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 ($\cdot O_2^-$, $\cdot OH$, OCl^-, H_2O_2, etc.) in the respiratory process. Cells are equipped with a family of enzymes that catalytically scavenge the superoxide free radical through the disproportionation $\cdot O_2^- + \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (McCord and Fridovich, 1969), and therefore help to protect the cell against the toxic $\cdot O_2^-$ 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,

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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. *Debaryomyces hansenii* is a normal marine yeast, which has provided the principal model for study of salt tolerance in marine organisms. *D. hansenii* is strongly halotolerant. 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 (Norkranz 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 60 l 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 ($2250 \times g$, 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 ($2250 \times g$, 8 min at 4°C). The supernatant was mixed with 300 g/l of K_2HPO_4 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 $CuSO_4$ 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 M 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 M NH_4Cl, 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 $CuSO_4$, 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 dabsylation labelling reaction (Beckman); separation and quantification was carried out by HPLC in a System Gold (Beckman). These analyses were done at the Institut de Genetique et de Biologie Moleculaire et Cellulaire Peptide Service (IGBMC, Strasbourg, France).

Protein assay

The protein content in the different extracts was determined by the Lowry method (Lowry *et al*., 1976) using bovine serum albumin fraction V (Sigma) as a standard.

Enzyme assay

SOD activity was determined according to Beauchamp and Fridovich (1971) using NBT in the presence of a sensitizing dye (riboflavin). For this, 2 ml of reaction mixture (0·1 mM EDTA, 13 μM methionine, 0·75 mM NBT, 20 μM riboflavin in phosphate buffer 50 mM, pH 7·8), plus 0–100 μl of the enzyme extract were placed under fluorescent light for 10 minutes, or until A_{560} in control tubes reached 0·2–0·25 OD. The enzyme volume necessary to inhibit 50% of NBT reduction was determined and defined as a unit of enzyme activity (U). Specific activity (units per milligram of protein) was calculated on the basis of the protein content of the extracts by using a computer program (Vázquez-Juárez *et al*., 1993).

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 Cu^{++} and Zn^{++}. 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 20 000°C and finally cleaned by 10 s at 2200°C. Zinc content was determined by the flame absorption method in a Analyst-100 apparatus (Perkin-Elmer) 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 Miniprotean 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 H_2SO_4 as anode buffer. IEF broad range standards (BioRad) pI 4·45–9·6 and (Pharmacia) pI 3·5–9·3 were used.

Table 1.   Summary of the purification of Cu–Zn SOD from *D. hansenii*.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (%)	Yield (%)
Cell homogenate (So)	444	64 617	146	100	100
Solvent extraction (Sf)	10	41 709	4162	2870	64
IMAC chromatography (superose HR)					
Peak A	1·35	6291	4660	3214	10
Peak B	1·56	26 413	15 702	10 829	41

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/µl) was made with the corresponding buffer system (pH 3–10) to get a final enzyme concentration of 10 µg/µl. 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 H_2O_2 (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 semidry 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 $Na_2HPO_4 \cdot 7H_2O$, 2·4 g/l KH_2PO_4, 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, $3·58 \pm 0·34$ mg/ml and $153 \pm 47·5$ U/mg of protein for So extracts, and $4·5 \pm 0·35$ mg/ml and $4414·1 \pm 569·74$ 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 NH$_4$Cl from the IMAC column. Peak B (fractions 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, VSGVVNFEQSSESDPT, 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. AFO 16383; 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 GVVNFE). 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

