Copper–Zinc Superoxide Dismutase from the Marine Yeast *Debaryomyces hansenii*

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We have isolated the cytosolic form of Cu–Zn superoxide dismutase (SOD) from the marine yeast *Debaryomyces hansenii*. This enzyme has a subunit mass of 18 kDa. The preparation was found to be heterogeneous by IF electrophoresis with two pI ranges: 5.14–4.0 and 1.6–1.8. The enzyme preparation had a remarkably strong stability at pH 6.0–7.0, surviving boiling for 10 min without losing more than 60% of activity. On Western blots, this enzyme was recognized by antibodies raised in rabbits against *D. hansenii* extracts, while only a weak cross-reaction could be detected using antibodies generated against either *Saccharomyces cerevisiae* or bovine erythrocyte Cu–Zn SODs. In sequencing analysis, a peptide obtained by trypsin digestion was found to have 85% identity to the *S. cerevisiae* Cu–Zn SOD. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — marine yeast; superoxide dismutase; *Debaryomyces hansenii*

INTRODUCTION

Although oxygen is an essential element in respiring living cells, it also causes potential problems by the generation of highly reactive oxygen species (·O₂⁻, ·OH, OCl⁻, H₂O₂, etc.) in the respiratory process. Cells are equipped with a family of enzymes that catalytically scavenge the superoxide free radical through the disproportionation

\[ \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]  

(McCord and Fridovich, 1969), and therefore help to protect the cell against the toxic ·O₂⁻ species. Such enzymes, denoted as superoxide dismutases, SOD (EC 1.15.1.1), are ubiquitous in oxygen-metabolizing. They are metalloproteins with different prosthetic groups. Fe SOD is present in subcellular organelles and in the periplasmic space of prokaryotes, Mn SOD is present in the mitochondrial matrix of eukaryotes and in the cytosol of prokaryotes, and the Cu–Zn SOD is generally present in eukaryotic cell cytoplasm, mammalian body fluids and plant chloroplasts (Amano et al., 1990). Cytosolic Cu–Zn SOD is found as a dimer (Hong et al., 1992), whereas the extracellular form is a tetrameric glycoprotein (Marklund, 1984). Both forms of Cu–Zn SOD have a monomer of 16 kDa (Marklund, 1984; Tibell et al., 1987), and are believed to derive from a common ancestor gene that is different from that of Mn and Fe SODs (Fridovich, 1989).

SODs have been detected and isolated from a number of microorganisms, plants and animals (Donnelly et al., 1989). In some cases, it has been possible to induce their synthesis for large-scale purification and use in medicine by manipulating the culture conditions (Hassan and Fridovich, 1977; Moody and Hassan, 1984). The interest in using SODs for clinical purposes and specific applications in the food industry stems from the overwhelming evidence of the importance of oxygen-free radicals in a variety of pathological symptoms and food spoilage (Donnelly et al., 1989). It is isolated commercially using human or bovine erythrocytes, bovine liver (Huber and Shulte, 1973), brewer’s yeast and other common microorganisms (Johansen, 1983; Scott et al., 1987). Because of the importance of free radical generation and scavenging in aerobic organisms,
free radicals and derivatives have been implicated as causative agents in numerous human diseases including cancer, emphysema, immunologic impairments and, recently, neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) (Rosen et al., 1993) and also in the normal ageing process. Thus, it is not surprising that Cu–Zn SOD (SOD-1) has been the focus of considerable attention. This protein from moulds and yeasts (except from Saccharomyces cerevisiae) has not been studied extensively. It can grow in 0–24% (0–4·13 m) sodium chloride media (Adler, 1986; Hernández-Saavedra et al., 1995). When growth in high salt conditions, this organism excretes sodium and selects for potassium uptake (Norkrans and Kylin, 1969; Hobot and Jennings, 1981). Polyhydroxy alcohols are also produced and accumulated within the organism to counterbalance the decreased osmotic potential of the environment (Adler and Gustafsson, 1980; Hernández-Saavedra et al., 1995). This report describes the isolation and characterization of a Cu–Zn SOD enzyme from the marine yeast D. hansenii, strain C-11, isolated from seawater off the west coast of Baja California Sur, México (Hernández-Saavedra, 1990).

