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Molecular Cloning and High-Level Expression of Human Cytoplasmic Superoxide Dismutase Gene in *Escherichia coli*

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사람의 세포질 Superoxide Dismutase 유전자의 클로닝과 대장균내에서의 대량발현에 관한 연구

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ABSTRACT: Complementary DNA (cDNA) coding for human cytoplasmic superoxide dismutase (SOD1) (superoxide: superoxide oxidoreductase E.C.1.15.1.1) was isolated from human liver cDNA library of λgt11 by *in situ* plaque hybridization. The insert cDNA has the 5' untranslational region (UTR) and 3'UTR of SOD1 gene. Polymerase Chain Reaction (PCR) method was used for subcloning of SOD1 structural gene. Using synthetic sense strand primer (24 mer) containing a start codon and antisense strand primer (24 mer), SOD1 structural gene was selectively amplified. Amplified DNA was directly cloned into the *Hin*cII site of pUC19 plasmid. Insert cDNA was subcloned into M13 mp19 and sequenced by dideoxy chain termination method with Sequenase. The nucleotide sequence of insert cDNA had an open reading frame (ORF) coding for 153 amino acid residues. The structural gene of cytoplasmic SOD was placed under the control of bacteriophage λP_L regulatory sequences, generating a highly efficient expression plasmid. The production of human SOD1 in *E. coli* cells was about 7% of total cellular proteins and recombinant human SOD1 possessed its own enzymatic activity.

KEY WORD Human SOD1, Cloning, Expression

Superoxide dismutase (superoxide: superoxide oxidoreductase E.C.1.15.1.1) is the enzyme that catalyzes the removal of superoxide radicals which are generated in a various biological oxidation (Fridovich, 1975). It is thought to be the first line of cellular defense mechanism against the oxidative damage mediated by superoxide anion radicals. This enzyme acts by catalyzing the dismutation of two superoxide radicals to yield hydrogen peroxide and oxygen.

$$2\ O_2^- + 2\ H^+ \longrightarrow H_2O_2 + O_2$$

Two distinct types of SODs possessing different molecular masses, amino acid sequences and different metal ions at the active site have been found in mammalian cells. Cu-Zn containing enzyme is found principally in the cytosol (McCord and Fridovich, 1969), whereas the manganese containing enzyme is found exclusively in the mitochondrial matrix (Weisiger and Fridovich, 1973). Recenly, another type of Cu-Zn SOD was found in human extracellular fluids (Marklund, *et al.*, 1982; Marklund, 1984). In human, these two enzymes are encoded by two separate genes on the chromosomes. The gene coding for cytoplasmic Cu-Zn SOD is located on chromosome 21 (Tan *et al.*, 1973), whereas that for Mn SOD is on chromosome 6 (Creagan *et al.*, 1973). Location

of extracellular SOD gene has not been determined. The human cytoplasmic superoxide dismutase (SOD1) is a dimeric protein composed of apparently identical noncovalently linked subunits (Briggs and Fee, 1978; Jabusch *et al.*, 1980) of known amino acid sequences, each with a molecular weight of 16,000-19,000 dalton (Hartz and Deutsch, 1972; Lieman-Hurwitz, 1981). Mammalian Cu-Zn SOD dimer is an unusually stable protein. It is more thermostable than any characterized globular protein including those from thermophiles (Stellwagon and Wilgus, 1978), remains active in 8M urea and is not dissociated by 4% SDS (Forman and Fridovich, 1973). Moreover, it is resistant to a variety of protease and chemical (Friedman *et al.*, 1978; Malinowski and Fridovich, 1979). Superoxide dismutase has been shown to be anti-inflammatory and to substantially reduce reperfusion damage to the heart, kidney and other organs (Flohe *et al.*, 1985; McCord, 1974). Since every biological macromolecule can serve as a target for the damaging action of the abundant oxygen radicals, interests have evolved in the therapeutic potential of SOD. A wide range of clinical applications has been suggested. These include prevention of oncogenesis and tumor promotion, reduction of the cytotoxic and cardiotoxic effects of anticancer drugs (Oberley and Beuttner, 1979), anti-inflammatory action (Huber and Menander, 1980) and protection against reperfusion damage of ischemic tissues (Fridovich, 1983). In addition, there are much interests in studying the effect of SOD on the aging process (Talmasoff *et al.*, 1977). In this report, the cloning and expression in *E. coli* of cDNA gene encoding human cytoplasmic SOD (SOD1) were performed to understand its function in relation to therapeutic potential.

