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Expression of Cu, Zn-Superoxide Dismutase Gene from *Saccharomyces cerevisiae* in *Pichia pastoris* and Its Resistance to Oxidative Stress

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Abstract

The gene for the Cu, Zn-superoxide dismutase (SOD) from the yeast *Saccharomyces cerevisiae* was cloned, characterized, and overexpressed in the methylotrophic *Pichia pastoris*. The *sod* gene sequence obtained is 465 bp and encodes 154 amino acid residues. The *sod* gene sequence was cloned into the pPIC9K vector, yielding pAB22. The linearized pAB22 DNA, digested with restriction enzyme *Sac*I, was transformed into the genome of the GS115 strain of the yeast *P. pastoris*. The SOD was purified from the cultured yeast by ammonium sulfate precipitation and DEAE-cellulose column chromatography. This relatively simple purification method produced a single band on analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The overexpressed SOD protein was shown to have immunologically biologic activity and to be enzymatically active. The yeast overexpressing Cu, Zn-SOD appeared to be more resistant to oxidative stress such as paraquat, menadione, and heat shock.

Index Entries: Cu, Zn-superoxide dismutase gene; polymerase chain reaction; sequence analysis; similarity comparison; oxidative stress.

Introduction

Superoxide dismutase (SOD) is an abundant enzyme present in most aerobic organisms and many anaerobic ones. Its known activity is the

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catalysis of the disproportionation of the superoxide radical O_2^- to give dioxygen and hydrogen peroxide *(1–3)*. It is widely assumed to provide protection in vivo against this reactive metabolic byproduct, although this has not been conclusively proven. SOD exists in several forms in different organisms. Prokaryotes have two forms, one containing Fe and one containing Mn. Eukaryotes have an Mn-containing form in their mitochondria and a Cu- and Zn-containing form in their cytoplasm (Cu, Zn-SOD). The Mn and Fe proteins are related to each other, whereas the Cu, Zn-SOD is unrelated to both. SOD has been shown both to be anti-inflammatory and to reduce substantially reperfusion damage to the heart, kidney, and other organs *(4,5)*. Because every biologic macromolecule can serve as a target for the damaging action of the abundant oxygen radicals, there is interest in the therapeutic potential of SOD. A wide range of clinical applications has been suggested. These include the prevention of oncogenesis and tumor promotion, the reduction of the cytotoxic and cardiotoxic effects of anticancer drugs *(6)*, and protection against reperfusion damage of ischemic tissues *(7)*. SOD has been overexpressed in yeast acetylated at the N-terminus. By contrast, SOD in bacteria cannot be acetylated *(8–10)*. Efficient procedures for isolating SOD are important for clinical applications, and, therefore, the overexpression of SOD and simple isolation procedures with high specific activity are of interest. Stress conditions including heat shock and oxidative stress are deleterious to normal cellular function. Heat-shock treatment of murine macrophage resulted in an enhanced capacity to release superoxide anion *(11)*, and paraquat is known as a superoxide-generating agent. Thus, it is very interesting to know whether overexpression of SOD would protect the cells from the damage of stress conditions.

In the present study, SOD was overexpressed and purified with high specific activity from a recombinant yeast by a simple method. The yeast that overexpressed SOD was tested for resistance to the oxidative stresses paraquat, menadione, and heat shock.

Materials and Methods

Bacterial Yeast Strains, Plasmids, and Reagents

Table 1 provides the bacterial and yeast strains and plasmids used. All media were prepared using Invitrogen protocols. The Geneclean Kit, the specific rabbit antibody against SOD, and the goat antirabbit immoglobulin G (IgG) antibody–horseradish peroxidase (HRP) conjugate were purchased from Invitrogen. The restriction enzymes and Marker DL2000 were purchased from TaKaRa Biotech (Japan). *Escherichia coli* DH5α and the yeast *Pichia pastoris* GS115 were used for propagation of the constructed plasmids and as the host strains of the expression of heterologous *sod* gene.

