



**HAL**  
open science

## A novel, thermostable manganese-containing superoxide dismutase from

Ivanka Petrova Boyadzhieva, Miroslava Atanasova, Elka Emanuilova

► **To cite this version:**

Ivanka Petrova Boyadzhieva, Miroslava Atanasova, Elka Emanuilova. A novel, thermostable manganese-containing superoxide dismutase from. *Biotechnology Letters*, 2010, 32 (12), pp.1893-1896. 10.1007/s10529-010-0368-8 . hal-00614503

**HAL Id: hal-00614503**

**<https://hal.science/hal-00614503>**

Submitted on 12 Aug 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Section: Microbial and Enzyme Technology**

**A novel, thermostable manganese-containing superoxide dismutase from *Bacillus licheniformis***

**Ivanka Petrova Boyadzhieva<sup>\*</sup>, Miroslava Atanasova<sup>1</sup>, Elka Emanuilova**

Department of Extremophilic Bacteria, Institute of Microbiology, Bulgarian Academy of Sciences, 26, Acad. G. Bonchev str., 1113 Sofia, Bulgaria

<sup>\*</sup>Corresponding author: E-mail: [petrovaim@abv.bg](mailto:petrovaim@abv.bg); Tel: (359 2) 979 31 10; Fax: (359 2) 8 700 109

<sup>1</sup>Present address: Department of Molecular Biology and Biotechnology, ANFACO – CECOPESCA Crta. Colegio Universitario 16, 36310 VIGO (Pontevedra), Espana

**Abstract** A new, thermostable superoxide dismutase (SOD) from *Bacillus licheniformis* M20, isolated from Bulgarian mineral springs, was purified 11-fold with 11% recovery of activity. From native PAGE and SDS-PAGE, the enzyme was composed of two subunits of 21.5 kDa each. The SOD was inhibited only by NaN<sub>3</sub>, which suggested that this SOD is of the manganese superoxide dismutase type. The purified enzyme had maximum activity at pH 8 and 55°C. The half-life of the SOD was 10 min at 95°C.

**Keywords:** *Bacillus licheniformis*; Enzyme thermal stability; Manganese-containing superoxide dismutase; Purification

## **Introduction**

Superoxide dismutases (SODs, EC 1.15.1.1) are metalloenzymes, classified into three types: Mn-, Fe- and Cu/Zn-SOD according to their metal cofactors (Whittaker 2003). They are involved in the detoxification of the superoxide radicals ( $O_2^-$ ) to  $H_2O_2$ , which in turn is broken down to  $H_2O$  by catalases and peroxidases. The superoxide radicals are generated by a single electron transfer to  $O_2$  and can damage DNA, proteins and lipids (Halliwell 2007).

An important requirement for SODs intended for industrial applications is their thermal stability as thermal denaturation is a common cause of enzyme inactivation. In this connection, an increasing interest to the SODs from thermophiles and hyperthermophiles were observed (Wang 2008). Thermostable SODs have also a higher resistance to most chemical denaturants and could be very useful in the industries of pharmaceuticals, cosmetics, food, and biotechnology and in environmental protection. SODs are crucial components in the physiological response to oxygen toxicity and are actively investigated as potential therapeutic agents in pathological conditions related to oxidative stress. Moreover, SODs offer a great potential for the sensitive quantification of  $O_2^-$  in electrochemical biosensors (Campanella et al. 2001).

To our knowledge, studies describing SOD from *Bacillus licheniformis* are not available. Therefore, in the present work, the purification and biochemical properties of a novel, thermostable SOD from *Bacillus licheniformis* M20 are reported for the first time.

## **Materials and methods**

### Microorganism and growth conditions

*Bacillus licheniformis* M20 isolated from Bulgarian thermal springs (Dolna banja region), identified by 16S rRNA analysis and deposited in the EMBL GenBank DDBJ under accession number FN597717, was used. The strains was cultivated as described previously (Boyadzhieva and Emanuilova 2005).

### SOD assay

SOD activity was determined by the method of Beauchamp and Fridovich (1971). The reaction mixture in 3 ml was composed of 20 mM phosphate buffer (pH 7.8), 13 mM L-methionine, 75  $\mu$ M NBT (Nitroblue Tetrazolium), 2  $\mu$ M riboflavin and 20  $\mu$ l purified enzyme. The test tubes were placed 30 cm below a fluorescent lamp (40 W). The reaction was initiated by turning the light on and reduction of NBT was determined by following the reading  $A_{560}$  for 5 min. One unit of SOD activity was defined as the amount of the enzyme causing one half of the maximum inhibition of NBT reduction.