MATERIALS AND METHODS

Materials

The enzymes used in this study were purchased from suppliers as follows; Taq DNA polymerase from Cetus, restriction endonuclease from New England Biolabs (NEB), KOSKO and Boehringer Mannheim. T4 DNA kinase from NEB. Nitrocellulose filter papers were purchased from Schleicher & Schuell and Gelman Sciences. DNA sequencing kit containing Sequenase was purchased from U.S. Biochemical. Radioactive materials (α-^{32}P-ATP, γ-^{32}P-ATP) were purchased from Amersham. MacConkey agar was obtained from Difco. 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma. Other materials were obtained from Sigma and Aldrich. All materials used in this experiment are laboratory grade.

Bacterial strains and growth

E. coli Y1090 (ΔlacU169, pro A, Δ lon, araD139, supF) was used for infection with λgt11 containing human liver cDNA library from Clontech co.. HB101 (F^-, r^-, m^-, recAB, lacY1), JM83 (ara$^-$, Δ(lac-proAB), rpsL, thi, ψ 80, lacZ ΔDM15) were generally used for plasmid growth and preparation. JM101 (supE, thi, Δ(lac-proAB), F^-, $LacI^q$, Δ DM15) was used for the selection of colonies containing recombinant DNA on X-gal, IPTG plates and transfection with M13 phage. *E. coli* Y1090 was grown in LB containing 0.2% maltose and transformed cells grown in LB containing appropriate antibiotics (Maniatis *et al.*, 1982). JM83 was used for the selection of colonies containing recombinant plasmid DNA by color on MacConkey agar plate. HB101-clts, HB101 containing a plasmid with cI857 gene, was used for the expression of human cytoplasmic SOD (SOD1).

Oligonucleotide primers and probes

Oligonucleotides were synthesized with an automated DNA synthesizer (Beckman) at KIST, Korea. Primers were designed for *in situ* plaque hybridization and polymerase chain reaction (PCR). The primer1 (P1) containing ATG codon of SOD1 gene and the primer3 (P3) possessing 3'UTR region of SOD1 gene were used in PCR. P2 containing internal sequence of the structural gene was used in Southern blotting and sequencing with P3. The sequences of primers are follows;

P1: 5'-CTAGCGAGTTATGGCGACGAAGGC-3' (24-mer)

P2: 5'-GGATTAAAGTGAGGACCTGC-3' (20-mer)

P3: 5'-CAGGATACATTTCTACAGCTAGCA-3' (24-mer)

In situ plaque hybridization

In situ plaque hybridization of the cDNA library was carried out as described (Maniatis *et al.*, 1982), with a slight modification. Radioactive probes were prepared by the 5'end labeling of the oligonucleotide with T4 polynucleotide kinase and (γ-^{32}P)-ATP (3000 Ci/mmole). Nitrocellulose filters, containing the transferred phages, were prehybridized in prehybridization solution containing 6X SSC, 5X Denhardt's solution, 0.1% sodium dodecyl sulfate, 100 μg/ml salmon sperm DNA and hybridized in hybridization solution containing 6X SSC, 1X Denhardt's solution, 100 μg/ml yeast RNA, 0.05% sodium pyrophosphate (20X SSC; 3M NaCl, 0.3M sodiumcitrate: 50X Denhardt's solution; 1% ficoll, 1% polvinyl-

pyrrolidone, 1% bovine serum albumin) at 42 °C for 18 hours, and then washed five times in 6X SSC/0.1% sodium pyrophosphate at 50 °C. The washed filters were dried and exposed to X-ray film overnight. The secondary screening was carried out as same procedure.