Table 1
Bacterial and Yeast Strains and Plasmids

Strain or plasmid	Relevant characteristic(s)	Reference of source
Pichia pastoris GS115	*HIS4*	Invitrogen
LY21	GS115*HIS4AOX1 pAB22Sod*	This study
LY35	GS115*HIS4AOX1 pAB22Sod*	This study
LY47	GS115*HIS4AOX1 pAB22Sod*	This study
LY56	GS115*HIS4AOX1 pAB22Sod*	This study
LY68	GS115*HIS4AOX1 pAB22Sod*	This study
LY71	GS115*HIS4AOX1 pAB22Sod*	This study
Saccharomyces cerevisiae	Wild type	Kindly provided by Hangzhou West Lake Beer Factory
Escherichia coli DH5α	*supE*44Δ*lac*U169(ϕ80*lacZ*Δ*M*15) *hsdR*17*recA*1*endA*1*gyrA*96*thi* –1*relA*1	
Plasmids		
pPIC9K	*E. coli-P. pastoris* shuttle vector, Amp^rHis^+	Invitrogen
pAB22	pPIC9KΩ*sod*	This study

Cloning of Cu, Zc-SOD Gene by Polymerase Chain Reaction

Saccharomyces cerevisiae was inoculated in 100 mL of YPD medium and incubated for 24 h at 28°C with shaking. After the cells were harvested by centrifuging for 10 min at room temperature at 4000*g*, the genomic DNA was isolated as described previously *(12)* and used as the template for polymerase chain reaction (PCR) amplification. PCR amplification of Cu, Zn-SOD gene from *S. cerevisiae* was done using the specific upstream primer 5'-TAATGAATTCATAATGGTTCAAGCAG-3' and the specific downstream primer 5'-AGTGAATTCTCATTAACATTAGTTGGTT-3' including an *Eco*RI site for cloning into the *P. pastoris* expression vector pPIC9K. The PCR system consisted of 5 μL of 10X buffer, 34 μL of H_2O, 2 μL of upstream primer, 2 μL of downstream primer, 1 μL of dNTPs, 4 μL of Mg^{2+}, 0.2 μL of *Taq* polymerase (5 U), and 1 μL of DNA sample. The DNA sample was replaced with sterile water for the control. The PCR procedure consisted of denaturation at 93°C for 5 min followed by 35 cycles of the following: denaturation at 93°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min. A final elongation step was performed at 72°C for 8 min. The PCR products were electrophoresised on a 1% agarose plate (Fig. 1) and purified with a Geneclean Kit according to the manufacturer's specifications. The sequence of the purified PCR products was confirmed using a Sequenase 2.0 DNA sequencing kit from United States Biochemical as recommended by the supplier. Similarity analysis at the amino acid level was conducted using Blast software from the GenBank, NCBI database.

Plasmid Construction

Plasmid pAB22 was constructed by insertion of the *sod* gene engineered by PCR with compatible restriction ends into the *Eco*RI site of pPIC9K. The reaction system consisted of 10 μL of *sod* gene fragment, 5 μL of pPIC9K fragment, 1 μL of ligase, and 4 μL of ddH_2O. The reaction was performed at 16°C overnight. Plasmid pAB22 was constructed to create an in-frame fusion between *sod* gene and the 2-mating factor pre-peptide secretion signal present on plasmid pPIC9K. The *sod* gene in plasmid pAB22 was sequenced following PCR amplification to confirm its sequence and to establish that it was in frame with the pre-pro peptide secretion signal.

P. pastoris *Expression System*

The *P. pastoris* expression system uses the tightly regulated, alcohol-inducible *AOX1* (alcohol oxidase) promoter to express the gene of interest for heterologous protein expression. Other essential components of the expression vector include the *HIS4* gene for selection of recombinants and 5' and 3' sequences of the *AOX1* locus to allow for integration at the *AOX1* locus in the chromosome. In a transcriptionally regulated response to methanol induction, several of the enzymes are rapidly synthesized at high levels. Because the promoters controlling the expression of these genes are among the strongest and most strictly regulated yeast promoters, the methylotrophic yeast has become very attractive as a host for industrial production of recombinant proteins.

