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CHEMICALLY-SYNTHESIZED GENE ENCODING MODIFIED HUMAN SUPEROXIDE DISMUTASE: ITS CONSTRUCTION, EXPRESSION AND PROPERTIES OF THE PRODUCT

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The gene encoding modified human superoxide dismutase (h-SOD) with 153 amino acid residues was constructed by chemical synthesis using the phosphoramidite method. The gene was designed so as to use bacterial codons for expression in prokaryotes and to introduce several unique restriction sites for further mutagenesis by the cassette exchange method. The distance between Shine-Dalgarno sequence and initiation codon was adjusted to maximum expression by using synthesized oligonucleotide. In addition, Cys 6 of h-SOD was changed to Ala to improve instability of native h-SOD.

Synthesized structural gene of h-SOD was expressed in *E. coli* after induction of isopropyl β-D-thiogalactoside by inserting the gene into the expression vector pKK223-3 having *tac* promoter. The gene that has 10 base pairs between Shine-Dalgarno sequence and initiation codon showed the most efficient expression. The gene produced three active SOD isomers as revealed by chromatofocusing.

The main isomer was purified to homogeneity and characterized. The h-SOD-Ala[6] showed similar properties to those of native h-SOD with respect to molecular weight, subunit structure, absorption spectrum, but the modified SOD was more resistant to heat denaturation than was native h-SOD; half-denaturing temperature was shifted by 10 °C. Thus, the exchange of Cys 6 to Ala of h-SOD increased a stability of the enzyme.

KEY WORDS: Superoxide dismutase, gene, mutagenesis, chemical synthesis, protein engineering, cystein residue.

INTRODUCTION

Superoxide dismutase (SOD) reduces the steady state concentration of the superoxide radical in cells by catalyzing its disproportionation, thus protecting cells from the toxic effects of superoxide itself and of other superoxide derived-reactive oxygen species, i.e. hydrogen peroxide, the hydroxyl radical, and excited singlet oxygen. Involvement of superoxide in many diseases has been well recognized, and hence considerable interests have been focused on the therapeutic use of SOD.[1] Since the human enzyme has the potential therapeutic value, human CuZn-SOD (h-SOD) has been produced by recombinant techniques in *E. coli*,[2–4] yeast[5] and mammalian cells.[6] Mutagenesis of h-SOD by molecular engineering has also been reported.[7]

h-SOD, *in vitro*, is relatively unstable compared to CuZn-SOD from other organisms, but the reason for this is not elucidated. In addition, the presence of charge isomers of h-SOD is noticeable,[8] but its generation mechanism is not clear. h-SOD has four cysteine residues at the positions of 6, 57, 111 and 146.[9,10] Among them, the Cys 57 and Cys 146 form an intramolecular disulfide bond which is a structural feature of all CuZn-SODs sequenced so far, but the Cys 6 and Cys 111 remain as free SH

groups. Briggs and Fee suggested that either of free SH group is reactive and forms an adduct complex.[8] Thus, it seems possible to improve instability of native h-SOD by changing Cys 6 and/or Cys 111 to other amino acid residues.

In this paper, we designed and constructed the h-SOD gene by chemical synthesis based on the known nucleotide sequence,[11] expressed the gene in *E. coli*, and characterized the gene product. Furthermore, in an attempt to address the problems of instability of h-SOD, we attempted to substitute Cys 6 of h-SOD to Ala, since CuZn-SOD from most plants and fungi have Ala at this position.[12] This selection is based on the assumption[12] that plant CuZn-SODs have evolved under the severe pressure of the photo-oxidative stress, which is easily realized in the environments that plants grow.