Phage DNA preparation and subcloning

Recombinant phage DNA was prepared by procedure of Maniatis *et al.* (Maniatis *et al.*, 1982). The isolated recombinant phage DNA was digested with *Eco*RI and analyzed by 0.8% agarose gel electrophoresis. The insert cDNA was subcloned into pUC19 vector. Transformation of *E. coli* JM83 was carried out according to the procedure of Maniatis *et al.*

Southern blotting

Southern transfer was carried out as described by Maniatis *et al.* For Southern blot analysis, DNA was separated by 0.8% agarose gel electrophoresis, transferred onto nitrocellulose filter paper and hybridized with (^{32}P)-end labeled probes (P2). Hybridization was carried out as described previously in aqueous solution.

Polymerase chain reaction (PCR)

The PCR was performed by a modification of the method originally described by Saiki *et al.* (Saiki *et al.*, 1985). Template DNA (100-1000 ng) was diluted into 100 μl of a solution containing 200 μM (each) dATP, dGTP, dCTP, and dTTP; 1 μM of each primers (P1 and P3), 50 mM KCl, 10 mM Tris (pH 8.4), 2.5 mM $MgCl_2$, 0.02% gelatin (w/v). After overlaying with 100 μl of light mineral oil (Janssen Chimica), the mixture was heated at 94 °C for 3 minutes, then the mixture slowly cooled to 37 °C, Taq DNA polymerase was added. Sequentially, primer extension at 70 °C for 5 minutes, heat denaturation at 94 °C for 1.5 minutes and primer annealing at 42 °C for 3 minutes were carried out 20 cycles. Amplified cDNA fragment was shown by 1% agarose gel electrophoresis.

Nucleotide sequencing

Amplified cDNA fragment was subcloned into M13mp19 phage using *Pst*I, *Eco*RI. Sequencing was performed with a slight modification of protocols from U.S. Biochemical. Single stranded recombinant phage DNA was purified (Kim *et al.*, 1988) and sequenced by the chain termination reaction method (Sanger *et al.*, 1977) using either M13 universal primer (NEB) or specific oligonucleotide primer P2 or P3. Single stranded DNA of 1 μg was added to reaction buffer with 0.5 pmole of primer (40 mM Tris-Cl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl). The mixture was heated for 2 minutes at 65 °C, then cooled slowly to room temperature. Labeling reactions were accomplished by Sequenase at room temperature for 5 minutes with (α-^{32}P) dATP (600 Ci/mmole). Extension reaction was terminated by adding each dideoxynucleotide. After adding the stop solution, the mixture was heated to 75 °C for 2 minutes and immediately loaded to 6% sequencing gel. The samples were runned at 2000 volts for 4-6 hours.

Induction of expression

E. coli strain HB101-clts, harboring SOD1 expression plasmid, was grown at 32 °C in LB containing the appropriate antibiobics until OD_{600} = 0.7. Induction was performed by a temperature shift to 42 °C for various time intervals. In induction experiments, the culture medium was supplement with $CuSO_4$ and $ZnSO_4$ to final concentrations 200 ppm, 2 ppm respectively. Crude extracts of bacteria containing plasmid pSOD1-PL were analyzed on a 12% SDS-polyacrylamide gel before and after induction. Also, cells were harvested by centrifugation and suspended in 50 mM potassium phosphate buffer (pH 7.3). Harvested cells were sonicated for 5 × 30 seconds in a sonicator and partitioned to soluble and insoluble fractions by centrifugation at 10,000g for 5 minutes. Soluble fraction was assayed by pyrogallol Method to detect SOD1 activity (Marklund and Marklund, 1974).

Enzymatic assays of SOD1

Gel electrophoresis was performed in 10% polyacrylamide slab gel. Gel and running buffer were without SDS and samples were not heated prior to electrophoresis. Superoxide dismutase was localized by soaking the gels in 1 mM nitrobluetetrazolium (NBT) for 10 minutes, followed by immersion for 15 minutes in a solution containing 20 mM tetramethylethylenediamine, 0.25 μM riboflavin and 36 mM potassium phosphate at pH 7.8. The gel was then placed in a glass tray and illuminated for 5-15 minutes with fluorescence light. During illumination the gel became uniformly blue except at positions containing superoxide dismutase. Illumination was discontinued when maximum contrast between the clear zone and the blue formazan region had been achieved.

