes were verified by DNA sequencing. The following oligonucleotides were used for mutagenesis of ChoR gene as shown in Fig. 1:

Q145EF: 5'GCATCAAGGTCTAC**G**A**A**GGCCGCGGCGTC 3' (Mutagenesis site)

Q145ER:5'GACGCCGCGGCC**T**T**C**GTAGACCTTGATGC 3'(Mutagenesis site)

Q145F: 5' TATAGAATTCATGACTGATAGCCGGGCC 3' (*Eco*RI cut site)

Q145R: 5'TACCAAGCTTTCACTGGATGTCGGACGAGA 3'(*Hin*dIII cut site)

Cholesterol oxidase activity determination: To determine cholesterol oxidase activity, two methods were used. In the first method, the absorbance of quinoneimine dye formed by coupling with 4-aminoantipyrine, phenol and peroxidase was measured at 500 nm by spectrophotometry. One unit of activity was defined as the formation of 1 μmol L^{-1} of hydrogen peroxide (0.5 μmol L^{-1} of quinoneimine dye) per minute at 37°C and pH 7.0. In some experiments, 4-cholesten-3-one, which is the product of isomerization from 5-cholesten-3-one, was measured at 240 nm by spectrophotometry. One unit of activity was defined as the formation of 1.0 μmol L^{-1} of 4-cholesten-3-one per minute at 37°C and pH 7.0. The protein concentration was determined with the Bradford method (Bradford, 1976) using human albumin as a standard.

Q 145ER Q 145R

Q 145F Q 145EF

Fig. 1: Arrangement of annealing site of oligonucleotides were used for mutagenesis

Expression of cholesterol oxidase in *E. coli*: The wild-type and mutant plasmids were extracted from *E. coli* NovaBlue and then transformed into the BL21(DE3)pLysS strain. Recombinants were grown in LB medium. Overexpression of the cholesterol oxidase gene was induced when OD at 600 nm was 0.6 by adding IPTG to a final concentration of 0.4 mM. Incubation was continued for 4 h at 30°C, then the cells were harvested and the cell extract was prepared as a crude enzyme.

One-step purification of recombinant cholesterol oxidase: Cell pastes of 1 L of wild-type and mutant culture medium were washed twice with 50 mM Tris-HCl pH 8.0 and then lysed with a solution containing 1 mM EDTA, 100 mM NaCl, 0.01 g L^{-1} and 50 mM PMSF in 50 mM Tris-HCl pH 8.0. Cells were lysed after 20 min at 25°C. Cell debris was removed by centrifugation at 20000 g for 15 min. To purify the recombinant enzyme, 500 mg cholesterol was added to 50 mL of crude extract and then incubated for 2 h at 4°C. Suspended cholesterol was collected by centrifugation at 10000 g for 10 min. The supernatant was discarded and the collected cholesterol was washed twice with 50 mM Tris-HCl pH 8.0. To remove loosely bounded proteins, the cholesterol was washed with 0.5 M NaCl in Tris-HCl buffer. The protein was solubilized with 1.0% Triton X-100 in Tris-HCl buffer. Cholesterol oxidase activity and protein concentration were measured for each step.

RESULTS

Expression and purification of cholesterol oxidase: Among various transformants tested, those possessing the highest intracellular cholesterol oxidase activity were

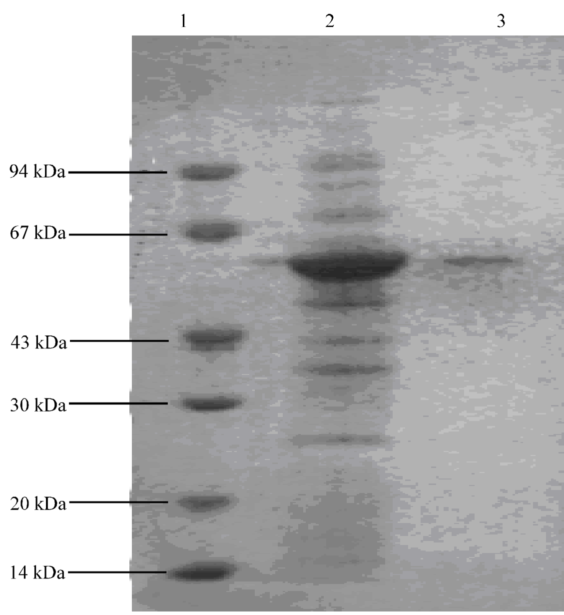

Fig. 2: SDS-PAGE analysis of mutant cholesterol oxidase. Lane 1: Molecular weight markers (Low Molecular Weight standard size markers, Pharmacia), Lane 2: Whole extract from cells bearing pET23 (ChoR), Lane 3: Purified cholesterol oxidase