Transformation of Linearized Plasmid DNA into Yeast Spheroplasts

Linearized pAB22 DNA obtained by digestion with the restriction enzyme *Sac*I was used to transform spheroplasts (prepared using the Invitrogen protocol) of yeast *P. pastoris* strain GS115, a histidine auxotroph that allows transformants to be selected by growth on complete media without histide. His^+ transformants appearing in 3–7 d following transformation were picked and streaked onto minimal medium with methanol (MM) and minimal medium with dextrose (MD). Differential growth rates on these two media were used to identify whether the transformants resulted from integration at the *AOX1* locus (Mut^s, inability to utilize methanol as a sole carbon source, and poor growth on MM) or from integration at the *his* locus (Mut^+, ability to utilize methanol as a sole carbon source, and equal growth on MM and MD).

PCR Analysis of P. pastoris *Transformants*

The *P. pastoris* transformants were analyzed by PCR to confirm the site of integration of the plasmid pAB22. The genomic DNA was isolated from the *P. pastoris* transformants by using a standard protocol (Invitrogen). The genomic DNA was used as the template in PCR using primers 5' *AOX1* and 3' *AOX1* (Invitrogen), corresponding to sequences present upstream and downstream, respectively, of the *sod* gene on the yeast-integrating plasmid

pAB22. These primers are also present in the wild-type *AOX1* yeast gene. PCR reactions using *Taq* polymerase (1.25 U) were performed in 50-μL solutions containing genomic yeast DNA (1 μg), primers (500 ng), dNTP (500 μ*M*), $MgCl_2$ (2 m*M*), and *Taq* polymerase (1.25 U). The amplification cycle employed a hot start at 94°C for 2 min followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 8 min to complete the reaction.

Expression of Recombinant P. pastoris *Strains and Enzymatic Assays of SOD*

The *P. pastoris sod* integrant clones were analyzed for expression of the SOD protein by using the following protocol. One hundred milliliters of minimal glycerol medium was incubated with an integrant cloned at 28°C in a 1-L baffle flask with vigorous shaking (250 rpm) until the optical density at 600 nm (OD_{600}) was between 10.0 and 14.0 (36–48 h). The cells were harvested by centrifuging for 10 min at room temperature at 4000*g*. The supernatant was discarded, and the pellet was resuspended in 20 mL of MM in a 100-mL baffle flask. The flask was covered with two layers of sterile gauze instead of a cap to allow maximal aeration. The flasks were incubated at 28°C with vigorous shaking (250 rpm) for an additional 3 d. The cells were induced with fresh methanol every 24 h to a final concentration of 0.5%. At the end of 3 d, the cells were centrifuged at 4000*g* for 10 min, and the pellet washed with cold phosphate-buffered saline and centrifuged as just described. The cells were suspended by sonication in 10 m*M* Tris-acetate with 0.1 m*M* phenylmethylsulfonyl fluoride and 0.75 *M* sucrose and incubated for 45 min. The suspension was centrifuged as just stated, and the supernatants were analyzed for the SOD protein. SOD activity was determined by the method of McCord et al. *(13)* and by the nitroblue tetrazolium (NBT) method. The NBT method was as follows: Gel electrophoresis was performed in a 10% nondenaturing polyacrylamide gel. The SOD was identified by soaking the gels in 1 m*M* NBT for 10 min, followed by immersion for 15 min in a solution containing 20 m*M* TEMED, 0.25 μm of riboflavin, and 36 m*M* potassium phosphate at pH 7.8. The gel was then placed in a glass tray and illuminated for 5–15 min with fluorescent light. During illumination the gel became uniformly blue except at positions containing SOD. Illumination was discontinued when the maximum contrast between the clear zone and the blue formazan region was achieved.

