Expression of a novel psicrophyl Cu/Zn superoxide dismutase from *Deschampsia antarctica* Desv. in *Escherichia coli*

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Submitted to: Molecular Biotechnology ?, Microbial Cell Factories?

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Abstract

Superoxide dismutase (SOD) converts the potentially dangerous superoxide radical ($O_2 \cdot^-$) into water and hydrogen peroxide. *Deschampsia antarctica* is a plant that grows in Antarctic territory and survives to extreme low temperature and high UV radiation, thus it is an ideal model to study its antioxidant effects. A cDNA Cu/Zn *SOD* gene from *D. antarctica* was cloned into a pET vector and expressed in *Escherichia coli* BL21-SI. 154 mg/mL of recombinant Cu/Zn SOD was attained in batch cultures in bioreactor. Using his-tag affinity gel chromatography, the recombinant Cu/Zn SOD was recovered with a purity of 90% and a specific enzyme activity of 749 U/mg at 25°C. However, zymogram test showed that the enzyme was more active at 4°C than 25°C. This SOD could be used reduce the oxidation of refrigerated and frozen foods.

Key Words: antioxidant, plant, psychophysics enzyme, photo-oxidation,

Introduction

Reactive oxygen species (ROS) are molecules produced during the metabolism and under stress conditions [1]. The main biological ROS are singlet oxygen ($^1O_2$), hydroxyl radical ($OH\cdot$), hydrogen peroxide (H_2O_2) and the superoxide radical ($O_2\cdot^-$) [2, 3]. Although ROS occur naturally during the metabolism, exposure to ultraviolet (UV) radiation and other types of stress can overwhelm the antioxidative response and they lead to the damage of intracellular lipids, DNA and proteins [4-6].

Superoxide Dismutases (SOD's) are the first line of defense against the ROS. SOD converts the superoxide radical to molecular oxygen and hydrogen peroxide in a two-step reaction [7]. There are four types of SODs, each one has a distinct metal ion in its active site: Mn, Fe, Ni and Cu/Zn-SOD [7, 8]. SOD's have been isolated from organisms in all kingdoms [9-13] including several thermophylic SOD's [14-17] and psychrophilic bacteria such as *Pseudoalteromonas haloplanktis* and *Marinomonas* sp. NJ522 [18, 19]. However to acknowledge this is the first Cu/Zn SOD from a psychrophilic vascular plant expressed by recombinant technology.

Deschampsia antarctica Desv. is one of the only two vascular plant species native to Antarctica. Due to the conditions of its habitat that include frozen ground, and ice/snow cover, deficient precipitation, incidence of low illumination during the winter and high UV radiation during summer. High levels of ROS are expected to be present in this plant.

In this work, we report the cloning, expression and purification of a cold active Cu/Zn superoxide dismutase from *D. antarctica* (*Da*SOD) in *Escherichia coli*. The recombinant protein was purified using affinity chromatography and its activity was assessed.

Materials and Methods

Bionformatic analysis

The aminoacid sequence of the *Da*SOD was inferred by translation of the nucleotide sequence. Then a BLAST analysis was performed using the non-redundant database of the GeneBank. The sequences with the highest scores were aligned with the aminoacid sequence of *Da*SOD through ClustalW software [20] and the alignment was used to build a phylogenetic tree using Mega software v 4 [21] choosing the neighbor joining method and the bootstrap was calculated from 1,000 replications. The theoretical molecular weight and the isoelectric point of *Da*SOD protein were calculated using MacVector software v 10.0.2 (MacVector Inc.).

Bacterial strains and plasmids

E. coli XL1-Blue (Gibco) was used as a host for general cloning *E. coli* BL21-SI, which contains the T7 RNA polymerase gene driven by the *pro*U promoter inducible by NaCl was used as a host for expression [22]. The pGEM-T Easy vector (Promega) was used for cloning and the pET-28a (Novagen) for expression. The pP2 is a pGEM-T (Promega) vector containing the open ready frame of the *DaSOD* gene.