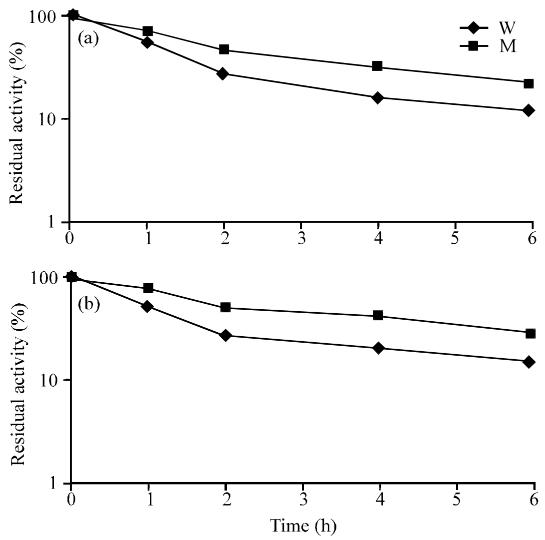

Fig. 3: Effect of temperature on stability of wild-type (W) and mutant (M) cholesterol oxidase. The enzyme solution was incubated at 45°C (a) and 55°C (b) for 6 h. The activity was assayed under standard conditions

selected. SDS-PAGE analysis of an IPTG-induced culture of *E. coli* BL21(DE3)pLysS harboring mutant plasmid showed a strong band at the molecular weight of about 55 kDa (Fig. 2). The recombinant cholesterol oxidases

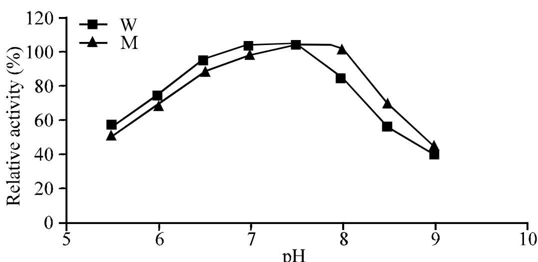

Fig. 4: Effect of pH on wild-type (W) and mutant (M) cholesterol oxidase activity. Enzyme activity was assayed under standard conditions except for changing pH of the buffer. Buffers used were dipotassium-monopotassium phosphate for pH 6.0-7.5, Tris-HCl for pH 8.0-9.0

were purified using a simple one-step purification method. The specific activities of the wild type and mutant were found to be 50.8 and 30.1 U mg^{-1}, respectively.

Comparison of the wild-type and mutant ChoR: The thermal stability of the mutant ChoR was compared with that of the wild-type enzyme (Fig. 3). The improved thermal stability of Q145E was particularly obvious. After 1 h more than 80 and 71% of the activity of mutant enzyme were remained in 45 and 55°C, respectively while only 54 and 55% of activity were remained in wild-type enzyme in that time. The mutant ChoR had the same optimum temperature (50°C) for its activity as that of the wild-type enzyme. The effects of pH on the stability and activity of the mutant and wild-type ChoR were also compared. The mutants displayed pH stabilities similar to that of the wild-type enzyme (data not shown). However, the pH profile of Q145E differed from that of the wild-type ChoR (Fig. 4). The optimum pH of Q145E was shifted to alkali and encompassed a wide-ranging between acid and alkali.

DISCUSSION

In this study, successful enhancement of thermal activity of cholesterol oxidase from *Rhodococcus* sp. PTCC 1633 was described. The *choR* has been previously expressed and characterized.

The three-dimensional structure of ChoR based on its homology to ChoB provides a rational initial point for analysing the structure-activity relationships of the wild-type and mutant enzymes. Substitution of the Q145 by E could introduce not only a hydrogen bond between E145 and D134, but also a salt bridge between E145 and R147 (Nishiya *et al*., 1997). These newly formed attractive interactions could contribute to the stabilization of the conformation and increase the thermal stability of Q145E. Alteration in pH profile displayed by Q145E is likely to

have been caused by introduction of the negatively charged amino acid glutamate. This makes the mutant enzyme suitable for clinical purposes, because various buffers and pHs are encountered depending on the assay reagents.

In conclusion the recombinant cholesterol oxidase described here is highly stable in comparison with wild-type cholesterol oxidase. Therefore, this recombinant cholesterol oxidase might improve the usability of cholesterol oxidase for reactions in harsh condition.

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