MATERIALS AND METHODS

Materials

Cyt *c* (horse heart, type III), h-SOD (erythrocyte) and isopropyl-β-D-thiogalactoside (IPTG) were obtained from Sigma, xanthine oxidase (bovine milk) was from Boehringer, dimethoxytrityl nucleoside phosphoramidites from Applied Biosystems, expression vector pKK223-3 from Pharmacia, Nensorb 20 from Dupont, IsoGel plates from FMC. Restriction endonucleases, T4 DNA ligase and enzymes used in DNA work were purchased from Takara Co. Ltd., Kyoto. Kit of M13 cloning and dideoxy sequencing using 2′-deoxy-7-deazaguanosine triphosphate was obtained from Toyobo Co. Ltd., Osaka. Rabbit antiserum against h-SOD was obtained from Cosmobio Co. Ltd., Tokyo.

Methods

Deoxyoligonucleotides were chemically synthesized by the phosphoramidite method[13] using an Applied Biosystems DNA synthesizer, model 380A, and purified by a reverse phase HPLC. Agarose and polyacrylamide gel electrophoresis of DNA, electroelution of DNA from gel, purification of DNA, plasmid preparation, ligation, Western blotting, transformation of bacteria were performed according to Maniatis *et al.*[14] and Davis *et al.*[15] M13 cloning and dideoxy sequencing were performed according to the instruction manual of supplier's.

SOD assay and definition of the activity, polyacrylamide gel electrophoresis (PAGE), SDS-PAGE and determination of protein were performed as described previously.[16] The sequence of the NH_2-terminal region was determined using an Applied Biosystems protein sequencer, model 477A.

RESULTS AND DISCUSSION

Construction of CuZn-SOD Structural Gene

The sequence of cDNA for h-SOD has been reported by Sherman *et al.*[11] Based on this sequence, we designed the gene of h-SOD as shown in Figure 1. In our strategy, bacterial codons were used for expression in *E. coli* and several unique restriction sites were introduced at the position of every 30–40 bases for further mutagenesis by