RESULTS AND DISCUSSIONS

Cloning of human superoxide dismutase cDNA

Isolation of human cytoplasmic superoxide dismutase (SOD1) cDNA in human liver cDNA library of λgt11 was carried out by *in situ* plaque hybridization (Maniatis *et al.*, 1982) with mixed oligonucleotide probe (P1, P2 and P3 in Materials and Methods). Among 100,000 phages, four showed positive signals, but only one clone was found to contain a

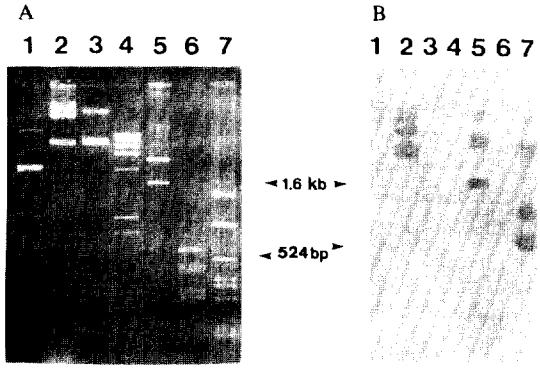

Fig. 1. *Restriction endonuclease mapping and Southern blotting of pSOD1.6K.*
(A): Agarose gel electrophoresis of pSOD1.6K digested with restriction endonucleases. lne 1; pUC19, lane 2; pSOD1.6K, lane 3; pBR322, lane 4; laboratory linear size marker, lane 5; pSOD1.6K + *Eco*RI, lane 6; pUC19 + *Hae*III, lane 7; pSOD 1.6K + *Mae*I. (B): Southern blotting with [^{32}P]-end labeled P2.

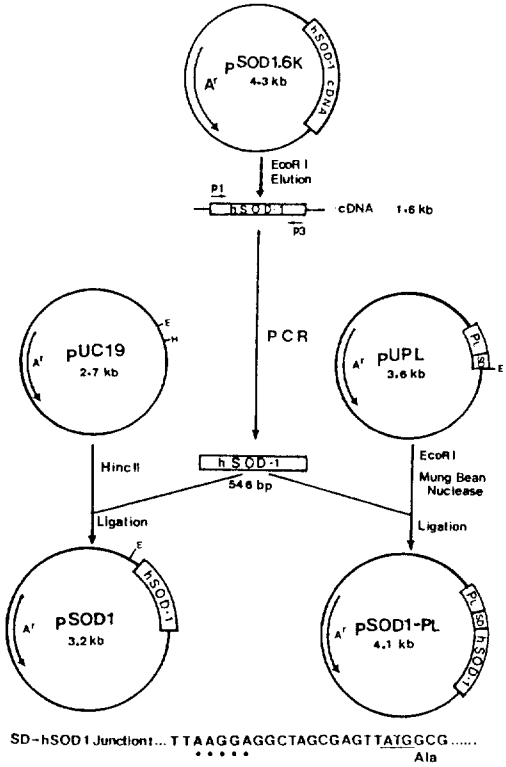

Fig. 2. *Construction of pSOD1 and pSOD1-PL.*
The following abbreviations are used: E, *Eco*RI; H, *Hin*cII; A^r, ampicillin resistant gene; PCR, polymerase chain reaction; PL, λP_L promoter-operater; SD, Shine-Dalgarno sequence. The sizes of fragments and plasmids are not exactly to scale.