MATERIALS AND METHODS

The microorganism

Debaryomyces hansenii strain C-11 was obtained from the CIBNOR Marine Yeast Collection (Hernández-Saavedra, 1990).

Chemicals and enzymes

Most of chemicals were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.), all of which were of analytical grade. Several SOD enzymes were used as reference: Cu–Zn SOD enzymes from Saccharomyces cerevisiae (CARLBIOTECH; Copenhagen, Denmark); Bos taurus erythrocytes (Sigma, St Louis, MO, U.S.A.); and liver (DDI Pharmaceutical, San Francisco, U.S.A.); and Fe and Mn SOD enzymes from Escherichia coli (Sigma, St Louis, MO, U.S.A.).

Cell biomass production

Cell biomass was produced in filtered seawater media containing glucose 20 g/l, peptone 10 g/l, and yeast extract 5 g/l at pH 5·6, according to the method described by Ochoa et al., 1995. The culture was made in sterile 601 Nalgene carboys filled up to half their volume with sterile culture medium, adding 10 ml of 10% FG10 antifoam agent (Dow Corning) and 2 ml/l of a 5% chlorine dioxide solution (Halox®). After incubation, the cell biomass was removed by continuous centrifugation (rotor JCF-Z Beckman; 8000 rpm at 5°C). The cell pellet was washed with phosphate buffer (50 mM, pH 7·8) before use.

SOD extraction procedure

For cell disruption, 35 g wet biomass was added to a Bead Beater (Biospec Products) container immersed in an ice bath, and containing 200 ml of phosphate buffer (50 mM, pH 7·8) and 200 ml of 0·45 mm glass beads. While keeping the temperature between 1 and 4°C, 10 pulses of 30 s were applied with 30 s intervals between pulses. The homogenate was centrifuged (2250 × g, 15 min at 4°C) (JA20 Beckman rotor) and the supernatant (So) was recovered and then mixed with chloroform: ethanol (0·15:0·25 v/v) while stirring (15 min at 4°C). The resulting mixture was centrifuged (2250 × g, 8 min at 4°C). The supernatant was mixed with 300 g/l of K2HPO4 to obtain a new supernatant after centrifugation. The new supernatant was mixed with 0·75 vol. of cold acetone (−20°C), the resulting precipitate was recovered by centrifugation as above, and then redissolved and dialysed against phosphate buffer (50 mM, pH 7·8). This semipurified extract contains the copper–zinc enzyme, and subsequently is referred to as Sf.

SOD purification

Semipurified enzyme extracts (Sf) were further purified by chelate chromatography (IMAC); (Michalski, 1992). IMAC was done using a copper-saturated chelating Superose HR 100/2 column (100 mm × 20 mm, Pharmacia LKB) activated with 0·2 m CuSO4 in distilled water and connected to a FPLC system (Pharmacia Biotech). The sample first was dialysed in buffer A (10 mM sodium phosphate, 0·75 mM NaCl, pH 6·8), and then 500 µl was loaded on to the column (1·0 mg of protein). After protein injection, 100% buffer A for 20 min (two column volumes), then 35 min of 20% B (10 mM sodium phosphate, 0·75 mM NH4Cl, pH 7·8), 25 min of 45% B, and finally 25 min of 100% B was passed through the column. The flow
rate was constant at 1 ml/min and fractions were collected every minute. SOD activity in every fraction was determined by a qualitative Nitroblue Tetrazolium (NBT) microassay in 95-well plates. The NBT technique (Beauchamp and Fridovich, 1971) was modified as follows: to every well were added 50 µl of each fraction plus 50 µl of a mixture of 1 mM EDTA, 0.13 mM methionine, 7.5 mM NBT and 0.2 mM riboflavin in 50 mM phosphate buffer, pH 7.8. Fractions showing activity were pooled and dialysed against TRIS buffer (10 mM TRIS–HCl, 15 µM CuSO₄, pH 7.5) and stored at 4°C until use.

