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# THERMOSTABILIZATION OF LACTATE OXIDASE BY RANDOM MUTAGENESIS

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## SUMMARY

A lactate oxidase (LOD) gene from *Aerococcus viridans* was cloned and sequenced to generate thermostable LOD. One mutant LOD selected from a set of variants created by random mutagenesis had a half life of 6.2 min at 65°C, approximately three times longer than that of the wild type LOD. This mutant exhibited an Asn to Asp point mutation at position 212 in the amino acid sequence.

## INTRODUCTION

Lactate oxidase (LOD) is a kind of flavin enzyme which catalyzes the oxidation of lactate to pyruvate, with a reduction of $O_2$ to $H_2O_2$. LOD is widely employed in biosensors to measure lactate concentration in blood and other body fluids (Bardeletti *et al.*, 1986, Wang and Heller, 1993); improving the thermostability of LOD would prolong the life-time of such lactate biosensors. This, in fact, has been the purpose of our current study.

LOD is known to be produced by several microorganisms (Ducan *et al.*, 1989). Although the structure gene for LOD from *Aerococcus viridans* has been sequenced (European Patent EP 0376163 A2, 1989), its three-dimensional structure has yet to be clearly determined, and thus, in order to generate thermostable LOD, we had to employ random mutagenesis, in this case, polymerase chain reaction (PCR) random mutagenesis .

## MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions**

*A. viridans* IFO12219 was purchased from the Institute for Fermentation (Osaka, Japan) and grown aerobically in tryptic soy broth (Difco) at 30°C. *Escherichia coli* DH5 and plasmid pBR322 were used for the gene cloning. *E. coli* JM109 and pKK223-3 were used for producing the LOD. *E. coli* was grown in L broth (1% tryptone (Difco), 0.5% yeast extract, 0.5% NaCl) supplemented when necessary with ampicillin (100 μg/ml). Gene expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside. An LOD activity assay medium (LL broth) was prepared by dissolving 1 unit/ml of horseradish peroxidase, 0.01% (w/v) 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate (6)), and 50 mM L-lactate (lithium salt) in L broth.

**Cloning and sequencing of LOD gene**

We employed standard recombinant DNA techniques, as described by Sambrook *et al.* (1989). Genomic DNA from *A. viridans* was digested with *Eco*RI, and the fragments were inserted into the *Eco*RI site of pBR322. *E. coli* DH5 cells were transformed with the ligation mixture as described by Dower *et al.* (1988). In order to facilitate the selection of transformants which express LOD, the cells were cultured on an LL plate containing ampicillin at 37°C. Transformants producing LOD developed purple color around their colonies as a result of their LOD activity. We used a QIAGEN column (Diagen GmbH) to purify a plasmid from one of the colored colonies and designated this plasmid pBRLOD. Sequencing was conducted by the method of Sanger *et al.* (1977), using appropriate fluorescein isothiocyanate-labeled primers, a BcaBest Sequencing Kit (Takara Shuzo Co., Ltd., Japan), and a DNA sequencer SQ-3000 (Hitachi Ltd., Japan).

**Production and purification of LOD**

The structure gene for LOD carried on pBRLOD was amplified by PCR using two oligonucleotides, PrN (5'AATAACAATGACATTGAATATAATGCACCT3') and PrC (5'GCCTAAATCTAGTATTCATAACCGTATGGG3') as 5'- and 3'-primers, respectively. The amplified LOD gene was dephosphorylated, blunted, and ligated to the blunted *Eco*RI site of pKK223-3, and the construct was designated pLODwt. LOD was purified from *E. coli* JM109 transformed with pLODwt by means of a slight modification of the method of Ducan *et al.* (1989).

**Random mutagenesis and isolation of thermostable mutant LOD**

In conducting our random mutagenesis, we followed a PCR method which was a modification of that proposed by Leung *et al.* 1989. The LOD gene on the pLODwt was used as a PCR template, and PrN and PrC were used as primers. The mutagenized fragments were blunted and ligated to the blunted *Eco*RI site of pKK223-3, and then used to transform *E. coli* JM109. The transformants were spread on an LL plate and incubated at 37°C. Purple-bordered colonies were transferred onto an L plate and cultured at 37°C for 12 hours. After a one hour heat treatment at 70°C, the plate was stained with LOD activity assay solution (see below), and purple-bordered colonies were selected. The mutant LOD was purified by the same method as for the wild type LOD.