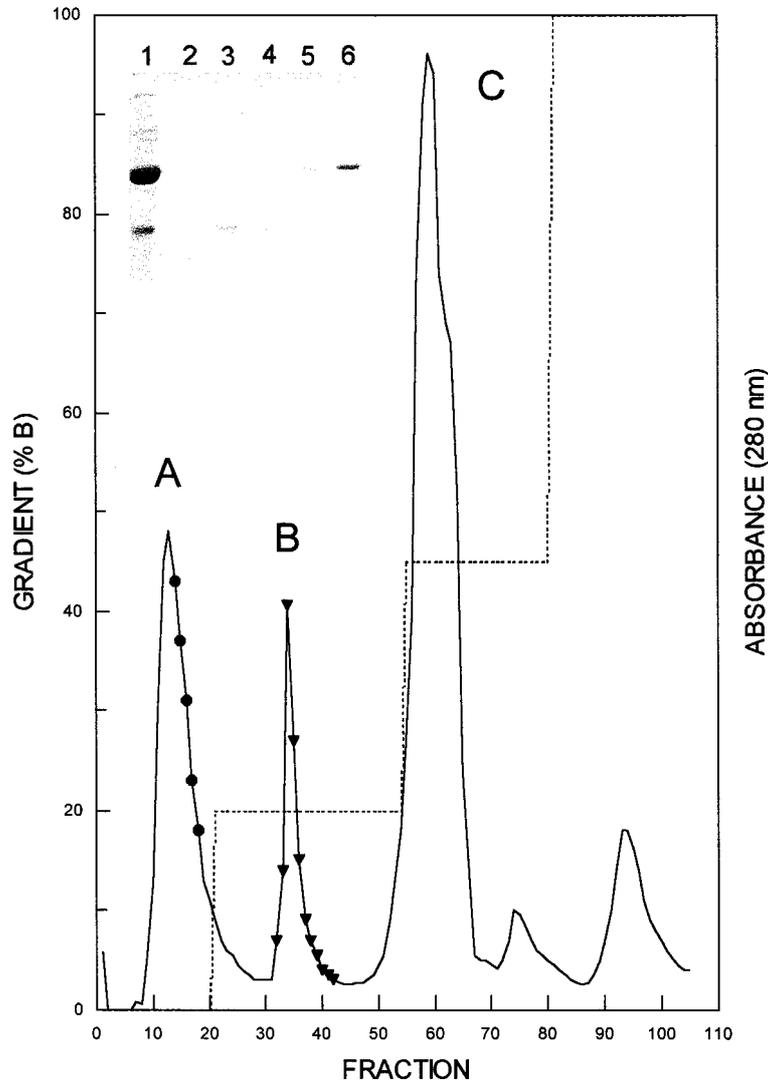Figure 1.   IMAC chromatography of Sf extracts on Superose HR. Continuous line represents the elution profile of proteins recorded at Abs_{280}. The dotted line represents the percentage of buffer B. Black marks represent fractions showing SOD activity. Filled circles correspond to peak A, and filled 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.

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 *S.* *cerevisiae* (Swiss Protein Data Bank Accession No. P00924).

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 *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 *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 *D. hansenii* (lane 1), one band migrates like Mn-SOD from *E. coli* (lane 4), but two other bands are intermediate between Cu–Zn SOD mobility from *S. cerevisiae* (lane 2) and *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 (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 *D. hansenii* is possible.

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.

Molecular mass

To determine the subunit molecular mass of the *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

Table 2. Amino acid content of Cu–Zn SOD from yeasts.

Amino acid	SOD *Sc*[a]	SOD *Dh*[b]
Alanine	13	15
Cysteine	2	—
Aspartic acid	10	**
Glutamic acid	9	*
Phenylalanine	6	6
Glycine	22	22
Histidine	6	6
Isoleucine	4	7
Lysine	10	7
Leucine	6	9
Methionine	1	—
Asparagine	9	**
Proline	8	6
Glutamine	3	*
Arginine	4	8
Serine	11	10
Threonine	11	13
Valine	17	13
Tryptophan	0	—
Tyrosine	1	2
*	12	11
**	19	19

[a]*S. cerevisiae*; data from Swiss Protein Data Bank, Accession No. p00445.
[b]*D. hansenii*; data from protein analysis; ID not identified.
*Glutamine plus glutamic acid residues.
**Asparagine plus aspartic acid residues.
—, Undetected.

(16 kDa ± 0·18), whereas proteins from *S. cerevisiae* and *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.

Isoelectric point

The IEF of Sf extracts from *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 *E. coli*.

Table 3.   Effect of inhibitors on SOD activity.

Inhibitor	Residual activity (%)				
	D. hansenii[a]	*S. cerevisiae*[a]	*Bos taurus*[a]	*E. coli*[b]	*E. coli*[c]
5 mM NaCN	0·0	0·9	0·0	83·9	76·1
10 mM H$_2$O$_2$	1·3	0·3	2·2	0·2	83·9
1 mM SDS	97·3	84·4	95·1	0·0	0·2

[a]Cu–Zn SOD. [b]Fe SOD. [c]Mn SOD.

Inhibition tests

The effect of several SOD inhibitors (Table 3) suggest that Cu–Zn SOD activity is strongly inhibited by NaCN and H$_2$O$_2$ and only marginally affected by SDS. Fe SOD shows a decreased activity with SDS and H$_2$O$_2$, 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 H$_2$O$_2$.

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.

The thermal stability of *D. hansenii* Cu–Zn SOD was analysed and compared with that of *S. cerevisiae* and bovine erythrocytes (Figure 3B and 3C). In this case, bovine erythrocyte SOD lost activity rapidly after 1 min boiling, whereas the Cu–Zn SODs from *S. cerevisiae* and *D. hansenii* retained 80% of their original activity. Even after 10 min

boiling, Cu–Zn SODs from yeast retain more than 30% of activity (Figure 3C). These results are similar to those observed in assays of material kept at several incubation temperatures for 20 min before measuring activity (Figure 3B), showing that sensitivity of bovine erythrocyte enzyme is higher compared with the other two enzyme sources.