Protein analysis

A sample of Cu–Zn SOD protein isolated from the marine yeast D. hansenii and further purified by SDS–PAGE was digested with trypsin according to Rosenfeld et al. (1992) to obtain internal peptide sequence. A similar sample was used to obtain amino acid composition and N-terminal sequencing. Amino acid analysis was done by acid digestion and dansylation labelling reaction (Beckman); separation and quantification was carried out by HPLC in a System Gold (Beckman). SOD activity was determined according to the method of Davis (1964) in a 7.5% polyacrylamide gel stained with 0.05% of 1 mM bromophenol blue). SDS–PAGE standards (low range, BioRad) pI 4.45–9.6 and (Pharmacia) pI 3.5–9.3 were used.

Metal analysis

Two different protein batches, obtained after IMAC chromatography, were dialysed against bi-distilled water and then concentrated by centrifugation using Ultrafree-CL filters (Millipore) until 0.032 mg/ml of protein; this theoretically corresponds to 2 nm of each Cu²⁺ and Zn²⁺. Copper was determined in a graphite oven under the following conditions: dry 20 s at 100–130°C, carbonization 20 s at 700–900°C, atomization 10 s at 2000°C and finally cleaned by 10 s at 2200°C. Zinc content was determined by the flame absorption method in a Analyst-100 apparatus (PerkinElmer) using a gas mixture of acetylene–air, 14 psi:35 psi respectively. These analyses were done at the Faculty of Veterinary Medicine and Zootechny, Toxicology Laboratory (UNAM, México).

Electrophoretic analysis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was done on 7.5% acrylamide gels according to the method of Davis (1964) in a Miniprotein II chamber (BioRad). SOD activity was detected in gels by the photochemical NBT stain (Beauchamp and Fridovich, 1971). The subunit molecular mass of the enzyme was determined by electrophoresis on a 12% polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS–PAGE). Before electrophoresis, samples were boiled for 10 min in reducing loading buffer (62.5 mM TRIS, pH 6.8, 5% glycerol, 2% SDS, 5% 2 mercaptoethanol, 12.5 µg/ml bromophenol blue). SDS–PAGE standards (low range, BioRad) used for calibration were: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soy bean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Isoelectric focusing (IEF) determination

The isoelectric point was determined both in a ROTOFOR apparatus (BioRad) using 0.2% ampholine (Pharmalyte 3–10, Sigma) and 2.5 mg of enzyme as sample, and by 5% polyacrylamide gel isoelectric focusing. Electrophoresis was done at constant voltage of 250 V and 150 mA at 4°C for 3 h, using 1 M NaOH as cathode buffer and 0.05 M H₂SO₄ as anode buffer. IEF broad range standards (BioRad) pH 4.45–9.6 and (Pharmacia) pH 3.5–9.3 were used.
Effects of pH and temperature on SOD activity

The stability of the enzyme as a function of pH was determined by quantifying the residual activity after 24 h preincubation at various pHs and constant temperature (20°C). For this, a 1:100 dilution of an enzyme stock (1 mg/ml) was made with the corresponding buffer system (pH 3–10) to get a final enzyme concentration of 10 μg/ml. After the incubation time, 0, 10, 25, 50 and 75 μl of the enzyme solution were transferred to 15 × 100 mm glass tubes and filled up a volume of 100 μl with the respective assay buffer system (phosphate buffer 50 mM, pH 7.8). After this, the NBT reaction was done as described before.

Thermostability was determined both by heating the samples at 95°C for various times (1, 2, 3, 4, 5 and 10 min) and by preincubation of the enzyme at various temperatures (20–65°C in increments of 5°C) for 20 min before the assay for activity. The protein concentration was adjusted to 15 μg/ml, and the pH of the activity assay was 7.8 using 50 mM phosphate buffer.