Purification of Expressed SOD Protein

SOD was purified from the recombinant *Pichia* by the method of Kumagai et al. *(14)* with some modification. Solid ammonium sulfate was added with stirring to the yeast extracts (50 mL) to make a 90% saturated solution. The pH of the ammonium sulfate solution was adjusted to 5.0 with acetic acid, and an equal volume of methanol was added slowly with

Table 2
Purification of SOD from Recombinant Yeast

Step	Volume[a] (mL)	Protein (mg)	Total activity[b] (units)	Specific activity (units/mg)	Yields (%)
Total cell extract	49	537	44528	83	—
Ammonium sulfate precipitation	103	17	38895	2300	88
DE52 cellulose	9	8.5	30970	3644	73

[a]Starting with 10 g of cell paste.
[b]SOD activity was determined according to the NBT method.

stirring. The resulting mixture was vigorously stirred for 15 min. Centrifugation (5000*g*, 10 min) of the mixture resulted in a separation of liquid and solid phases. The liquid phase contained active SOD. The supernatant was carefully transferred and concentrated to 10 mL at 4°C in an Amicon Ultra filtration system. The concentrated sample was dialyzed against 10 m*M* potassium buffer (pH 6.5)–0.01 m*M* EDTA (buffer A). The resulting sample was applied to a DE-52 cellulose column (40 × 1.6 cm). The SOD was eluted with 20 mL of a 0–0.2 *M* KCl linear gradient in buffer A. The fractions containing SOD protein were collected and combined. This sample method allowed the enzyme to be isolated readily with high specific activity. Table 2 summarizes the results of the purification procedure. The content and the specific activity of the SOD protein in the total cell extract were 537 mg and 83 U/mg, respectively. After purification according to the aforementioned simple procedure, the final yield was 8.5 mg of SOD with a specific activity of 3644 U/mg of protein and a total activity of 30,970 U. The purified SOD showed a single band with a molecular weight of about 16.9 kDa on sodium dodecyl sulfate (SDS) polyacrylamide gel (Fig. 5). The SOD protein was shown to be enzymatically active.

Analysis of Yeast Clone Supernatants for Production of SOD Protein

The yeast cell extracts, together with purified SOD protein, were analyzed by electrophoresis on a tricine-SDS-polyacrylamide gel with a 12% separating gel and a 4% stacking gel *(15)*. The electrophoresed gel was detected by staining with Coomassie brilliant blue. For Western blotting, the purified SOD protein was used. The electrophoresed SOD protein purified from the yeast supernatants was transferred onto a nitrocellulose membrane in 10 m*M* cyclohexylaminopropane sulfonic acid transfer buffer (pH 10.5)–15% methanol according to the procedure described in Harlow and Lane *(16)*. Gel transfer was done at 15 V overnight at +4°C. The membranes were blocked in a 5% skim milk buffer for at least 6 h at +4°C. The membranes were subsequently washed with a Tris/Tween-20 buffer, incubated for 1 h at ambient temperature with rabbit antibody against SOD

diluted 1:500, washed again, and finally incubated for 20 min with a goat antirabbit IgG antibody–HRP conjugate. After a final wash cycle with Tris/Tween-20 buffer, the membranes were reacted for 1 min with NEN Renaissance enhanced Western blot chemiluminescence reagent, blotted dry, and exposed to Kodak X-Omat blue X-ray film.

Oxidative Stresses and Heat Shock

Recombinant yeast cells were harvested by centrifuging at 4000*g* for 5 min and resuspended in 100 mL of potassium phosphate buffer (pH 7.4) containing appropriate amounts of stressors (5 m*M* paraquat, 5 m*M* menadione, 5 m*M* H_2O_2). Cell survival was monitored by sampling at 15-min intervals, diluting in 100 m*M* phosphate buffer (pH 7.4), and plating aliquots onto YEPD plates. Heat stress was induced by incubating cultures at 48°C. Samples were taken at 15-min intervals, diluted in phosphate buffer, and plated on YEPD to obtain viable cell mounts. All plate counts were done in duplicate and all experiments were repeated at least three times.

Nucleotide Sequence Accession Number

The nucleotide sequence for the *sod* gene from *S. cerevisiae* is in the GenBank database under accession no. AY690619.