The *Nco*I-*DaSOD-Hind*III insert was obtained by polymerase chain reaction (PCR) using the pP2 as template and the forward 5'- CCATGGTGAAGGCTGTAGCTGTG-3' and the reverse 5'-AAGCTTGCCCTGGAGCCCGATG -3' primers. The amplified fragment was subcloned in pET28a to generate the expression vector p*Da*SOD. The amplification mixture for 50 µL contained 0.75 U of *Tli* DNA polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 25 mM $MgCl_2$, 0.2 mM for each dNTP, 10 pmol of forward and reverse primers. PCR was performed in an iCycler (BioRad) using the following program: 94°C for 3 min, 30 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 1 min, and finally an extension step at 72°C for 8 min. The integrity of the p*Da*SOD was verified by restriction analysis and sequencing. The final construction has a his-tag at the C-terminus region to facilitate the purification by affinity chromatography.

Culture media

E. coli BL21-SI/p*Da*SOD cells were grown routinely in LBON agar plates containing 100 mg/mL ampicillin. The minimal medium contains per liter: 5 g glucose, 3.5 g $(NH_4)_2HPO_4$, 3.5 g KH_2PO_4, 1.0 g $MgSO_4$, 40 µg thiamine and 50 mg kanamycin. The pH was adjusted to 7.0 prior autoclaving for 15 min at 121°C. For all experiments, preinocula were cultured overnight at 250 rpm and 37°C, using 100 ml of minimal medium plus 0.5% yeast extract (Difco Labs, Franklin Lakes, NJ).

Batch culture

Batch cultures were performed in a 1.3-l bioreactor (Applikon) equipped with two six-blade Rushton turbines and stirred at 300 rpm. The cultures were started with 1 l of

minimal medium with the sufficient amount of the overnight-grown bacteria to achieve an initial optical density at 600nm (OD_{600nm}) of 0.2. The batch cultures were performed 37°C until the OD_{600nm} of 0.6 was attained. Then, the expression was induced with 0.3 M NaCl and the temperature of post-induction was lowered to 32.5°C. The pH was maintained at 7.0 and dissolved oxygen at 20% during the experiments using an ADI-1030 Bio-controller (Applikon) and the BioXpert v1.3 software (Applikon). Culture samples collected from the bioreactor were harvested by centrifugation, resuspended in PBS and sonicated for subsequent analysis of protein and SOD activity.

Analytical procedures

Cell growth was monitored at OD_{600nm} using a spectrophotometer (Varian Cary BIO-50, Palo Alto, CA). Biomass concentration was determined as dry cell weight with a calibration curve. Total protein concentration was analyzed by the Lowry method using bovine serum albumin (BioRad, Hercules, CA) as the standard. The proteins were separated by 4-20% gradient SDS-PAGE and visualized with 0.1% (w/v) Coomassie Brilliant Blue R250 (BioRad). Densitometry analysis of polyacrylamide gels and zymograms was carried out using the Quantity One™ v4.5 software (BioRad).

Protein purification

Cell suspension from the bioreactor were separated by centrifugation and resuspended in PBS and incubated on ice during 30 min. Then cells were sonicated in an Ultrasonic processor GE 505 (Sonics, Newtown, CT) using 10 pulses of 10 s at 30% amplitude and 10 s resting between each cycle. The soluble fraction was recovered by centrifugation in a

centrifuge RC5C plus (Sorvall) at 5000 rpm 15 min at 4°C. Protein purification was carried out using Ni-NTA affinity columns with the ProBond Purification System (Invitrogen) following the instructions provided by the manufacturer.

*Da*SOD *zymogram and enzyme activity*

*Da*SOD activity was assayed with the Beauchamp & Fridovich [23] staining method as follow: 10 µg of total proteins were electrophoreted in a 10% SDS-PAGE. The electrophoresis was carried at 4°C and 100 V for 4 h. After the gels were washed two-times at room temperature with 50 mL of 10mM Tris-HCl buffer (pH 7.9) containing 25% v/v of isopropanol for 25 min. Then, the gels were incubated 25 min in 50 mM phosphate buffer (pH 7.8), 25 min in a 50 mM phosphate buffer (pH 7.8) containing 2.5 mM *p*-nitro blue tetrazolium (NBT, USB Co, Cleveland OH), washed briefly and incubated 15 min with 25 ml of 50 mM phosphate buffer (pH 7.8) with 1.1 mM Riboflavin (Sigma, St. Louis, MO) and 1.4 mM TEMED (Sigma), washed 3 times in distilled water and exposed to source of light for 1 h. To determine the activity in cold, the gel was incubated all time at 4°C and following the process described above. Bovine Erythrocyte SOD (Sigma) was used as a control to determine activity. The specific enzyme activity was measured using the method of McCord & Fridovich [24]. One Unit of activity was defined as the amount of enzyme needed to attain the half of total inhibition of the reduction of NBT at 25°C [24].