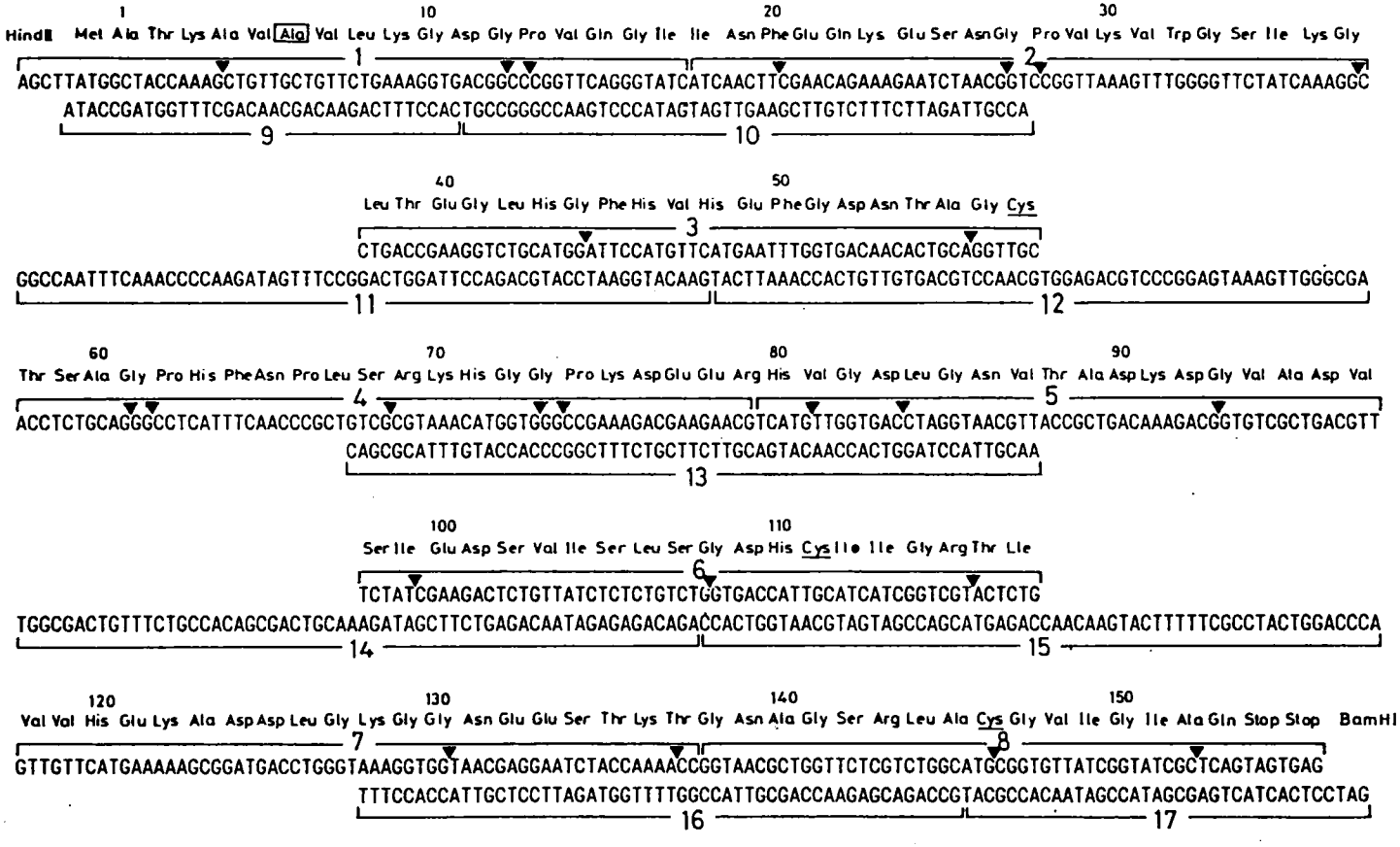

FIGURE 1 Nucleotide sequence of the chemically-synthesized human CuZn-SOD. The sequence, based on the cDNA for human CuZn-SOD reported by Sherman *et al.*,[11] was divided into 17 parts and each part was chemically synthesized as described in the text. Box indicates the substituted amino acid residue (Cys → Ala). Cys residues are underlined. Several unique restriction sites (▼) were introduced at the position of every 30–40 bases for further mutagenesis by cassette exchange.

cassette exchange. As mentioned in Introduction, h-SOD is rather unstable, and to improve instability of native h-SOD, we substituted Cys 6 of h-SOD to Ala.

Seventeen oligonucleotides consisting of 35–60 bases for both strands shown in Figure 1 were chemically synthesized. After purification by HPLC and phosphorylation of 5′ terminus of each oligonucleotide except oligonucleotides 1 and 17 which are located at the NH_2- and COOH-terminals of SOD gene, three to four oligonucleotides were assembled to make a block by annealing and ligation with T4 DNA ligase. The blocks were separated by polyacrylamide gel electrophoresis and purified by Nensorb