Inhibition tests

To compare and discriminate different types of SOD enzymes, samples were preincubated in phosphate buffer (50 mM, pH 7.8) containing 5 mM NaCN, 1 mM SDS or 10 mM H2O2 (5 min at 20°C) before addition of the substrate. The residual activity is considered as the percentage of remaining activity compared to a control.

SOD antibodies

Antibodies were raised in New Zealand white rabbits (2.5 kg and 6 weeks old) against the Cu–Zn SOD from S. cerevisiae, D. hansenii and Bos taurus (bovine erythrocytes). The primary injection in complete Freund’s adjuvant (day 0) and the second and third in incomplete Freund’s adjuvant (days 7 and 42) were given intramuscularly (1 mg of antigen for each injection per rabbit). Bleeding occurred at days 87 and 164 after immunization.

Western blot analysis

SDS–PAGE gels were transferred to nitrocellulose membranes (0.45 mm, Millipore) with a semidy electroblotter (Jancos, Denmark). Immobilized proteins were stained with 0.2% Ponceau dye in 3% glacial acetic acid and destained with water. All reactions took place in phosphate-buffered saline (PBS: 80 g/l NaCl, 2 g/l KCl, 14.4 g/l Na2HPO4·7H2O, 2.4 g/l KH2PO4, pH 7.4) containing 2% evaporated milk and 0.05% Tween 20 at room temperature. The primary antibodies were used for the first test at 1:1000, and for the second test at 1:5000 dilutions. Reacting proteins were visualized by staining with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) as a secondary antibody.

RESULTS AND DISCUSSION

SOD extraction

Protein and activity yield for 10 biomass lots were, on average, 3.58 ± 0.34 mg/ml and 153 ± 47.5 U/mg of protein for So extracts, and 4.5 ± 0.35 mg/ml and 4414 ± 569.74 U/mg of protein for Sf extracts. Accordingly, it appears that the methods employed for cell disruption and enzyme isolation and fractionation are reproducible and may be considered adequate for the extraction of SOD from D. hansenii. Protein content and specific activity were variables used in subsequent experiments to monitor the quality of different cell biomass batches of D. hansenii as a source of SOD.

According to the data in Table 1, the number of SOD enzyme units found in the crude extract of
D. hansenii (146 U/mg of protein) is higher than the previously reported values for yeast belonging to the genus Candida (2–5 U/mg of protein), and the genera Saccharomyces, Pichia and Kluyveromyces (14–38 U/mg of protein) (Kujumdzieva-Savova et al., 1991; Nedeva et al., 1993). However, differences in culture conditions may be responsible for differences in SOD levels, in addition to intrinsic physiological characteristics of the marine yeast D. hansenii. As compared to other SOD sources, where 50% loss of activity after 96 h has been documented (Matsumoto et al., 1991), the SOD enzyme in fraction Sf was more stable, because no loss in activity was detected after 6 months storage at 4°C in phosphate buffer, 50 mM, pH 7.8 (data not shown).

Enzyme purification

Table 1 shows the purification steps of the Cu–Zn SOD isolated from D. hansenii. As indicated, there is a dramatic increase in specific activity by fractionation using different organic solvents (Crapo et al., 1978). The Sf fraction, containing the Cu–Zn SOD enzyme, represented only 0.65% (in weight) of the original protein extract, but 64.5% of SOD units, which is equivalent to an approximately 2870% purification obtained by using a convenient single-step procedure. Such separation was possible because of the solubility of the Cu–Zn SOD in chloroform, whereas most of accompanying proteins precipitated. It is thus possible that the reduction of almost 35.5% of the total enzyme units found in the crude extract could correspond to Mn SOD, which precipitates with contaminant proteins.