Results

Bioinformatic analysis

The open reading frame of the cDNA *DaSOD* gene and the inferred aminoacid sequence for the protein is shown in the Fig 1. The *DaSOD* gene contains some codons referred as low used for *E. coli* such as CCC (2), GGG (5), GGA (9), but it does not contain the codons AGA, AGG, ATA, AGT, CTA, which are the most critical codons that could affect the translation in *E. coli* [25]. Therefore, no significant troubles of the gene expression are expected. The *Da*SOD is a protein with 152 residues of aminoacids with a calculated molecular weight of 15,131 Da and an estimated pI of 6.16. The complete sequence of the *Da*SOD was examined for similarity to Cu/Zn SODs from some vascular plants (Fig. 2). The alignment showed identity values between 84-86.8%. The identities compared with those from *Oryza sativa* ABF95937, *Populus suaveolens* ABF48717, *Zea mayz* NP001105704, *Populus trichocarpa* ABK94197, *Manihot esculenta* AAT77951, *Pennisetum glaucum* ABP65325 were 85.5, 86.8, 85.5, 85.5 and 84.9 respectively. *Bos taurus* AAI02433 was included as external issue and the identity was 57.9. The nearest sequence is the Cu/Zn SOD from *Zea mays* |NP_001105704| with an identity of 86.8%. Despite the low identity, the seven amino acid residues that coordinate with the copper and zinc atoms and two cysteine residues that form a disulfide bridge were conserved in all sequences (Fig. 2). Although the primary structure of the Cu/Zn SOD is high conserved in several plants, the phylogenetic analysis of the Cu/Zn SOD from sequences showed in the Fig. 2 and other, separated the *Da*SOD from the main cluster indicating an important difference in the primary sequence with respect to the other Cu/Zn SODs (Fig. 3).

DaSOD production in bioreactor

The fragment *DaSOD* gene was cloned in a pET vector to yield p*Da*SOD and the *E. coli* BL21-SI was selected as cell host, which has been used successful for the expression of recombinant proteins using NaCl as inducer [26, 27]. Typical batch culture of *E. coli* BL21-SI/p*Da*SOD is shown in the Fig. 4. For this culture, the biomass concentration increased exponentially at a specific growth rate of 0.17 h^{-1} to reach a maximum concentration of 1.2 g/L, and thereafter it remained constant (Fig. 2). The maximum production of the recombinant protein was attained 3 h after induction with 0.3 M NaCl. For this culture, the *Da*SOD concentration increased from 0 to 112 mg/L (Fig. 4) and the protein patterns of the total cell extract is shown in the Fig. 5. It can be observed that the recombinant protein is the main protein expressed and the molecular weight was approximately 16 kDa, which corresponds well with that calculated for the native protein (15.13 kDa) plus the molecular weight due to the 6xHis-tag (0.84 kDa).

Purification of recombinant Cu/Zn SOD

The recombinant *Da*SOD had a low affinity towards the Ni-NTA column; most of it was eluted with 5mM imidazole at pH 8.0, lowering the pH to 7.0 had no observable effect on its affinity (data not shown). The densitometry analysis revealed that the purified band represented at least 90% of the visualized content (Fig. 6).

Cu/Zn SOD from *D. antarctica* is cold active

After the purification the activity of the recombinant *Da*SOD was assessed in a zymogram. The assay was performed at 4 and 25°C (Fig. 7). It can be seen, the *Da*SOD

showed activity at both temperatures, whereas the bovine erythrocyte SOD was active only at 25°C, this reflects a broader temperature adaptation towards the colder environments than the mesophilic bovine SOD counterpart. Additionally, the time of exposure to 32.5°C of the enzyme during the fermentation is an insight that it withstands greater temperatures as well. The purified recombinant *Da*SOD showed an enzyme activity of 749 U/mg at 25°C. Table 1 summarizes some the specific enzyme activities of different Cu/Zn SODs reported. Despite the *Da*SOD showed best performance at 4°C, we only could measure the specific enzyme activity at 25°C since, the production of superoxide from fotooxydation of the riboflavin is not efficient at 4°C.