Results

Cloning and Sequencing of Cu, Zn-SOD Gene

Electrophoresis of the PCR products showed a band of the correct length in lane 1, and no band in the negative control (Fig. 1). The *sod* gene sequence obtained is 465 bp and encodes 154 amino acid residues (Fig. 2). The SOD protein deduced from it has a calculated molecular weight of 16,940 Daltons, with methionine and asparagine at the NH_2- and carboxyl-terminal amino acids, respectively. The GenBank, NCBI database was searched for other proteins with amino acid sequence similarity to the open reading frame (ORF) product. The sequence of the SOD protein showed high identity with that of SODs from other microorganisms (Fig. 3).

Transformation of Linearized Plasmid DNA into Yeast Spheroplasts

The constructed pAB22 plasmid containing *sod* under the control of the alcohol-inducible promoter AOX1 was digested with the restriction enzyme *Sac*I to generate linearized DNA for transformation. Between 3 and 10 μg of digested DNA was used to transform yeast strain GS115 spheroplasts. His^+ transformants appeared on d 3–7 following transformation. In two experiments, 72 and 96 His^+ transformants were identified. Twenty-one from the first experiment and 24 from the second experiment were of the Mut^s phenotype (29.1 and 25%, respectively) (the manufacturer suggests an expected frequency of 5–35%). To confirm further the site of integration, six of these Mut^s strains (designated for strain LY21, LY35, LY47,

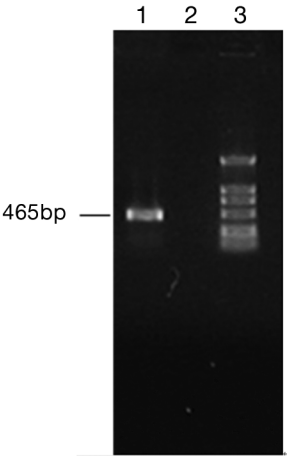

Fig. 1. Electrophoretic map of PCR products. Lane 1, PCR product; lane 2, negative control (no template); lane 3, marker DL2000 (from TaKaRa Biotech).

