Molecular Cloning of Manganese Catalase from *Lactobacillus plantarum*

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A genomic clone encoding manganese-containing catalase has been isolated from lactic acid bacterium *Lactobacillus plantarum*, sequenced, and expressed in *Escherichia coli* cells with an inducible expression system. The primary structure of the enzyme deduced from the nucleotide sequence, that comprises 266 amino acid residues, showed no significant homology with that of any other proteins registered on the available data bases. No peptide motifs conserved among active sites of proteins including manganese-containing enzymes were found. The *E. coli* cells carrying an expression construct, in which the 5’-noncoding region of the manganese catalase gene was replaced with the *lac* promoter, highly induced a protein reacting with the antiserum to manganese catalase.

The prediction of secondary structure from the deduced primary structure suggested that the *L. plantarum* manganese catalase, that is classified as a novel protein on the basis of its primary structure, has a main structural motif formed by four near parallel helices between which is the catalytic site manganese.

Catalases (EC 1.11.1.6), that catalyze the disproportionation of \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) and \( \text{O}_2 \), are classified into two groups. The majority of the enzymes, isolated from varieties of organism, contains a heme prosthetic group in the active site, and their amino acid sequences are yet unavailable. To assign the catalases arises due not only to the considerable utilities, but to the structural specificities. The refined structures based on their amino acid sequences are yet unavailable. To assign the amino acid residues as possible ligands to the catalytic site manganese, the data of primary structures based on DNA sequences are increasingly important. In this communication, we report the gene structure of *L. plantarum* manganese catalase and discuss the structural properties of the enzyme.

EXPERIMENTAL PROCEDURES

Materials and Organisms—*L. plantarum* ATCC14431 was obtained from the American Type Culture Collection, Rockville, MD. This organism was aerobically grown at 37°C in APT medium (BBL). All the oligonucleotides were provided by Nihon Techno Service. The nylon membrane for Southern blotting and colony screening was from Amer-sham (Hybond-N+). Restriction enzymes and *Escherichia coli* JM109 competent cells (Competent High) were purchased from Toyobo Biochemicals. Plasmid vector pUC18 (14), used here for both cloning and expression, was obtained from Pharmacia Biotech Inc. (Smal-linearized) and Toyobo Biochemicals (circular form).

Enzyme Assay and Protein Determination—Catalase activity was measured using an oxygen electrode, or visualized by activity staining on gel (15). Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories) with bovine serum albumin as a standard.

Enzyme Purification—The manganese catalase from *L. plantarum* was purified essentially according to the previous report (4). The purity of the enzyme sample was examined by SDS-PAGE according to Laemmli (16).

Microsequencing of Manganese Catalase Intact Subunit and V8 Protease Fragments—Purified enzyme, electrophoresed on a SDS-polyacrylamide gel (5–20% precast gel, ATTO), was electroblotted (ATTO Hoize-Blot apparatus) onto a polyvinylidene difluoride membrane (Millipore) in a Tris-glycine buffer containing 20% methanol. The protein was stained with 0.01% Coomassie Blue, and the 28-kDa band was excised and analyzed with an Applied Biosystems 476A Protein Sequencer. For internal sequencing, 20 μg of *Staphylococcus aureus* V8 protease (Sigma) was added to 40 μg of purified enzyme that had been heat-denatured (110°C, 3 min) in 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 5% glycerol, and 1% β-mercaptoethanol, and the mixture was incubated at 37°C for several hours. The resulting peptide fragments were electrophoresed on a SDS-polyacrylamide gel (15% gel, ATTO) and sequenced as above.

Cloning, Sequencing, and Expression—The methods used here were essentially based on those of Maniatis et al. (17) and on the manufacturer’s instructions. *L. plantarum* chromosomal DNA was isolated essentially according to Berna and Thomas (18) and to Marmur (19), and plasmids were extracted by the alkaline lysis method (17). DNA extraction from agarose gel (SeaKem™ GTG Agarose, FMC) was performed with the QiAquick Gel Extraction Kit (Qiagen). DNA ligation was performed with Takara DNA Ligation Kit Version 2. PCR was carried out using Takara dNTP Mixture and Stratagene Tag Plus™ DNA Polymerase. DNA transfer onto the membrane was done by electroblotting with the same apparatus as for protein blotting described above. Protein-based oligonucleotides, together with the ECL system of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D87070.