**Enzyme and protein assays**

LOD activity was determined by a peroxidase-coupled spectrophotometric method (Ducan, *et al.*, 1989). Assays were started by the addition of LOD to an activity assay mixture containing 1.5 mM 4-aminoantipyrine, 3.3 mM phenol, 9.9 mM L-lactate (lithium salt), 40 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, and 6 units of horseradish peroxidase in a final volume of 3 ml at pH 7.0. Assays were conducted at 37°C, and absorbance at 500 nm was monitored. We defined one unit of LOD activity as the amount of enzyme that catalyzes the production of 1 μmol $H_2O_2$ per minute. Protein concentrations were measured with a BCA Protein Assay Regent Kit (Pierce, U.S.A.) using purified bovine serum albumin as a standard. For Michaelis constant (Km) determination of the mutant and the wild type LODs for L-lactate, the concentrations of L-lactate were varied from 77 μM to 9.9 mM.

**Thermal inactivation**

Irreversible enzyme inactivation was measured at a protein concentration of 20 μg/ml in a 50 mM phosphate buffer, pH7.0. Some of the samples were incubated at various temperatures for 10 minutes, while others were incubated at 65°C for various time periods. They were then removed from the source of heat and placed on ice. Residual enzyme activities were measured by the method described above.

**Amino acid sequence determination**

N-terminal amino acid sequences were determined by means of an Auto Protein Sequencer PSQ-1 (Shimadzu Co., Japan).

## RESULTS AND DISCUSSIONS

### Sequence of LOD gene

The nucleotide sequence of the LOD-structure gene, including its flanking region, is shown in Fig. 1. The N-terminal amino acid sequence deduced from the largest open reading frame (Fig. 1) agreed with results obtained by amino acid sequencing of LOD purified from the transformants. The molecular weight for the peptide corresponding to the open reading frame is 40930. The molecular weight for active LOD purified from the transformants was estimated to be 160000 by gel filtration (data not shown). These data support the results described by Ducan *et al.* (1989) in which LOD was reported to be a tetramer of identical subunits.

In Table 1., we have listed the discrepancies between our sequencing results and those described in European Patent EP 0376163 A2, 1989. In both our experiment and the patent study, the same strain of *A. viridans* was used for cloning the LOD gene, and amino acid sequences of the same length were deduced. We have confirmed the sequence results shown in Fig. 1 by several sequencings of both strands of the cloned fragments, and we therefore consider the sequence of LOD gene described in the patent is inaccurate.

The thermostable mutant LOD had an A to G base change at position 634 (underlined in Fig. 1) from the start codon in the nucleotide sequence. This base transition resulted in the substitution of an Asn (AAC) by an Asp (GAC) at position 212 in the amino acid sequence.

Table 1. Discrepancies between LOD sequences

| Position | Nucleotide (amino acid) in present work | Nucleotide in published data[1)] |
|---|---|---|
| 126 | T (Ala 42)[2)] | C (Ala 42) |
| 695 | C (Ala 232) | G (Gly 232) |
| 763 | G (Ala 255) | C (Arg 255) |
| 764 | C (Ala 255) | G (Arg 255) |