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
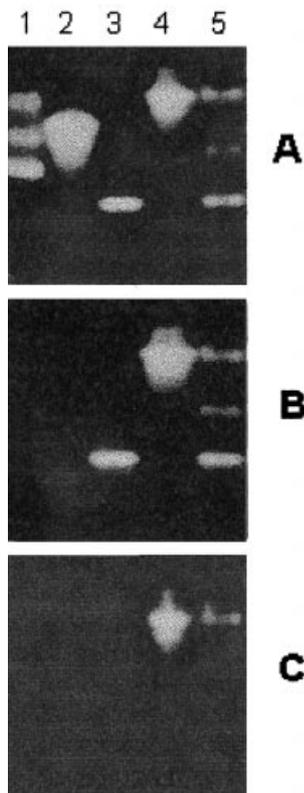
Figure 2. Non-denaturing 8% PAGE. Lane 1, Cu–Zn SOD enzyme from *D. hansenii*; lane 2, *Bos taurus* Cu–Zn SOD; lane 3, Fe SOD from *E. coli*; lane 4, Mn SOD from *E. coli*; and lane 5, crude extract from *E. coli* TOP 10. Gel A, normal stain condition; gel B stain mixture containing 5 mM NaCN and gel C 10 mM H_2O_2.

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
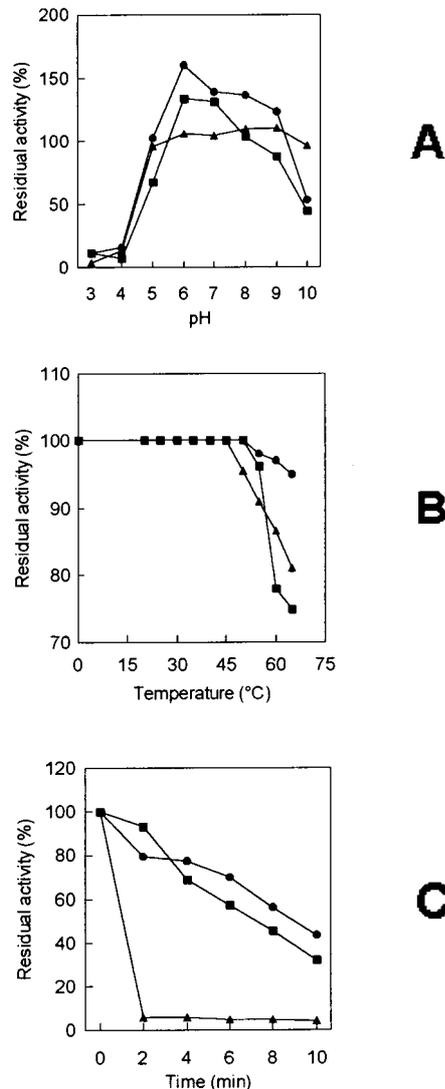

Figure 3. Thermal and pH stability of Cu–Zn SOD from several sources: *D. hansenii*, —■—; *S. cerevisiae*, —●—; *Bos taurus*, —△— (erythrocyte). (A) Effect of pH on the enzyme stability; the buffer systems used were: sodium citrate (50 mM, for pH 3–4), sodium acetate (50 mM, for pH 5), sodium phosphate (50 mM, for pH 6–7), TRIS·HCl (50 mM, for pH 8) and glycine–NaOH (50 mM, for pH 9–10). (B) Effect of incubation at several temperatures. (C) Effect of boiling time on SOD activity.

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.

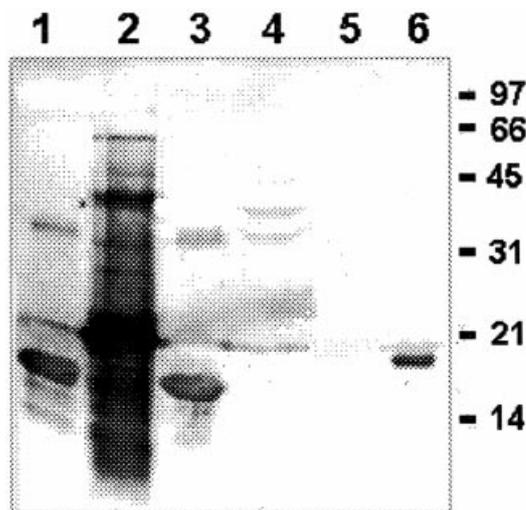The observed patterns of *D. hansenii* Cu–Zn SOD enzyme, compared with other SODs tested,

Figure 4. Western blot done using *S. cerevisiae* Cu–Zn SOD antiserum. The antigens used were: Cu–Zn SOD from *Bos taurus*, lane 1 (liver) and lane 3 (erythrocyte); lane 2, *S. cerevisiae* Cu–Zn SOD; lanes 4 and 5, Fe and Mn SOD from *E. coli*; lane 5, Cu–Zn SOD from *D. hansenii*. Antigens were immobilized on nitrocellulose membranes and specific proteins were visualized by staining with alkaline phosphatase-conjugated goat anti-rabbit IgG.

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 physicochemical 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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