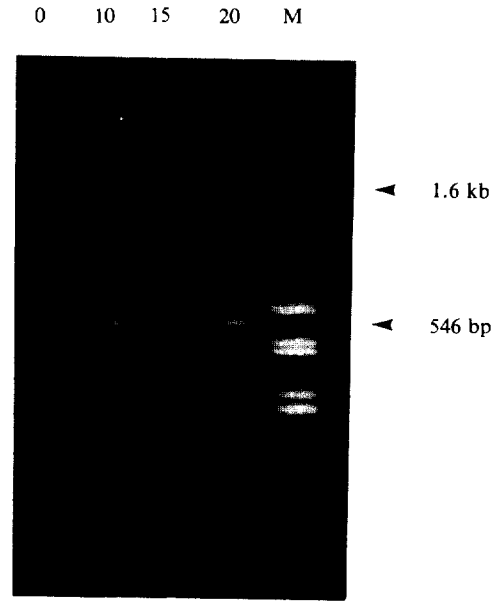

Fig. 3. *Agarose gel electrophoresis of the amplified SOD1 gene by PCR.*
A band of 546 bp fragment appears at the predicted location.

human cytoplasmic SOD cDNA by second screening. Recombinant phage DNA was purified, digested with *Eco*RI and subcloned into pUC19 vector. This plasmid containing cDNA insert was purified and digested with *Eco*RI. The insert cDNA size was about 1.6 kilo base pair (kb). The resulting plasmid was designated as pSOD1.6K. Restriction endonuclease mapping and Southern blot analysis of pSOD1. 6K was performed (Fig. 1). It showed that pSOD1.6K possessed a human cytoplasmic SOD gene (SOD1) and contained 5'UTR and 3'UTR of SOD1 gene. To subclone the structural gene of SOD1, polymerase chain reaction (PCR) was accomplished as described in Materials and Methods with primer P1 and P3 (Fig. 2). As a template DNA, electroeluted cDNA insert (1.6 kb) was used. After tewnty cycles of PCR reaction, aliquots of PCR mixture were runned in 1% agarose gel. As shown in Fig. 3, a band of the expected size was obtained. Southern blotting of PCR product was also carried out with [^{32}P]-labeled probe (data not shown). It was proved that PCR product contained a SOD1 gene. PCR product was directly subcloned to *Hin*cII site of pUC19 plasmid for sequencing and the *Eco*RI site of pUPL plasmid for the expression of human SOD1 gene in *E. coli* (Fig. 2).

Nucleotide sequence of SOD1 gene cDNA

The DNA fragment of pSOD1 codigested with

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                Mae I          +1
1       c tag cga gtt ATG GCG ACG AAG GCC GTG TGC GTG CTG AAG GGC      43
                      MET Ala Thr Lys Ala Val Cys Val Leu Lys Gly

44        GAC GGC CCA GTG CAG GGC ATC ATC AAT TTC GAG CAG AAG GAA      85
          Asp Gly Pro Val Gln Gly Ile Ile Asn Phe Glu Gln Lys Glu

86        AGT AAT GGA CCA GTG AAG GTG TGG GGA AGC ATT AAA GGA CTG     127
          Ser Asn Gly Pro Val Lys Val Trp Gly Ser Ile Lys Gly Leu

128       ACT GAA GGC CTG CAT GGA TTC CAT GTT CAT GAG TTT GGA GAT     169
          Thr Glu Gly Leu His Gly Phe His Val His Glu Phe Gly Asp
                *
170       AAT ACA GCA GGC TGT ACC AGT GCA GGT CCT CAC TTT AAT CCT     211
          Asn Thr Ala Gly Cys Thr Ser Ala Gly Pro His Phe Asn Pro

212       CTA TCC AGA AAA CAC GGT GGG CCA AAG GAT GAA GAG AGG CAT     253
          Leu Ser Arg Lys His Gly Gly Pro Lys Asp Glu Glu Arg His

254       GTT GGA GAC TTG GGC AAT GTG ACT GCT GAC AAA GAT GGT GTG     295
          Val Gly Asp Leu Gly Asn Val Thr Ala Asp Lys Asp Gly Val

296       GCC GAT GTG TCT ATT GAA GAT TCT GTG ATC TCA CTC TCA GGA     337
          Ala Asp Val Ser Ile Glu Asp Ser Val Ile Ser Leu Ser Gly

338       GAC CAT TGC ATC ATT GGC CGC ACA CTG GTG GTC CAT GAA AAA     379
          Asp His Cys Ile Ile Gly Arg Thr Leu Val Val His Glu Lys