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§ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; kb, kilobase(s).
chromosomal DNA was digested with several routinely used restriction enzymes and subjected to Southern blot analysis using three degenerate oligonucleotides described above. For each digest, NS-1b and NS-2 cross-hybridized strongly to one size of the DNA fragments, whereas NS-1a did not hybridize (data not shown). The HpaI fragments of approximately 1.5 kb were collected from agarose gel, ligated to Smal-digested and dephosphorylated pUC18, and transformed into E. coli JM109. Approximately 200 “white” transformants generated from this partial genomic library were screened by colony hybridization with NS-1b and NS-2, and eight positive clones were isolated. All of the positives carried 1.5-kb fragments hybridizable with NS-1b and NS-2 (data not shown).

**RESULTS AND DISCUSSION**

**Sequencing of Intact Subunit and V8 Protease Fragments**—The purified enzyme preparation migrated as a single protein band on SDS-PAGE, corresponding to an apparent molecular mass of 28 kDa (Fig. 1A), that is consistent with the previous report (4). S. aureus V8 protease digestion of the enzyme yielded several fragments with molecular masses less than 14 kDa (Fig. 1A). The N-terminal sequences determined for the intact subunit (upper) and for one of the proteolytic fragments (Lower) (A, lane 2, indicated by the arrow). C, three degenerate oligonucleotides designed for the N terminus of manganese catalase subunit (B, the corresponding regions are shown by divergent arrows).

**Sequencing of Intact Subunit and V8 Protease Fragments**

**FIG. 1.** N- and C-terminal and internal sequencing of manganese catalase subunit (A), the purified enzyme and its proteolytic fragments were electrophoresed on a SDS, 15% polyacrylamide gel. Lane M, size marker proteins; lane 1, purified enzyme; lane 2, proteolytic peptides of 4 µg of purified enzyme with 2 µg of S. aureus V8 protease. B, N-terminal sequences determined for the intact subunit (upper) and for one of the proteolytic fragments (Lower) (A, lane 2, indicated by the arrow). C, three degenerate oligonucleotides designed for the N terminus of manganese catalase subunit (B, the corresponding regions are shown by divergent arrows).

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**Nucleotide sequence of the manganese catalase gene and the deduced amino acid sequence of the enzyme.** The N-terminal and internal amino acid sequences that were chemically determined are indicated by solid underlines. Typical expression signals, a promoter sequence (−35 and −10 regions), a Shine-Dalgarno sequence (SD), and transcriptional termination signal(s) (convergent arrows) are also indicated. The thymine-rich sequence following a termination signal is shown as white letters. Palindrome sequences in the 5′-noncoding region are double-underlined.

**Cloning of the Gene for Manganese Catalase—**L. plantarum catalase: Gene Structure and Protein Characteristics

**Fig. 2.** Nucleotide sequence of the manganese catalase gene and the deduced amino acid sequence of the enzyme. The N-terminal and internal amino acid sequences that were chemically determined are indicated by solid underlines. Typical expression signals, a promoter sequence (−35 and −10 regions), a Shine-Dalgarno sequence (SD), and transcriptional termination signal(s) (convergent arrows) are also indicated. The thymine-rich sequence following a termination signal is shown as white letters. Palindrome sequences in the 5′-noncoding region are double-underlined.
polypeptide was 29,743 Da, that is similar to the molecular mass of 28 kDa estimated for purified L. plantarum enzyme by SDS-PAGE (Fig. 1A). The amino acid composition calculated (data not shown) was generally in good agreement with that determined early for the purified enzyme (4, 13).