Discussion

The cDNA of the *Da*SOD was cloned and expressed in *E.coli* using a NaCl inducible system. The recombinant enzyme showed to be active at 4 and 25°C, but it was more efficient at 4°C due to its psychrophil origin. It has been reported that psychrophilic enzymes have an increased catalytic efficiency at low or moderate temperatures without changing their binding and active site architecture [34]. This is accomplished by an improved flexibility that leads to a low thermal stability on heat, compared to its mesophilic counterparts [34]. This propriety of achieving at lower temperatures an increased activity than other thermophilic or mesophilic enzymes, makes them most useful for processes that are in the range of 0-30°C, this seems to be the case of cold active lipases that have a wide variety of industrial applications [35]. This is the first report of a plant cold active Cu/Zn SOD. Only iron SODs from Antarctic marine bacteria have been reported previously [19, 36]. The potential applications of the psychrophilic

SODs include the prevention of oxidative damage in the eyes and skin caused by UV radiation [37, 38], premature skin ageing [39], chilling damage caused by low temperature inactivation of antioxidant enzymes in tropical fruits [40], as well as protection from any other superoxide damage within their optimum range of temperature.

Acknowledgements

This work was financed by the grant Antarctica Des S-3607. Sergio A. Garcia thanks CONACyT for scholarship 209658.

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Table 1. Specific enzyme activities of some Cu/Zn SODs from different organisms.

Organism	Temperature (°C)	Specific activity (U/mg)	Reference
Deschampsia antarctica	25	749	This work
Humicola lutea	25	96.1	[28]
Cryptococcus liquefaciens	30	119.3	[29]
Saccharomyces cerevisiae	30	27.1	[29]
Homo sapiens	25	192	[30]
Epinephelus malabaricus	25	3883	[31]
Thermoascus aurantiacus	25	150	[32]
Bos taurus	25	3660	[33]

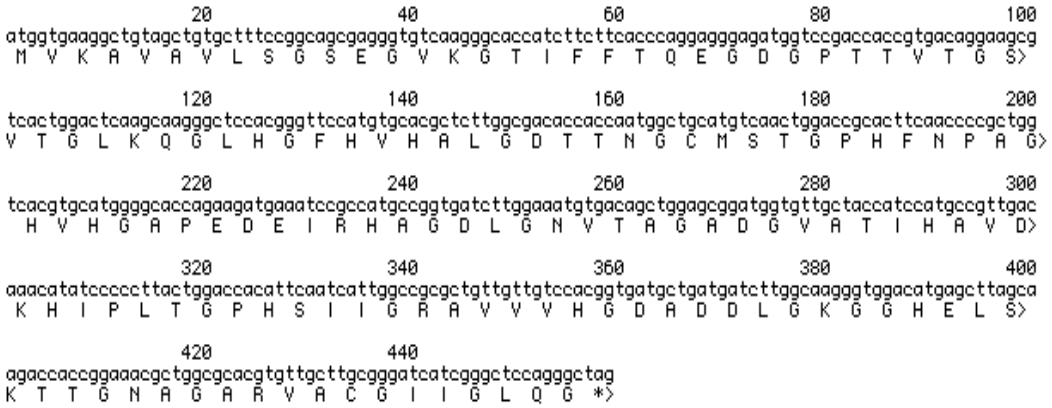

Fig 1. Nucleotide sequence of a *Da*SOD cDNA and the deduced amino acid sequence. The asterisk denotes the stop signal.