380       GCA GAT GAC TTG GGC AAA GGT GGA AAT GAA GAA AGT ACA AAG     421
          Ala Asp Asp Leu Gly Lys Gly Gly Asn Glu Glu Ser Thr Lys

422       ACA GGA AAC GCT GGA AGT CGT TTG GCT TGT GGT GTA ATT GGG     463
          Thr Gly Asn Ala Gly Ser Arg Leu Ala Cys Gly Val Ile Gly

464       ATC GCC CAA TAA aca ttc cct tgg atg tag tct gag gcc cct     506
          Ile Ala Gln TER
                                      Mae I
506       taa ctc atc tgt tat cct gct agc tgt aga aat gta tcc tg     546
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Fig. 4. *Nucleotide sequence and predicted amino acid sequence of the cloned human cytoplasmic SOD (SOD1).* The nucleotide sequence is identical to that by established by Sherman *et al.* except of one base at 175 (A instead of G). * shows a mismatched base with previous report.

*Eco*RI and *Pst*I was subcloned into the replication form (RF) M13mp19 DNA. The ligated DNA was transfected to JM101 as described (Kim, *et al.* 1988). Recombinant M13mp19 was selected on X-gal, IPTG plate by difference of color. Using the obtained single stranded recombinant phage DNA, DNA sequencing was performed as described in Materials and Methods with universal primer (NEB), P2 and P3. The nucleotide sequence of cloned human SOD1 cDNA was identical to that of previously reported one (Sherman, *et al.*, 1983) except one base change at 175 (G ⟶ A) but the amino acid sequence remained unchanged (Fig. 4).

Expression of human SOD1 in *E. coli*

The structural gene of human SOD1 (546 bp fragment) amplified by polymerase chain reaction was placed under control of bacteriophage λP_L regulatory sequence generating a highly efficient expression plasmid, pSOD1-PL (Fig. 2). The distance of between the Shine-Dalgarno sequence and the ATG translation start codon was then adjusted to 12 bp for the optimal expression in *E. coli*. The *E. coli* (HB101-clts) containing the plasmid produced human SOD1 protein to about 7% of total cellular proteins. The *E. coli* strain, HB101-clts, produces constitutively the thermolabile repressor cI857 at 32 °C binding to O_L and preventing transcription from the strong P_L promoter. Upon temperature shift to 42 °C, repression is

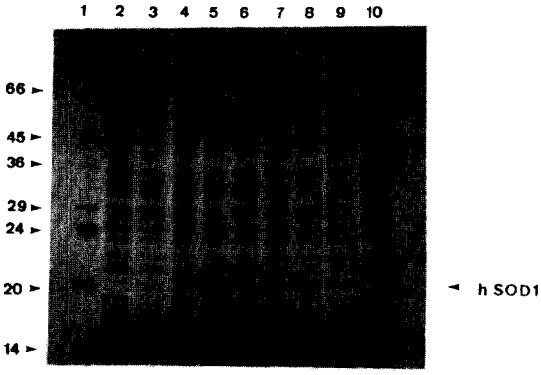

Fig. 5. *Induction of human SOD1 by heat shift.*
E. coli strain HB101-cIts, carrying pSOD1-PL, was grown at 32 °C in LB medium with 100 μg of ampicillin per m*l*. Heat shift to 42 °C was performed, when cells reach $OD_{550} = 1.0$. Uninduced sample was taken just prior to heat induction (lane 2). Induced samples were taken at time 10, 40, 70, 120, 240 minutes and 12 hours after heat shift (lane 3-8, respectively). After sonication, soluble fraction and insoluble pellet were also loaded (lane 9 and 10). Protein standards (in kDa) (Sigma) are indicated on the left (lane 1). All samples were electrophoretically analyzed on a 12% SDS-polyacrylamide gel. Gel was stained with Coomasie brilliant blue.