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TAATGAATTCATAATGGTTCAAGCAGTCGCAGTGTTAAAGGGTGATGCCGGTGTCTCTGGTGTTGTCAAGTTC
          M  V  Q  A  V  A  V  L  K  G  D  A  G  V  S  G  V  V  K  F
GAACAGGCTTCCGAATCCGAGCCAACCACTGTCTCTTACGAGATCGCTGGTAACAGTCCTAACGCAGAACGTGGG
E  Q  A  S  E  S  E  P  T  T  V  S  Y  E  I  A  G  N  S  P  N  A  E  R  G
TTCCACATTCATGAGTTTGGAGATGCCACCAATGGTTGTGTCTCTGCTGGTCCTCACTTCAATCCTTTCAAGAAG
F  H  I  H  E  F  G  D  A  T  N  G  C  V  S  A  G  P  H  F  N  P  F  K  K
ACACATGGTGCTCCAACTGACGAAGTCAGACATGTCGGTGACATGGGTAACGTAAAGACGGACGAAAATGGTGTG
T  H  G  A  P  T  D  E  V  R  H  V  G  D  M  G  N  V  K  T  D  E  N  G  V
GCCAAGGGCTCCTTCAAGGACTCTTTGATCAAGCTTATCGGTCCTACCTCCGTTGTAGGCAGAAGCGTCGTTATC
A  K  G  S  F  K  D  S  L  I  K  L  I  G  P  T  S  V  V  G  R  S  V  V  I
CACGCCGGCCAAGATGACTTAGGTAAGGGTGACACTGAAGAATCTTTGAAGACTGGTAATGCCGGTCCAAGACCA
H  A  G  Q  D  D  L  G  K  G  D  T  E  E  S  L  K  T  G  N  A  G  P  R  P
GCCTGTGGTGTCATTGGTCTAACCAACTAATGTTAATGAGAATTCACT
A  C  G  V  I  G  L  T  N
```

Fig. 2. Nucleotide sequence of cloned DNA fragment showing ORF encoding SOD. The amino acid sequence deduced from it is indicated beneath the DNA sequence. The initial codon and the stop codon sequences in the corresponding positions, respectively, are indicated in boldface.

LY56, LY68, and LY71, respectively) were randomly selected and analyzed by PCR.

PCR Analysis of P. pastoris *Transformants*

Six *P. pastoris* transformants were analyzed by PCR (Fig. 4). Except for the LY35 transformant, a specific 945-bp band was amplified in the transformants (496 bp from *sod* including partial 5'-flanking and partial 3'-flanking regions plus 449 bp from the pPIC9K vector). This was the only

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MVQAVAVLKGDAGVSGVVKFEQASESEPTTVSYEIAGNSPNAERGFHIHEFGDATNGCVSAGPHFNPFKKTHGAPTDEVRHVGDMGNVK.
**K****VR**SK*Q*T*H***E***A**TISW**E**DP**L****IHQ****************G*Q*******E********IS.
**Q****LR**SK*I*V*N***S***D**FITW**S**DA**L****VHT*****************T*E*******N********VT.
**Q****LR**SK*S*V*N***S***D**TITW**S**DA**L****VHT*****************T*E*******N********VT.

TDENGVAKGSFKDSLIKLIGPTSVVGRSVVIHAGQDDLGKGDTEESLKTGNAGPRPACGVIGLTN.
**GN*****T****LI****KD********V******Y****FED*KT*******P***VI**TQ.
**TS*****S****FV****QN********I******L****NAE*KK*******L***VI**TN.
**TS*****S****FV****QN********I******L****HEL*KT*******V***IN**QG.
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Fig. 3. Comparison of similarity of SOD protein from *S. cerevisiae* with that of SODs from other microorganisms at amino acid level. Organisms from top to bottom: *S. cerevisiae*, *Candida albicans*, *Debaryomyces hansenii*, *Udeniomyces puniceus*. Asterisks indicate identity with the *S. cerevisiae* SOD sequence at that position. The compared amino acid sequence data were taken from refs. *28–30* and the GenBank database.

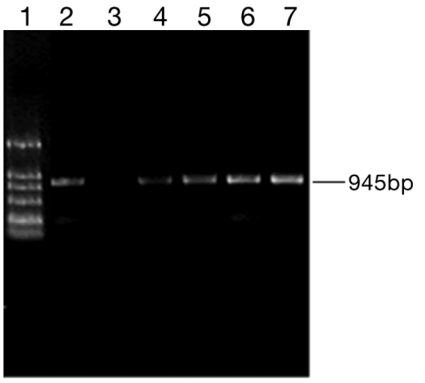

Fig. 4. PCR amplification from chromosomes of yeast clones (1% agarose gel). Lane 1, marker DL2000 (from TaKaRa Biotech); lanes 2–7, six *P. pastoris* transformants randomly selected for PCR analysis (from left to right was designated for strain LY21, LY35, LY47, LY56, LY68, and LY71, respectively).

band amplified in five integrants, indicating integration at the *AOX1* gene. Thus, of the strains identified as Mut^s strains by plating on the differential media, five strains were found to have integration at the *AOX1* gene by PCR.

Expression of Recombinant P. pastoris *Strains*