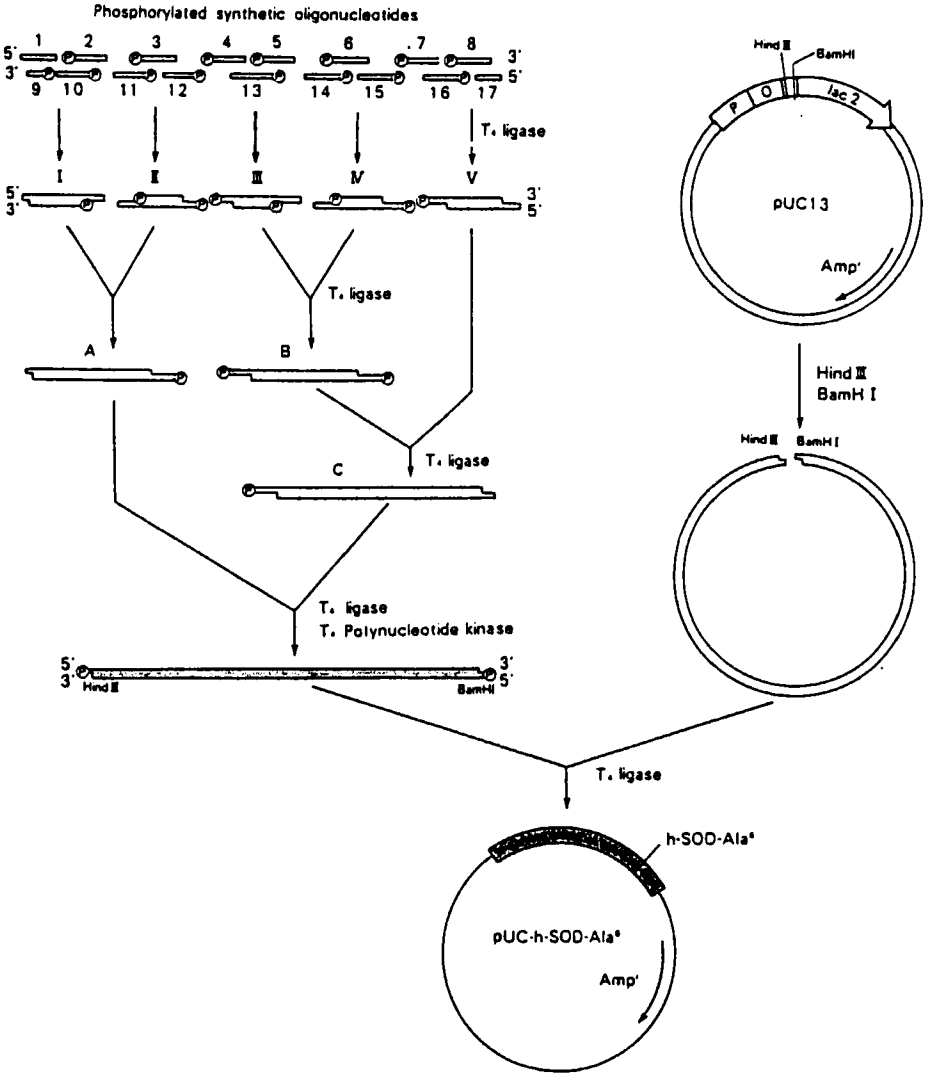

FIGURE 2 Schematic representation of the construction of human CuZn-SOD structural gene. Order of the block synthesis of the gene is shown. Numbers of oligonucleotides are given in Figure 1. Details are given in the text.

20. Finally, the blocks were similarly assembled to produce full-length SOD gene, and the gene was inserted into Hind III and Bam HI sites of pUC13 and cloned in *E. coli* JM109 on ampicillin plate. Thus, h-SOD-Ala^{6} gene encoding 153 amino acid residues was obtained (Figure 2). The sequence of the synthesized gene was confirmed by the M13 dideoxy methods.