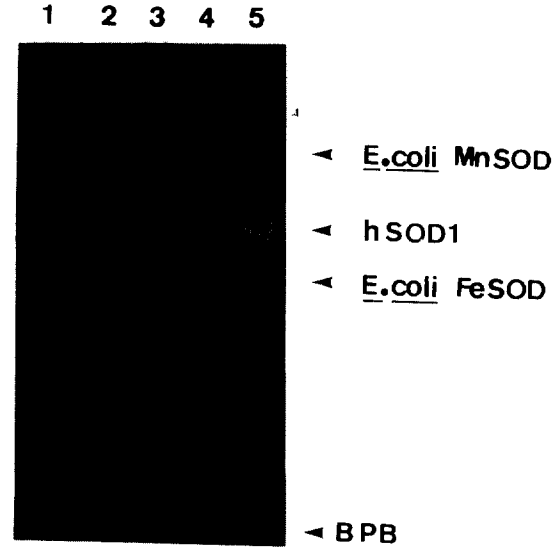

Fig. 6. *Activity of overproduced human SOD1*
Extracts were electrophoresed through 10% polyacrylamide gel under non-denaturing condition. Lane 1 is 1 μg of human erythrocyte cytoplasmic SOD (SOD1); Lane 2 and 3 are extracts of uninduced culture HB101-cIts/pSOD1-PL, grown in presence of Cu^{++} supplementation of 200 ppm; Lane 4 and 5 are extracts of induced culture under the same condition. Staining for SOD activity was performed according to NBT method.

abolished permitting transcription. Induced culture accumulated human SOD1 up to 4 hour, at which stage the expressed protein was about 7% of total proteins in HB101-cIts as determined by densitometric scanning (Fig. 5, lane 7). The expressed SOD1 has apparently a molecular mass of about 20 kDa in Coomassie blue stained gel shown in Fig. 5, which is slightly larger than that of the predicted molecular mass (16 kD) in nucleotide sequence. The induced band appears mainly in the soluble fraction (Fig. 5, lane 9) because the expressed SOD1 is soluble in the cytoplasm of *E. coli*. The soluble fraction of HB101-cIts/pSOD1-PL induced at 42 °C was electrophoresed on a polyacrylamide gel, and the position of the SOD activity was visualized by the NBT method (Beauchamp, C. and Fridovich, I., 1971). A new major SOD activity was appeared in lane of heat-induced HB101-cIts/pSOD1-PL soluble fraction (Fig. 6, lane 4 and 5), which was distinguishable from that of uninduced one. The activity of the expressed human SOD1 was at least 10 folds as much as that of total *E. coli* SODs. This shows that human SOD1 is highly expressed in *E. coli* and the expressed SOD1 possessed its own enzymatic activity.

적 요

생체내의 유해산소를 제거하는 superoxide dismutase (superoxide : superoxide oxidoreductase E.C.1.15.1.1) 중 세포질내에서 그 활성을 지니는 인체의 세포질 superoxide dismutase (SOD1) 유전자를 사람의 간 cDNA library로부터 동위원소로 표지된 oligonucleotide probe를 이용, *in situ* plaque hybridization 방법으로 선별 분리하여 대장균 벡터로 클로닝하였다. 이 클론은 SOD1 유전자의 5'UTR과 3'UTR을 포함한 1.6 kb 정도의 cDNA였다. SOD1 구조유전자만을 선택적으로 분리하기 위해서 ATG를 포함하는 sense strand primer와 3'UTR 부위의 antisense strand primer를 이용하여 중합효소연쇄반응 (Polymerase Chain Reaction) 방법을 써서 SOD1 구조유전자 부위만을 선택적으로 증폭시켰다. Taq DNA polymerase에 의해 증폭된 DNA를 벡터 pUC19의 multiple cloning site (MCS) 내의 *Hinc* II 위치에 넣었으며 이 insert DNA를 M13 mp19으로 옮겨 dideoxy chain termination 방법으로 sequenase를 사용하여 염기서열을 결정하였다. 클론닝된 cDNA는 153개의 아미노산을 코딩하는 하나의 open reading frame (ORF)을 가졌다. 중합효소연쇄반응에 의해 증폭된 SOD1 구조유전자를 λP_L 프로모터를 포함하고 있는 발현 벡터 pUPL에 옮긴 후 대장균에서 대량으로 발현시켰다. 이 때 발현된 단백질 SOD1은 고유의 효소활성을 가지고 있었다.

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