1) EP 0376163 A2, 1989
2) Corresponding amino acid

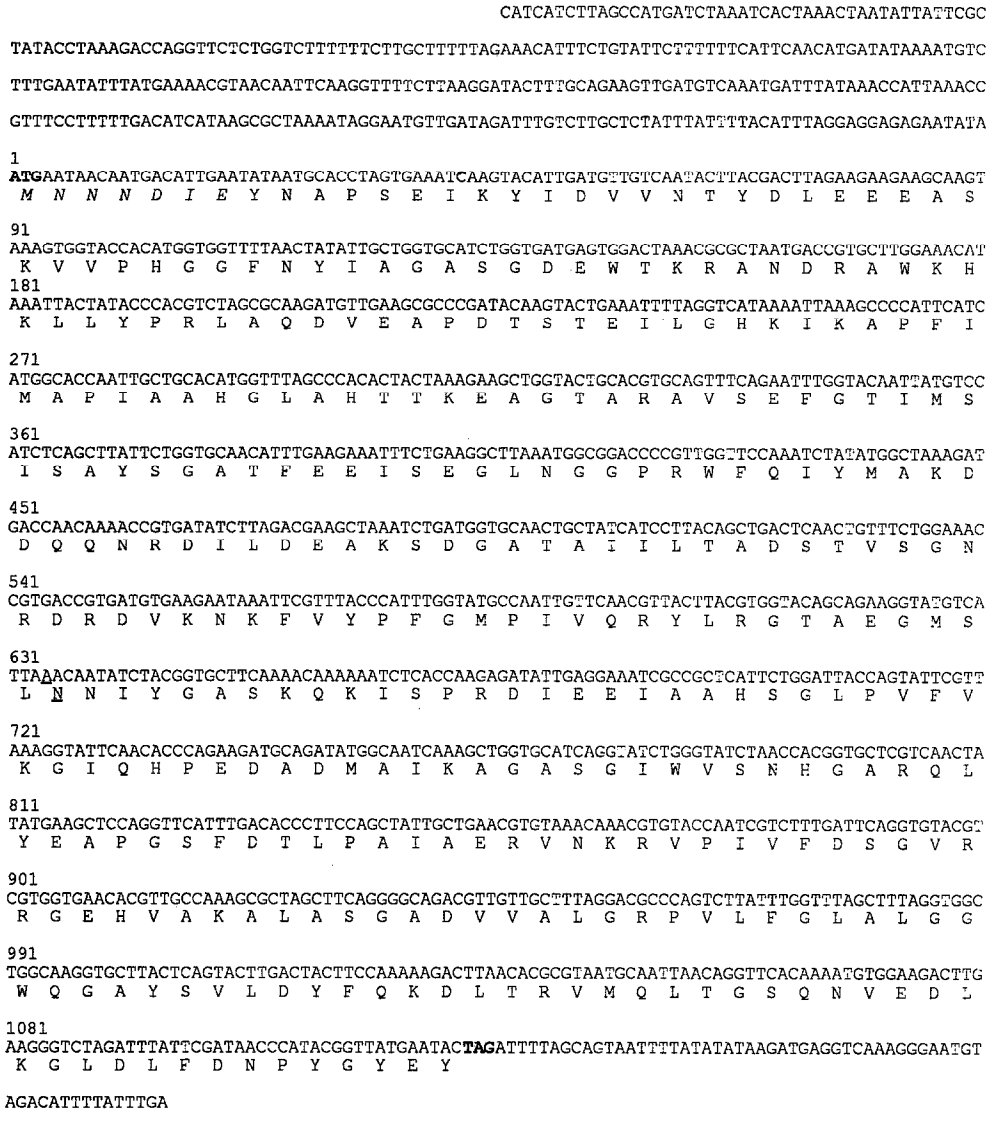


Fig. 1. Nucleotide and predicted amino acid sequence of the wild type LOD

The nucleotide sequence is numbered from the start codon. Start and stop codons are in **boldface**. The amino acid sequence is given below the nucleotide sequence. The sequence of the first seven amino acids (in *italics*) was experimentally determined. The thermostable mutant LOD carried an A to G base change at position 634 (underlined) in the nucleotide sequence.

Table 2. Kinetic parameters of the mutant and wild type LODs

| | Km for L-lactate (mM) | Specific activity (unit/mg protein) |
|---|---|---|
| Mutant LOD | 0.24 | 103 |
| Wild type LOD | 0.28 | 107 |

**Comparison of the thermostability and kinetic parameters between the wild type and the mutant LOD**

The wild type and mutant LODs purified from the *E. coli* transformants were assayed for their activities. The mutant showed no appreciable improvement thermostability at temperatures below 60°C under the experimental conditions (Fig. 2a). At 65°C, however, the half-life ($t_{1/2}$) of the mutant was about 3 times longer than that of the wild type (Fig. 2b). This characteristic of the mutant seems to have enabled us to select the mutant.

The optimal temperature for the catalytic activity of the mutant LOD seems to be almost the same as that of the wild type (Fig. 3). The Km value and specific activity of the mutant LOD for L-lactate also remained almost unchanged (Table 2). Moreover, after 30-day-incubation at 37°C, the mutant LOD retained 68% of the initial activity, while the wild type retained 29%. The mutant LOD is, therefore, functionally identical with the wild type LOD, and has the added merit of improved stability. This suggests, then, the feasibility of producing long-life lactate biosensors using the mutant LOD and following the same designs for lactate biosensors as have heretofore been applied with the wild type LOD as a component.