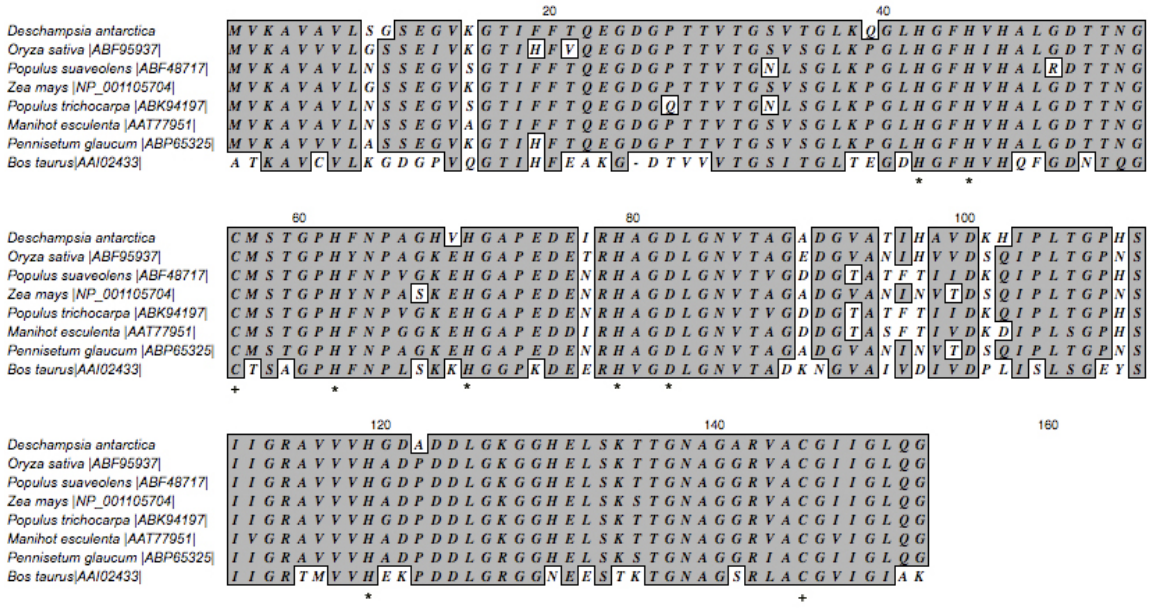

Fig 2. Comparison of the deduced amino acid sequence of the Cu/Zn-*Da*SOD with those from *Oryza sativa* ABF95937, *Opulus suaveolens* ABF48717, *Zea mayz* NP001105704, *Populus trichocarpa* ABK94197, *Manihot esculenta* AAT77951, *Pennisetum glaucum* ABP65325 and *Bos taurus* AAI02433 used as external control. The residues that coordinate copper and zinc atoms and that form the single disulfide bridge are indicated with asterisks and plus signs, respectively.

Fig 3. Cluster analysis of the Cu/Zn *Da*SOD, representative vascular plants and the Bovine Erythrocyte SOD. The phylogenetic tree was constructed with full-length Cu/Zn SOD amino-acid sequences using ClustalW program as described in Material and Methods. The bootstrap values in selected node are percentages of 1000 replications.

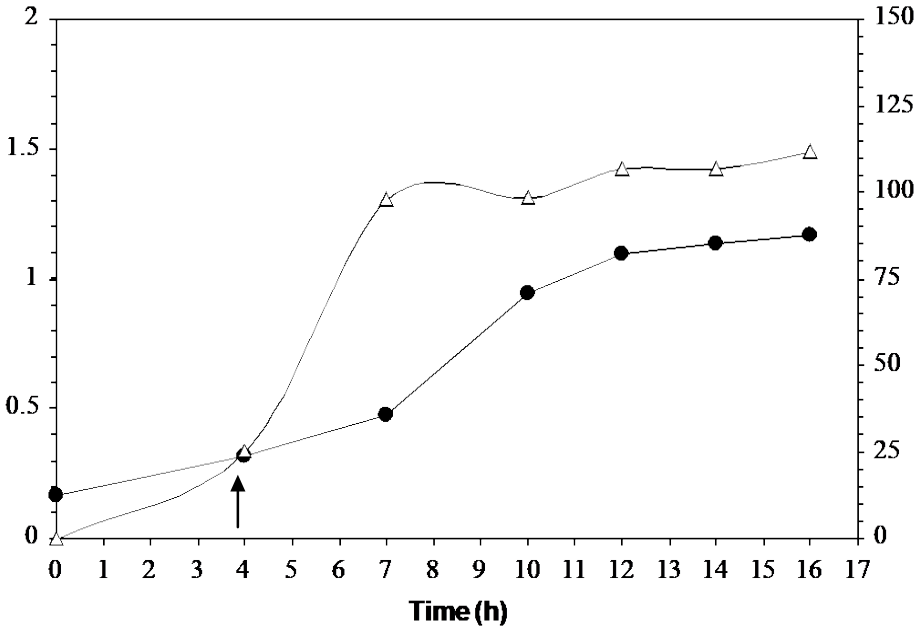

Fig 4. Growth kinetics of *E. coli* BL21-SI/p*Da*SOD in a batch culture using minimal medium. Biomasa conc. [●] (g/L) and *Da*SOD conc. [△] (mg/L). Arrow shows induction time with NaCl.