Protein concentration was estimated according to the Bradford method, using bovine serum albumin as standard.

All analyses of each sample were carried out in two repetitions and the data presented were obtained after three independent experiments, i.e. the data are mean values  $\pm$ SD, n=6.

## Results and discussion

### Purification of the SOD

A summary of the purification steps is given in Table 1. A purification of 11-fold with a yield of 11% was achieved. The SDS-PAGE and PAGE analysis of the purified enzyme revealed a single band (Fig. 1a). The molecular weight of the SOD was 22 kDa by SDS-PAGE. The native PAGE and the zymogram showed a single active protein band with molecular weight 43 kDa (Fig. 1b). These data indicated that the SOD from *B. licheniformis* M20 was dimeric. Most of the reported SODs are homodimers or homotetramers with subunits of about 20 kDa (Whittaker 2003). The purified SOD was completely inhibited by 20 mM  $\text{NaN}_3$ , but not by KCN and  $\text{H}_2\text{O}_2$ , suggesting that the enzyme belonged to Mn-SOD classification (Fig. 1c).

### Properties of the SOD

The SOD from *Bacillus licheniformis* M20 was catalytically active only in the presence of  $\text{Mn}^{2+}$  (Table 2). The purified SOD was optimally active at pH 7.5-9, with maximum activity at pH 8 (Fig. 2a). The enzyme exhibited 60% relative activity at pH 9.5, unlike other Mn-SODs, which rapidly lost its activity at pH over 7.8 (Seatovic et al. 2004). The optimum enzyme activity was established at 50-55°C (Fig. 2b). The purified SOD from thermophilic *Geobacillus kaustophilus* ATCC8005 is optimal active at the same temperature, but with respect to the enzyme activity, SOD from *B. licheniformis* M20 exhibited 9780 U  $\text{mg}^{-1}$ , compared to 1780 U  $\text{mg}^{-1}$  for

*Geobacillus kaustophilus* SOD (Huang 2009). SOD from *B. licheniformis* M20 was distinguished with a high thermal stability, as shown in Figure 3. The half-life of the enzyme was 10 min at 95°C. In comparison with the SOD from thermophilic *Rhodothermus sp.* XMH10, having half-life 30 min at 80°C (Wang et al. 2008) and the SOD from thermophile *Thermomyces lanuginosus* with half-life 30 min at 70-75°C (Li et al. 2005), the SOD from *B. licheniformis* M20 had exceptionally better thermal stability.

The studied SOD from *B. licheniformis* M20, able to catalyze the conversion of superoxide ions or radicals under the moderate to high temperature environments is exceptionally useful in their removing or subtracting and has valuable potentials in different biotechnological processes. An advantage of the proposed SOD in the present work is its superior thermal stability, which could be provided a relatively longer functional action in the applied processes. The new, thermostable purified Mn-SOD from *B. licheniformis* M20 with its important features to be active at a high alkaline pH of the reaction media (8; 9-9.5) and high temperatures (75-95°C) could be a candidate for industrial uses.

## References

- Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 44:276-278
- Boyadzhieva I, Emanuilova E (2005) Characteristics of bacterial strain, superoxide dismutase producer, isolated from Bulgarian thermal spring. *J Culture Coll* 4:61-67
- Campanella L, De Lusa S, Favero G, Tomassetti M (2001) Superoxide dismutase biosensors working in non-aqueous solvent. *Fresenius J Anal Chem* 369:594-600
- Halliwell B (2007) Biochemistry of oxidative stress. *Biochem Soc Trans* 35:1147-1150
- Huang SL (2009) Thermostable superoxide dismutase. United States Patent No US 2009/0311768 A1
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685
- Li DC, Gao J, Li YL, Lu J (2005) A thermostable manganese-containing superoxide dismutase from the thermophilic fungus *Thermomyces lanuginosus*. *Extremophiles* 9:1-6
- Seatovic S, Gligic L, Radulovic Z, Jankov RM (2004) Purification and partial characterization of superoxide dismutase from the thermophilic bacteria *Thermothrix sp.* *J Serb Chem Soc* 69:9-16

Wang X, Yang H, Ruan L, Liu X, Li F, Xu X (2008) Cloning and characterization of a thermostable superoxide dismutase from the thermophilic bacterium *Rhodothermus* sp. XMH10. *J Ind Microbiol Biotechnol* 35:133-139