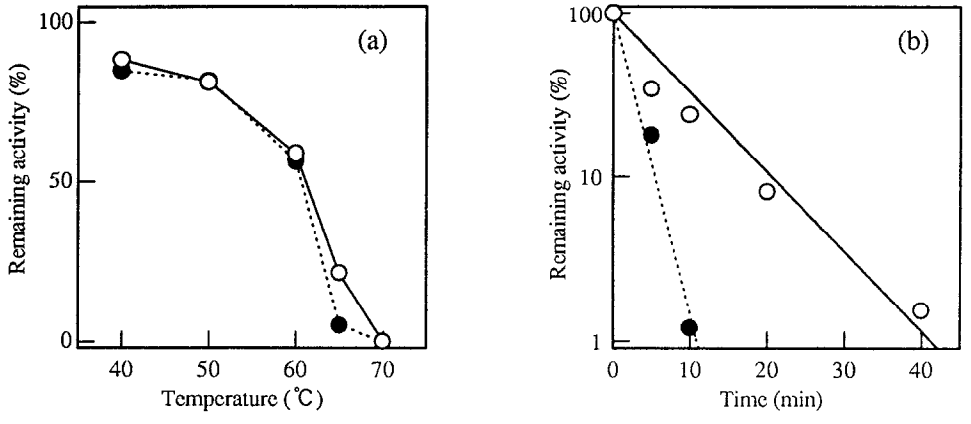


Fig. 2. Thermostability of mutant and wild type LODs

Remaining activities after heating at various temperatures for 10 min (a), or after heating at 65℃ for various time periods (b) are expressed as a percent of the original activity. ○, mutant LOD; ●, wild type LOD

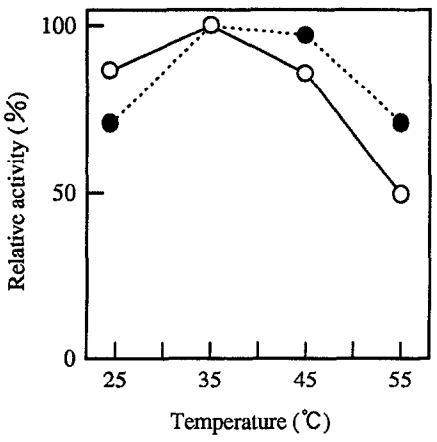


Fig. 3. Temperature dependence of mutant and wild type LODs

Activities for mutant (○) and wild type (●) LODs at various temperatures are expressed as a percentage of the activities at 35℃.

The amino acid sequence of LOD was similar to glycolate oxidase (Cederlund *et al.*, 1988), the three-dimensional structure of which has been reported by Lindqvist (1989). The homology between the LOD and glycolate oxidase (GOX) was approximately 40% (data not shown here). Because of the high amino acid sequence homology between LOD and GOX, the three-dimensional structure of LOD should be deducible from that of GOX by computer modeling (Blundell *et al.*, 1987). Further work is in progress to generate a model structure of LOD in order to reveal how the amino acid substitution (212Asn to Asp) contributes to improving thermostability.

**Nucleotide sequence accession number**: The sequence shown in Fig. 1 will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession number D50611.

## References

Bardeletti, G., Sechaud, F., and Coulet, P. R. (1986). *Analytica Chimica Acta.* **187**, 47-54.
Blundell, T. L., Sibanda, B. L., Sternberg, M. J. E., and Thornton, J. M. (1987). *Nature.* **326**, 347-352.
Cederlund, E., Lindqvist, Y., Söderlund, G., Brändén, C., and Jörnvall, H. (1988). *Eur. J. Biochem.* **173**, 523-530.
Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988). *Nucl. Acids. Res.* **16**, 6127-6145.
Ducan, J. D., Wallis, J. O., and Azari, M. R. (1989). *Biochem. Biophys. Res. Commun.* **164**, 919-926.
Leung, D.W., Chen, E., and Goeddel, D.V. (1989). *Technique-A journal of methods in cell and molecular biology*, **1**, 11-15.
Lindqvist, Y. (1989). *J. Mol. Biol.* **209**, 151-166.
Sanger, F., Nicklen, S., and Coulson, A. R. (1977). *Proc. Natl. Acad. Sci. USA.* **74**, 5463-5467.
Sambrook, J., Fritsch E. F., and Maniatis, T. (1989). *Molecular Cloning: A Loboratory Manual 2nd ed.*, N.Y.: Cold Spring Harbor Laboratory Press.
Toyo Jozo Co., Ltd. (1989). EUROPEAN PATENT. EP 0 376 163 A2.
Wang, D. L. and Heller, A. (1993). *Anal. Chem.* **65**, 1069-1073.