To determine whether the *P. pastoris sod* integrant clones produced SOD protein, one of the five clones identified by PCR to contain the *sod* gene integrated at the *AOX1* gene was tested for expression of the SOD protein in comparison to the control *P. pastoris* strain. We examined expression of the SOD protein in minimal medium. It was found that the expression of a methanol-induced protein was produced in a stable pattern in the minimal medium. The SOD protein was visible in cell extracts including the culture

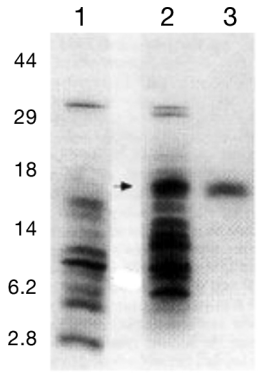

Fig. 5. Electrophoretic pattern of SOD protein in crude supernatants and purified. The sizes of the molecular weight standards are shown on the left in kilodaltons. Lane 1, control *P. pastoris* strain GS115; lane 2, crude supernatants of selected strain LY56 containing *sod* gene; lane 3, purified SOD from selected strain LY56.

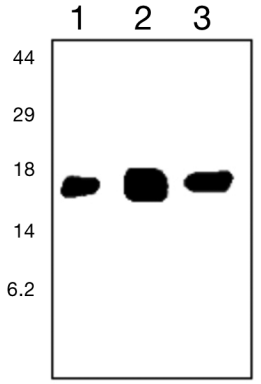

Fig. 6. Western blotting analysis of recombinant SOD protein expressed in *P. pastoris* using specific antibody raised against SOD. The sizes of the molecular weight standards are shown on the left in kilodaltons. The purified SOD was used. The amounts of purified SOD added to lanes 1, 2, and 3 were 20, 100, and 80 ng, respectively.

supernatants analyzed on tricine-SDS-polyacrylamide gels by 48-h postmethanol induction (Fig. 5). Protein production appears to be at a maximum at 72 h after induction. To test whether SOD has immunologically biologic activity, Western blot analysis was done with the specific antibody raised against SOD. The specific antibody raised against SOD bound to the purified SOD, creating a single band (Fig. 6), which indicated that the recombinant SOD protein expressed in the selected *P. pastoris* has immunologically biologic activity.

Expression of Cu,Zn-SOD Increases Resistance to Oxidative Stresses

The lethal effects of peroxide- and superoxide-generating agents on cultured yeast cells were apparently dependent on cell density. Cells enter-

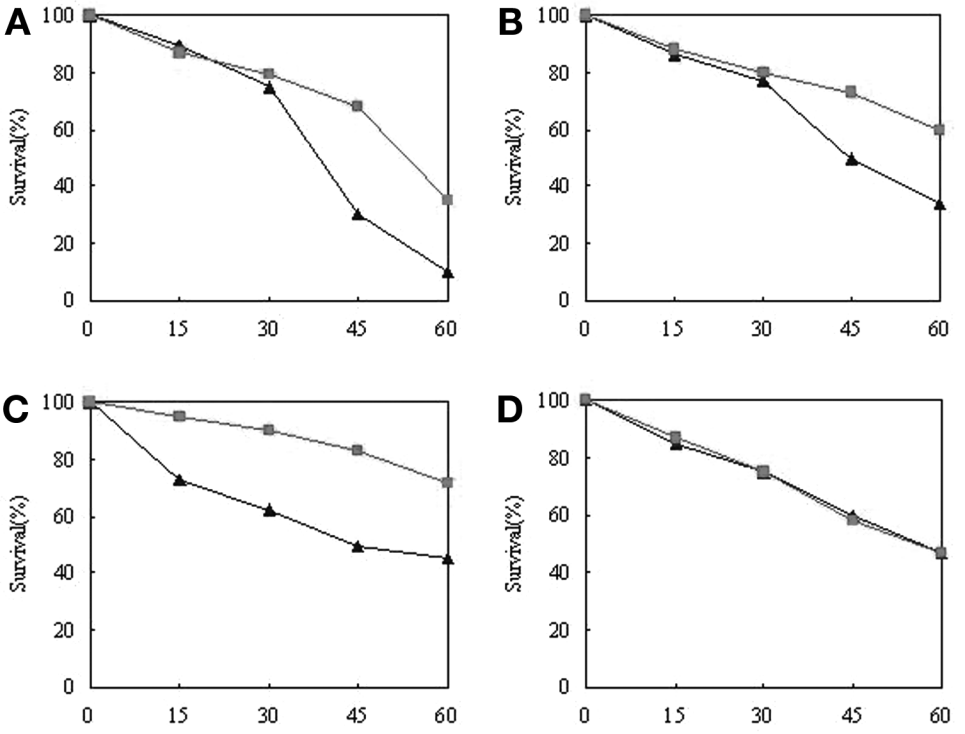

Fig. 7. Resistance to oxidative stresses. Cells were exponentially grown at 30°C to an OD_{600} of 1.0. This represents 3.0 H 10^7 cells/mL, which is the 100% survival value. Cells were treated with **(A)** 5 m*M* paraquat, **(B)** 5 m*M* menadione, **(C)** heat shock at 48°C, and **(D)** 5 m*M* H_2O_2 at various times. Samples were diluted and plated on YEPD solid media to monitor cell viability. (■) Cells containing SOD expression vector; (▲) control cells.

ing high density became more resistant to the stresses, including oxidative stress and heat shock. Early exponential–phase cultures used for this investigation were very sensitive to paraquat concentration higher than 1 m*M*. In subsequent experiments, a paraquat concentration of 5 m*M* was chosen to test for lethality (Fig. 7A). Heat stress was induced by incubating the culture at 48°C for various time intervals (Fig. 7C). There was a slight protection against paraquat stress during the first 30 min, but in the subsequent 30 min the more protective effect of expressed SOD was observed (Fig. 7A). When the yeast strain expressing SOD was treated with 5 m*M* menadione, an enhanced resistance similar to that seen with paraquat was obtained (Fig. 7B). With thermal stress, a protective effect of SOD was observed from the beginning and was higher than the stresses produced by paraquat or menadione. As a result, the recombinant yeast strain was more resistant to superoxide- generating agents (paraquat and menadione) and heat shock than the control cells. Survival was increased by about 50% when the yeast bearing SOD was subjected to paraquat, menadione, or heat shock. These results suggest that the SOD expressed in yeast protected against oxida-