Whittaker JW (2003) The irony of MnSOD. *Biochem Soc Trans* 31:1318-1321

**Table 1** Purification process of SOD from *B. licheniformis* M20

Purification steps	Total protein (mg)	Total activity (kU)	Specific activity (U mg <sup>-1</sup> )	Enzyme yield (%)	Purification ( fold )
Crude extract	390	351	900	100	1
40%-60% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	100	200	2000	57 ± 1.14	2.2
Phenyl- Sepharose	40	168	4200	48 ± 0.96	4.7
DEAE- Sepharose	10	60	6000	17 ± 0.34	6.7
Sephadex G - 100	5	41.75	8350	11.8 ± 0.35	9.2
Sephadex G - 50	4	39.12	9780	11.1 ± 0.28	10.8

After 5 h cultivation, the cells were collected by centrifugation (4500 x g for 15 min), and washed with 20 mM phosphate buffer (pH 7.8). The pellets were suspended in the same buffer and the cells were ruptured by ultrasonic disintegration (three times in an ice bath for 2 min at 40 V), and then the cell debris were removed by centrifugation (10000 x g for 15 min, 4°C). The resulting supernatant was precipitated with 40-60% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. After centrifugation (10000 x g, 30 min, 4°C), the precipitate was dissolved in 20 mM phosphate buffer and dialyzed against the same buffer overnight at 4°C. The sample was loaded to a Phenyl-Sepharose column (2.5 x 15 cm), pre-equilibrated with 20 mM Tris/HCl buffer (pH 7.8), containing 2.8 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The elution was carried out with the same buffer by stepwise decrease in the ionic strength of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ranging from 2.8 to 0.7 M. The fractions with SOD activity were pooled, dialyzed against 20 mM phosphate buffer and loaded to a DEAE-Sepharose column (2.5 x 20 cm), equilibrated with 20 mM phosphate buffer. Elution of SOD was done with a linear gradient from 20 to 100 mM phosphate buffer, pH 7.8. The active fractions were pooled and concentrated by ultrafiltration using PM-10 membrane (10 kDa; Amicon, Millipore, USA). The enzyme concentrate was applied to a Sephadex G-100 column, and then was loaded on Sephadex G-50 column. Gel filtration was carried out with 20 mM phosphate buffer, pH 7.8, on a self-packed column (2.5 x 25 cm)

**Table 2** Specific activity of native, apo- and metal-reconstituted apo-SOD from *B. licheniformis* M20

Sample	Recovery activity (%)
Native SOD	100
Apo-SOD	0
Reconstitution with 10 mM FeSO <sub>4</sub>	0
Reconstitution with 10 mM MnSO <sub>4</sub>	54
Reconstitution with 10 mM MnSO <sub>4</sub> + 10 mM FeSO <sub>4</sub> (1:1, v/v)	52

The initial activity of native SOD  $9700 \text{ U mg}^{-1} \pm 1.7 \%$  is taken as 100%. The SOD was denatured by dialysis into 8 M urea/10 mM EDTA/50 mM Tris (pH 8.0) for 12 h at 4°C. The sample was then dialyzed into 50 mM Tris (pH 8.0) for 12 h at 4°C, which resulted in catalytically inactive SOD apo-protein. The sample of SOD apo- protein was divided into three dialysis bags and dialyzed for 4 h at 4°C against 8 M urea-50 mM Tris buffer (pH 8.0), containing indicated above metal ions. After reconstitution, each sample was placed in 0.5 mM EDTA-50 mM Tris (pH 8.0) for 12 h at 4°C. The specific activity of each sample was measured by NBT reduction

### **Figure captions**

**Fig. 1** SDS-PAGE, PAGE and zymogram analysis of the purified SOD from *Bacillus licheniformis* M20.

**(a)** Coomassie Brilliant Blue staining. Lane 1: Protein marker, broad range (7-175 kDa, BioLabs, P7708S); Lane 2: SDS-PAGE; Lane 3: native PAGE. The electrophoreses were performed according to Laemmli (1970) on a 10% (w/v) acrylamide gel. The protein was stained 15 min in a 0.2% Coomassie Brilliant Blue R250 (ethanol/acetic acid/distilled water). **(b)** Zymogram. Lane 1: Activity staining with NBT was done according to Beauchamp and Fridovich (1971). For the detection of SOD activity in gels, proteins were separated on a 10% non-denaturing PAGE. The gel was soaked in 730  $\mu\text{M}$  NBT solution for 15 min in the dark, followed by immersion with illumination in 38 mM TEMED and 26  $\mu\text{M}$  riboflavin.