When fraction Sf was run on an IMAC separation system (Figure 1), two active fractions were obtained. Peak A (fractions 14–18), which was not retained by the column, represents only 0.3% of the original protein, but had a specific activity 3214% larger than the So sample. One protein peak containing SOD activity was eluted with 34.41%, which exceeds in specific activity all the other SODs reported so far in the literature (e.g. see: Amano et al., 1990; Hong et al., 1992; Matsumoto et al., 1991; González et al., 1991; Almansa et al., 1991) with a value of 15 700 U/mg of protein, represents 0.35% of the original protein. No other fractions show SOD activity, although samples of fractions 60–65 (major contaminant protein) were also analysed. On SDS-PAGE (Figure 1 inset), proteins from peaks A (lanes 2) and B (lanes 3 and 4) are observed as single bands, whereas in fractions 60 and 65, in addition to a major band, other minor bands are observed. The overall recovery of protein was 100%, and 78% in terms of SOD activity. Purification on IMAC indicated Cu–Zn SOD from the marine yeast D. hansenii strain C-11 is a heterogeneous enzyme. For analysis of amino acid content, fractions were analysed separately. On further analysis, peaks A and B were pooled.

Protein analysis

One stretch of 16 amino acid residues was obtained by peptide sequencing after trypsin digestion of a pure sample of Cu–Zn SOD from D. hansenii (from IMAC peak B). The sequence obtained, VSGVNFQSQSESDEPT, is near the N-terminal end, showing an 81% homology with the S. cerevisiae Cu–Zn SOD (Swiss Protein Data Bank, Accession No. P00445) and 100% with the sequence deduced from its own cloned cDNA (NCBI Data Bank, Accession No. AFO16383; Hernández-Saavedra et al., 1998). An identity >95% was observed with the same sequence, but on amino acid composition basis (Hernández-Saavedra, 1997).

Additionally, we know that the N-terminal end is free since a 20 amino acid sequence was obtained by sequencing (VKAVAVLRGDSKVS GVNVFE). When compared with the N-terminal end of corresponding cloned sequence (Hernández-Saavedra et al., 1998), we observe a 95% identity; only one change on sequence was observed—Q2 is substituted by K.

Amino acid composition of peak B (Table 2) is similar to that of S. cerevisiae Cu–Zn SOD. However, differences in residue number of some amino acids (Ile, Met, Arg, Val) are important, although not negatively affecting the catalytic activity of the enzyme. The number of His residues, which theoretically play a principal role in enzyme activity by acting as metal binding sites for Cu++ and Zn++, is conserved. By amino acid composition analysis it is not possible to discriminate between either aspartic acid and asparagine or glutamic acid and glutamine, since each pair of amino acids elutes as a single peak (Table 2, marked as ** and *, respectively). In the case of the methionine and cisteine residues, they can not be labelled by dabsylation (because of the presence of S), as well as tryptophan. The major contaminant protein in Sf
extracts (fractions 60–65 after IMAC chromatography) was identified as an enolase by peptide sequencing after trypsin digestion. Four sections were obtained: VDEFLLSLDGTPNK, NQIGT, TESIQAA and KIEESLGADAIYAGK, showing homologies of 79%, 100%, 71% and 53% with sequence of enolase 1 (EC 4.2.1.11) from \textit{S. cerevisiae} (Swiss Protein Data Bank Accession No. P00924).

**Figure 1.** IMAC chromatography of Sf extracts on Superose HR. Continuous line represents the elution profile of proteins recorded at $\text{Abs}_{280}$. The dotted line represents the percentage of buffer B. Black marks represent fractions showing SOD activity. Filled circles correspond to peak A, and filled triangles correspond to peak B. Inset: SDS 12.5% PAGE gel stained with Coomassie Blue B G-250: lane 1, Sf extract (major proteins ratio, 70:30 ENO:SOD); lane 2, peak A (fraction 14); lanes 3 and 4, peak B (fractions 34 and 38); lane 5, fraction 60; and lane 6, fraction 65.