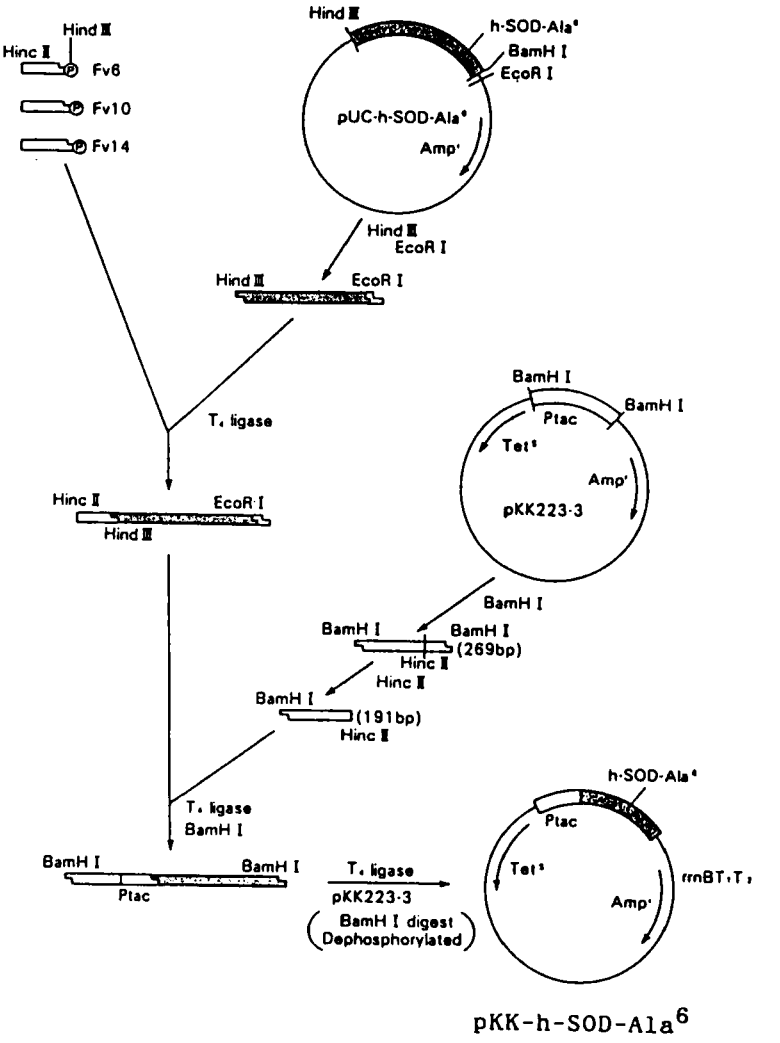

FIGURE 3 Schematic representation of the construction of expression vector, pKK-h-SOD-Ala^{6}. Fv6, Fv10 and Fv14 are synthesized fragments having Shine-Dalgarno sequence region and Hind III junction site as:

Fv6, – AGGAA Fv10, - AGGAAACAA
– TCCTTTCGA – TCCTTTGTTTCGA

Fv14, - AGGAAACAGCTAA
- TCCTTTGTCGATTTCGA Details are given in the text.

Expression of the gene in E. coli

We used the expression vector pKK223-3 having *tac* promoter to express the structural SOD gene in *E. coli*. It is well established that base numbers between Shine-Dalgarno (SD) sequence and ATG start codon affects the efficiency of translation.[17] Thus, in order to optimize the expression of the gene, three Hinc II-Hind III fragments of *tac* promoter of pKK223-3 differing in the length between SD and ATG, of 6, 10, and 14 bp, were chemically synthesized (Figure 3). Each fragment was linked with the structural SOD gene which was excised by Hind III and Eco RI from pUC-h-SOD-Ala^6 (Figure 3). The rest region of the *tac* promoter was excised as Bam HI fragment having 269 bp from pKK223-3, then Bam HI-Hinc II fragment (191 bp) was obtained by Hinc II digestion followed by separation on agarose gel. This fragment was ligated with the three sets of Hinc II-Eco R1 fragment then treated with Bam HI, producing the Bam HI fragments consisting of *tac* promoter and SOD structural gene. Finally, these Bam HI fragments were inserted into Bam HI sites of the expression vector pKK223-3 which was digested with Bam HI and dephosphorylated. *E. coli* JM105 were transformed with the plasmids pKK-h-SOD-Ala^6 and the clones were obtained on ampicillin plate.

We compared the amount of expressed h-SOD-Ala^6 after induction by 1 mM IPTG by Western blotting. The results showed that the plasmid having 10 bp between SD and ATG gave rise most efficient expression, followed by the plasmid having the length of 6 bp. Little expression was observed with the plasmid having the length of 14 bp (data not shown).