**(c)** The effect of inhibitors on the purified SOD. The samples were incubated for 60 min at 4°C with the inhibitors and then used for a zymogram analysis. Lane 1: as the control, no treatment; Lane 2: 10 mM KCN as inhibitor; Lane 3: 40 mM  $\text{H}_2\text{O}_2$  as inhibitor; Lane 4: 20 mM  $\text{NaN}_3$  as inhibitor

**Fig. 2** pH **(a)** and temperature profile **(b)** of the purified SOD from *Bacillus licheniformis* M20.

**(a)** SOD activity was measured under standard assay conditions at pH range of 5-12. The following buffers were used: 0.2 M Na-acetate- $\text{CH}_3\text{COOH}$  (pH 5-6), 0.2 M potassium-sodium phosphate (pH 6.5-7.5) and 0.2 M glycine- $\text{NaOH}$ - $\text{NaCl}$  (pH 8-12). The enzyme activity corresponding to 100% was 9670  $\text{U mg}^{-1}$ . Standard deviations of relative activities were in range of 0.5-1.4%. **(b)** SOD activity was measured under standard assay conditions at temperatures over the range 30-90°C in 20 mM phosphate buffer, pH 7.8. The enzyme activity corresponding to 100% was 9550  $\text{U mg}^{-1}$ . Standard deviations of relative activities were in range of 1.5-3.4%

**Fig. 3** Temperature stability of the purified SOD from *Bacillus licheniformis* M20. The enzyme was pre-incubated at various temperatures (95°C-squares; 85°C-triangles; 75°C-circles; 65°C-diamonds; 55°C-empty circles) and at defined time intervals, the residual activities were determined under standard assay conditions. The non-heated

enzyme was considered as 100% ( $9810 \text{ U mg}^{-1}$ ). Standard deviations of residual activities were in range of 0.7-2.7%

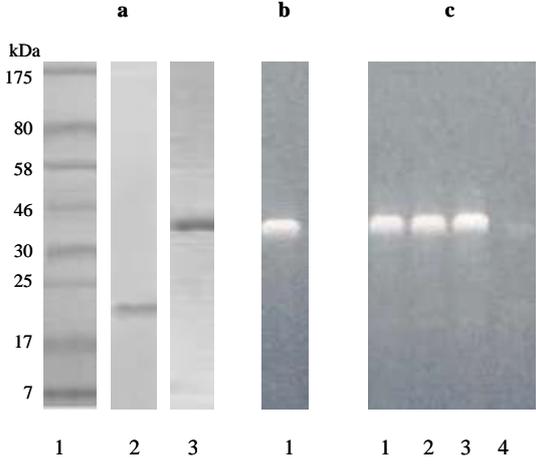


Fig. 1

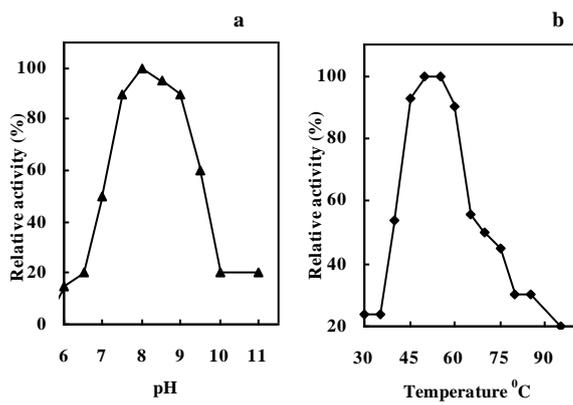


Fig. 2

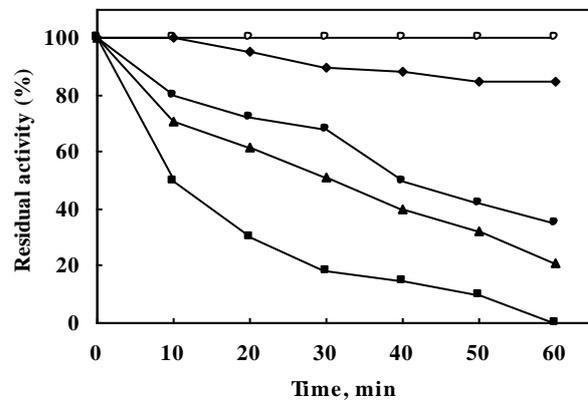


Fig. 3