**Degree of purification of the enzyme preparation**

Semipurified extracts derived from fractionation using organic solvents (Sf) yield preparations with...
two principal components that can be easily separated using IMAC chromatography. The purified Cu–Zn SOD enzyme of \textit{D. hansenii} (obtained by IMAC chromatography) was visualized by a non-denaturing PAGE and compared with other kinds of SODs and sources (Figure 2A). SOD activity on gels was revealed by NBT stain. For \textit{D. hansenii–SOD} preparations, three bands were detected, whereas for other SOD sources only a single band is visible. Mobility of each kind of Cu–Zn SOD is quite different. For Cu–Zn SOD from \textit{D. hansenii} (lane 1), one band migrates like Mn-SOD from \textit{E. coli} (lane 4), but two other bands are intermediate between Cu–Zn SOD mobility from \textit{S. cerevisiae} (lane 2) and \textit{Bos taurus} (erythrocyte and liver Cu–Zn SOD have the same mobility, data not shown). Microheterogeneity has been observed on human Cu–Zn SOD, in which two bands (top and bottom) correspond to homodimer enzyme forms (\textit{Edwards et al.}, 1978), whereas the middle band corresponds to heterodimer form. From this, we believe the existence of two genes (or alleles) that codify for Cu–Zn SOD enzyme of \textit{D. hansenii} is possible.

\section*{Metal analysis}

Metal analysis revealed a zinc content of 0.29 µg/ml and a copper content of 0.41 µg/ml. No other metal was detected, confirming, in addition to inhibition tests, the copper–zinc nature of the enzyme.

\section*{Molecular mass}

To determine the subunit molecular mass of the \textit{D. hansenii} Cu–Zn SOD, 12% SDS–PAGE was done under denaturing conditions (boiling 10 min under reducing conditions) to IMAC-derived protein samples. Each enzyme preparation yielded a single band of protein when stained with Coomassie Blue B (Figure 1). Molecular masses were calculated to be 18.0 kDa. Thus, one may assume the enzyme is composed of two identical subunits (in molecular mass). However, when stained for activity when subjected to non-denaturing electrophoresis, three bands were observed.

Data of molecular mass of SODs used as reference were determined by the same method. No significative differences in molecular mass between Cu–Zn SOD enzymes were found. SODs from bovine (liver and erythrocyte) have similar mass (16 kDa ± 0.18), whereas proteins from \textit{S. cerevisiae} and \textit{D. hansenii} showed larger mass (18 kDa ± 0.14). We found the molecular mass of the principal contaminant protein (purified by IMAC, fractions 60–65) was 30.91 kDa ± 1.29.

\section*{Isoelectric point}

The IEF of Sf extracts from \textit{D. hansenii} analysed by ROTOFOR shows that this preparation possesses several components with SOD activity, having isoelectric points of 5.14, 4.69, 4.2, 4.0, 1.8 and 1.6. IEF–PAGE 5% of purified Cu–Zn SOD showed protein bands corresponding to pH 4.48–5.09, and a minor band that is distinguished only by silver stain with a pI near 1.6. Commercial enzymes show similar microheterogeneity, except Cu–Zn SOD from bovine liver and Fe SOD from \textit{E. coli}.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>\textit{SOD Sc}\textsuperscript{a}</th>
<th>\textit{SOD Dh}\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Cysteine</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>10\textsuperscript{**}</td>
<td>—</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9 \textsuperscript{*}</td>
<td>7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Glycine</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Histidine</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Lysine</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Leucine</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Methionine</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Asparagine</td>
<td>9\textsuperscript{**}</td>
<td>—</td>
</tr>
<tr>
<td>Proline</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3\textsuperscript{*}</td>
<td>8</td>
</tr>
<tr>
<td>Arginine</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Serine</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Threonine</td>
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<td>13</td>
</tr>
<tr>
<td>Valine</td>
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<td>Tryptophan</td>
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</tr>
<tr>
<td>Tyrosine</td>
<td>1\textsuperscript{*}</td>
<td>2</td>
</tr>
<tr>
<td>*</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>**</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>

\textsuperscript{a} \textit{S. cerevisiae}; data from Swiss Protein Data Bank, Accession No. p00445.