Characterization of h-CuZn-SOD-Ala^6

We characterized the gene product using the plasmid having 10 bp between SD and ATG. The enzyme was produced in *E. coli* by IPTG induction and purified by ammonium sulfate fractionation, ion-exchange chromatography, gel-filtration, chromatofocusing and hydrophobic chromatography. On chromatofocusing, three isomers of h-SOD-Ala^6 occurred at a peak ratio of 5:1:1. Native h-SOD also shows three isomers. The SOD of the major peak (peak I) was further purified and characterized. The purified h-SOD-Ala^6 showed a single protein band on native PAGE, but was less migrated than native h-SOD, in accordance with the fact that NH_2-terminus of the native h-SOD is acetylated but the recombinant SOD is not. Sequencing of the NH_2-terminal region of the recombinant SOD by Edman degradation up to 14 residues confirmed the replacement of 6th amino acid residue with Ala and the identical sequence to the native enzyme except unblocked Ala at the NH_2-terminus. Isoelectric focusing revealed the pI of 5.22 for peak I, which is higher than those of native h-SOD (major isomer) by 0.2.

The purified enzyme also showed a single protein band on SDS-PAGE and had the same mobility as native h-SOD indicating that the recombinant and native SODs have the same subunit molecular weight of 16,000. Molecular weight of h-SOD-Ala^6 was determined to be 32,000 by gel filtration. Thus, h-SOD-Ala^6 is a homodimers without disulfide linkage between subunits as is native h-SOD. Absorption spectrum of h-SOD-Ala^6 was identical to that of the native enzyme, exhibiting the absorption peaks at 259, 265, 268, 279 and 670 nm (data not shown). Molar absorbance coefficient at 670 nm due to the copper chromophore was varied depending on the culture conditions in which the amounts of copper and zinc were changed. A linear relation-

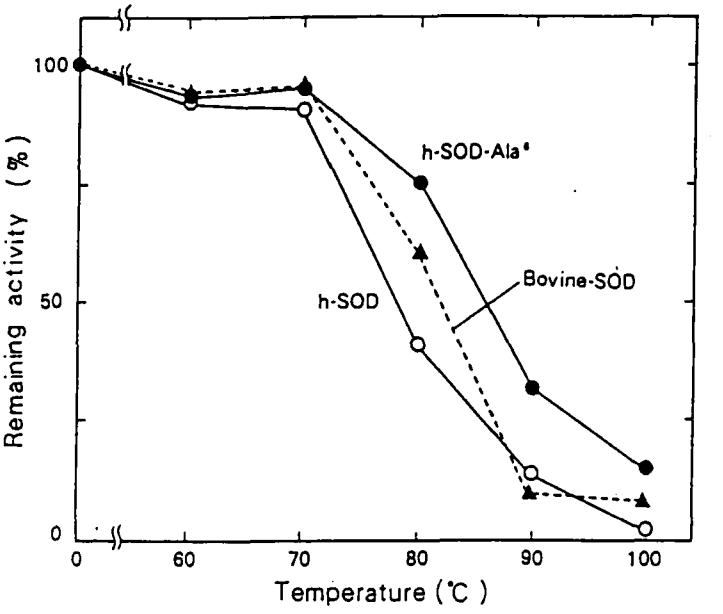

FIGURE 4 Heat stability of native and modified Cu-Zn-SODs. Each enzyme (10 units) in 20 μl of 10 mM potassium phosphate (pH 7.8) was incubated in a tube at the indicated temperatures. Aliquots were withdrawn after 10 min and the residual activity was assayed under the standard conditions at 25°C. h-SOD, native human CuZn-SOD; h-SOD-Ala^6, modified human CuZn-SOD; Bovine-SOD, bovine CuZn-SOD.

ship was observed between the specific activities and the coefficients at 670 nm of different preparations.

The effect of the substitution of amino acid at 6th residue of h-SOD was determined by the response against denaturing stress of heat. The h-SOD-Ala^6 was more resistant to heat denaturation than native h-SOD; half denaturing temperature was shifted by 10 °C (Figure 4). Thus, the exchange of Cys to Ala of h-SOD improved the stability of the enzyme. The elucidation for the mechanism of improvement is now under way.

In short, we constructed the h-SOD structural gene which has bacterial codons and several restriction sites every 30–40 bp. This will be a good source for further protein engineering of h-SOD.

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