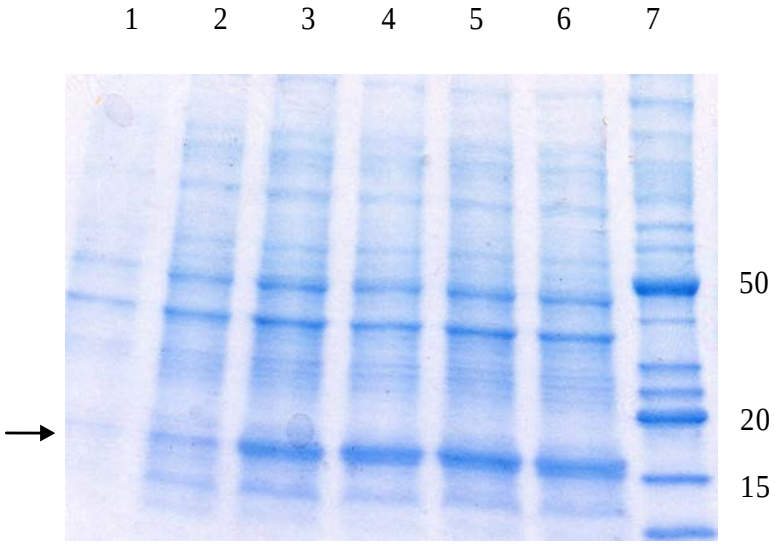

Fig. 5. Protein patterns of the total cell extract from the batch cultures of *E. coli* BL21-SI/pD*a*SOD in bioreactor. Lane 1, culture before the induction; lane 2-6, total cell proteins of five samples after induction; lane 7, protein ladder (Invitrogen). Arrow indicates the recombinant *Da*SOD.

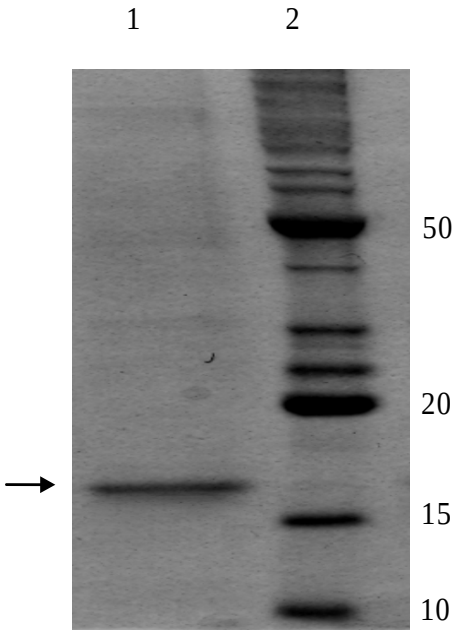

Fig. 6. Recombinant *Da*SOD after the Ni-affinity chromatofraphy. Lane 1, the recombinant *Da*SOD after the affinity chromatofraphy; lane 2, protein ladder (Invitrogen). Arrow indicates the recombinant *Da*SOD.

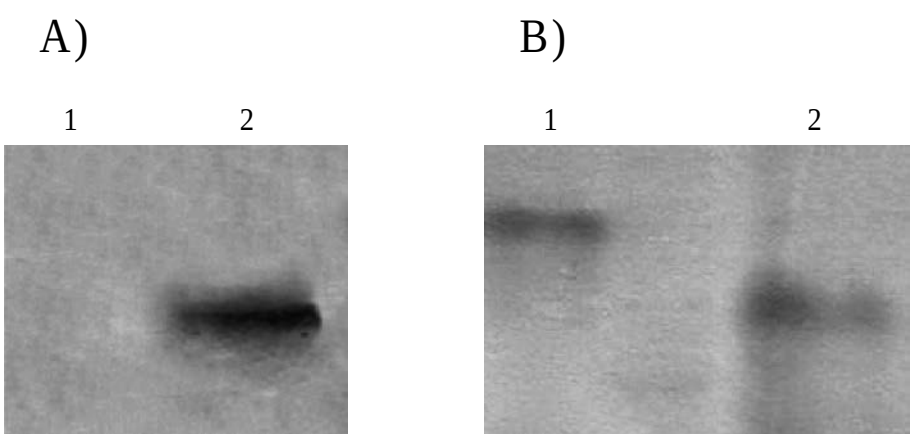

Fig 7. Zymagram showing the SOD activity assays at 4°C (A) and 25°C (B). For both gels: Lane 1, bovine erythrocyte SOD (Sigma); lane 2, recombinant *Da*SOD.