\textsuperscript{b} \textit{D. hansenii}; data from protein analysis; ID not identified.

\textsuperscript{*} Glutamine plus glutamic acid residues.

\textsuperscript{**} Asparagine plus aspartic acid residues.

—, Undetected.

Table 2. Amino acid content of Cu-Zn SOD from yeasts.
Inhibition tests

The effect of several SOD inhibitors (Table 3) suggest that Cu–Zn SOD activity is strongly inhibited by NaCN and H₂O₂ and only marginally affected by SDS. Fe SOD shows a decreased activity with SDS and H₂O₂, whereas Mn SOD was only affected by SDS. From these results, we conclude that the D. hansenii SOD studied here corresponds to the Cu–Zn form. Inhibition tests were done on the gel, resulting in the same observations (Figure 2B and 2C). The test on the gel helped us confirm that the three activity bands observed on non-denaturing PAGE are the result of three Cu–Zn SOD forms in D. hansenii, because these bands disappear in the presence of NaCN and H₂O₂.

Effect of pH and temperature on SOD activity

The effect of pH on the stability of different Cu–Zn SODs is shown in Figure 3A. Accordingly, all three kinds of enzyme completely retained their activity when treated, or stored, at pH 5–8 for short periods of time. However, D. hansenii and S. cerevisiae enzymes show enhanced activity at pH 6 and 7. No differences were observed in activity of bovine erythrocyte SOD from pH 5–9, in contrast with the other two sources. In general, it appears the enzymes lose activity rapidly at pH lower than 5 and higher than 9. Interestingly, D. hansenii SOD is 20% active at pH 3·0, whereas the other two enzymes tested are completely inactive.

Immunochemical analysis

Antigenic relationships among the different organisms were analysed by immunoelectrophoresis. All forms of SOD were recognized by their homologous antibodies, and by the antiserum to bovine liver, erythrocyte and S. cerevisiae SOD. In contrast, the SOD protein from D. hansenii was recognized only by its homologous antibody (data not shown). Thus, strong similarities between the Cu–Zn SOD of bovine and S. cerevisiae exist, but they all differ immunologically from the Cu–Zn SOD from D. hansenii. Using the antiserum obtained from the second bleeding (day 164 after immunization), the recognition of their corresponding protein antigens was studied by immunoblotting. The antigen proteins were immobilized on a nitrocellulose membrane from a 12% SDS–PAGE. In Figure 4, anti-S. cerevisiae SOD serum recognized its homologous antigen best (lane 2). The bovine SOD sources (lanes 1 and 3), the SOD from D. hansenii (lanes 6–9), and the Fe SOD (lane 4), were recognized weakly. A similar pattern is observed with anti-bovine erythrocyte SOD serum. This serum showed a good reaction with two bovine sources, and a rather good one with SOD from yeasts. The antibody tested also recognized Fe SOD from E. coli, but not Mn SOD. As expected, antibodies generated against D. hansenii SOD recognized homologous antigen best and, in a minor proportion, the monomer band of bovine SOD.

Table 3. Effect of inhibitors on SOD activity.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>D. hansenii</th>
<th>S. cerevisiae</th>
<th>Bos taurus</th>
<th>E. coli</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mm NaCN</td>
<td>0·0</td>
<td>0·9</td>
<td>0·0</td>
<td>83·9</td>
<td>76·1</td>
</tr>
<tr>
<td>10 mm H₂O₂</td>
<td>1·3</td>
<td>0·3</td>
<td>2·2</td>
<td>0·2</td>
<td>83·9</td>
</tr>
<tr>
<td>1 mm SDS</td>
<td>97·3</td>
<td>84·4</td>
<td>95·1</td>
<td>0·0</td>
<td>0·2</td>
</tr>
</tbody>
</table>

*Cu–Zn SOD. *Fe SOD. *Mn SOD.
S. cerevisiae and bovine Cu–Zn SODs, and Fe and Mn SOD (from E. coli).