There are putative promoter regions: −35, TAAACA (nucleotides 283 to 288), and −10, TATAAT (nucleotides 309 to 314), and a ribosome-binding site (Shine-Dalgarno sequence): GAAAGAGG (nucleotides 400 to 407), in the upstream sequence of the start codon (Fig. 2). Tandem inverted repeat sequences, that may form a stem and loop structure, were found in the 3′-noncoding region (nucleotides 1274 to 1305 and 1342 to 1371). The latter was followed by a thymine-rich sequence (Fig. 2), suggesting that this region functions as a transcriptional termination signal. Palindrome structures, potential operator sequences, were found in the 5′-noncoding region (nucleotides 360 to 369 and 384 to 397) (Fig. 2).

Expression of L. plantarum Manganese Catalase Gene in E. coli.—The region from the start codon to the 3′-terminus of the cloned sequence was amplified by PCR. Primers were designed to introduce EcoRI and BamHI sites at the 5′- and 3′-ends of the target sequence, respectively. Primer sequences were: 5′-GGATTTCCATGTTCAACATACGAAAGAAGTCG-3′ and 5′-GGATCCAAACACCATTGCG-3′. The EcoRI-BamHI fragment of amplified DNA was cloned into the corresponding sites of pUC 18. The resulting construct pUEMN directs the synthesis of manganese catalase under the lac promoter.

The E. coli JM109 cells harboring pUEMN or pUC 18 were cultured with or without IPTG, and the induction of manganese catalase in cell-free extracts was examined. The IPTG induction in the cells harboring pUEMN caused a large increase of the protein whose band corresponded to the manganese catalase on SDS-PAGE (Fig. 3A, indicated by the arrow), that was recognized by the antibody to manganese catalase (Fig. 3B, indicated by the arrow). Such a protein band was invisible in the cells without the induction or in the control cells carrying pUC 18 (Fig. 3). These results indicate that the isolated gene actually encodes the manganese catalase.

Characteristics of the Manganese Catalase Protein.—A computer search for proteins having homology to the manganese catalase was performed using the NBRF-PDB (release 46) database. None of sequences homologous to this enzyme was found in the protein data base. A motif search to the manganese catalase using the PROSITE (release 13) presented no peptide sequences that may be responsible for the catalytic domain and/or the involvement in the binding of manganese ion. The sequence alignment using the GENETYX-MAC program of L. plantarum manganese catalase and E. coli manganese superoxide dismutase (20), both of which contain manganese involved in redox (4, 21), showed that these two proteins are not closely related (data not shown). The manganese catalase is, if judged on the basis of its primary structure, able to be declared a novel enzyme, the catalytic domain of which may contain unknown peptide motifs.

The low-resolution x-ray crystal analysis has suggested that the T. thermophilus manganese catalase has four antiparallel α-helices in which two metal ions (possibly manganese) are embedded at a 3.6-Å distance (12). The L. plantarum catalase seems to have a similar structure (22). This “4-helical bundle” structure, that usually incorporates a prosthetic group in its “hydrophobic pocket,” has also been found in myohemerythrin, a monomeric non-heme oxygen transport protein (23), and in other “4-helical proteins” (24). Possible α-helical regions on the deduced primary structure of L. plantarum catalase (Fig. 2) were predicted according to Chou and Fasman (25) and to Robson and co-workers (26) (Fig. 4A). These predictions, al-
though with at most 50% accuracy, suggest that the *L. plantarum* catalase contains a nearly parallel arrangement of α-helices as well as *T. thermophilus* enzyme. Sequential four helices composed of longer peptide chains and connected by shorter loop regions are represented by “helical wheels” according to Schiffer and Edmundson (27) (Fig. 4B). The wheels show clusters of “hydrophobic arcs” between which are residues to be possible ligands to the manganese (28, 29: Asp, Glu, His, Lys). This raises the possibility that the bundle formed by packing adjacent four helices is to possess an “internal hydrophobic core,” in which is fixed the dimanganese. It has actually been proposed that the catalytic site of *L. plantarum* catalase is not accessible by solvents, but is via a hydrophobic channel (30). Here, the *L. plantarum* catalase is proposed to form a four-helical motif between which is the catalytic site manganese. This is the first report showing a primary structure of manganese catalase based on DNA sequence. The refinement of crystal structure for the *T. thermophilus* catalase to assign residues as ligands to the catalytic site manganese appears to be in progress. The data presented here will assist in investigating the structural detail of these unique catalases.

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