tive stresses and heat shock. However, the expression of SOD did not enhance the resistance to H_2O_2 (Fig. 7D), probably owing to neutralization by catalase. The oxidative stress response and its relationship to the heat-shock phenomena are also being intensely investigated in eukaryotic systems *(17)*. Heat-shock treatment of marine macrophages resulted in an enhanced capacity to release superoxide anions *(11)*. Most of the work has focused on the regulation of expression of genes coding for antioxidant defenses, and, at present, the signaling mechanisms are the main topic *(18–20)*. The introduction of SOD may be very important because a higher SOD level in the cell protects against more oxidative stresses *(21)*. The recombinant cells were easily cultured to high concentration, in contrast with the control cells, owing to increased resistance to oxidative stress.

Discussion

We have described the cloning and characterization of aCu, Zn-SOD gene, *sod*, from *S. cerevisiae*. We have also reported the amino acid sequences deduced from this gene sequence. The predicted mature form of SOD contains 154 amino acid residues with a calculated molecular weight of 16,940 Daltons. The *sod* sequence was cloned into the pPIC9K vector, yielding pAB22. We transformed linearized pAB22 digested by restriction enzyme *Sac*I into the genome of the methylotrophic *P. pastoris* GS115 and demonstrated the efficient expression of the *sod* gene in *P. pastoris*. In the experiments, we found that the expression of SOD is highly dependent on the composition of the growth medium and the density of the culture at the time of induction of protein expression. In recent years, several genes encoding SOD have been cloned and expressed in different host strains. Expression of the Mn-*sod* genes is strongly stimulated by the presence of dioxygen in *E. coli (22–24)* and in *S. cerevisiae (25,26)*. In *S. cerevisiae* and other eukaryotes, Mn-SOD is a mitochondrial protein and is regulated in coordination with other mitochondrial proteins *(13)*. Cu, Zn-SOD is constitutively expressed at a relatively high level. However, there are indications that the level is further increased by the presence of dioxygen or Cu *(25)*. Since Cu, Zn-SOD is a cytoplasmic protein, it would not necessarily be expected to be regulated in the same way as Mn-SOD, even though one would logically expect it to be regulated in response to dioxygen levels.

A number of enzymes exist whose function appears to be to protect the cell from oxidative damage: two forms of SOD, two forms of catalase, cytochrome-*c* peroxidase, and glutathione reductase. All appear to protect organisms against the toxicity of dioxygen species derived from them. Compared with the control cell, the recombinant cell containing SOD was more resistant to superoxide-generating agents. The expression of SOD and catalase genes may also have important implications in the determination of the metabolic potential or the total amount of oxygen consumed by the organisms during their lifetimes. It appears that the coexpression of SOD and catalase provides an efficient strategy for lowering the level of oxidative stress and extending lifespan *(27)*.

Acknowledgments

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