CONCLUSIONS

Besides differential inhibition test to discriminate among the three kinds of superoxide dismutase enzymes, we found three additional elements that let us conclude that the SOD enzyme from D. hansenii that we purified is a copper–zinc protein. First evidence comes from a peptide sequencing obtained after trypsin digestion. An identity of 81% was found when comparing with the Cu–Zn SOD from S. cerevisiae. In addition, in a previous report we cloned an encoding sequence of Cu–Zn SOD (by similarity) from D. hansenii, in this case, translated amino acid sequence shows 100% identity with the peptide sequence corresponding to the residues 13–28, and 95% identity on amino acid content (NCBI Data Bank, Accession No. AFO 16383; Hernández-Saavedra et al., 1998). Finally, by analysing the metal content we corroborated the enzyme prosthetic group.

The observed patterns of D. hansenii Cu–Zn SOD enzyme, compared with other SODs tested,
in terms of specific activity, amino acid composition, molecular mass, heat deactivation, pH storage stability and immunological characterization, suggest large differences among them. Additional evidence that the Cu–Zn SOD from *D. hansenii* is an uncommon protein arises from the lack of absorbance at 595 nm (data not shown) when the protein content was measured by the Bradford method (1976), in contrast to measurements done with the reference enzymes. Thus, our results were validated for protein quantification (including the reference enzymes) by use of the Lowry method (Lowry et al., 1976).

Differences in amino acid content did not affect the catalytic activity of the enzyme, when compared with *S. cerevisiae* protein. On the contrary, our results suggest a higher efficacy on scavenging superoxide radicals (300% increase on specific activity) of the pure *D. hansenii* SOD enzyme, that all other Cu–Zn SODs used as a reference (bovine erythrocyte and liver, and *S. cerevisiae* shows on average, 5000 U/mg of protein). This fact, by itself, could be considered as an advantageous characteristic of the marine Cu–Zn SOD over the commercially available Cu–Zn SOD preparations. Apparently, changes in some amino acid residues cause differences in globular charge, because the *D. hansenii* SOD mobility under non-denaturing PAGE yielded a pattern totally different to all other Cu–Zn SOD sources. Nevertheless, the Cu–Zn SOD from *D. hansenii* presents both an identical pattern and molecular mass to *S. cerevisiae* protein under SDS–PAGE.

Characteristics of Cu–Zn SOD from *D. hansenii* are different because it has qualities intermediate between the yeast and bovine enzymes, in addition to three isoforms (revealed under native PAGE) not reported before for other sources except human (Edwards et al., 1978; Borchelt et al., 1995) and *Schistosoma mansoni* (Hong et al., 1992). With the presented data, it is not possible to distinguish between the presence of two alleles or two genes, because the genetics of this species is little known. This is the first report about a protein purification from this marine yeast, although this species has been used extensively as ideal model system for studying the mechanisms of salt tolerance in marine organisms. The non-common specific activity of *D. hansenii* SOD is perhaps due to its marine origin. In the open sea, the conditions stress the cell because of the osmolarity of seawater and availability of carbon sources and other nutrients, although we ignore the particular physico-chemical conditions that influence this species in natural environments at 50 m depth (Hernández-Saavedra, 1990). This hypothesis is reinforced by the discovery of an important second role for the Cu–Zn SOD in *S. cerevisiae*, namely acting as metal chelator to avoid poisoning by heavy metals (Cirolo et al., 1994; Culotta et al., 1995). Additionally, this hypothesis is supported because it has been observed that an increase in concentration of divalent metals such as Cu++ may affect the expression of Cu–Zn SOD enzymes in yeast, through its corregulation with a metallothionein system via ACE1 factor (Carri et al., 1991; Gralla et al., 1991). Knowledge and understanding of genetics and regulation of some processes of this species are the subject of our future research.

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