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Purification and partial characterization of superoxide dismutase from the thermophilic bacteria *Thermothrix* sp.

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Abstract: Superoxide dismutase (SOD; EC 1.15.1.1.), a high molecular weight component of the antioxidant defense system, provided promising results in the treatment of oxidative damage. *Thermothrix* sp., isolated from thermal spa water in Serbia, showed high superoxide dismutase activity. The SOD, from cell free extract, was purified to homogenity by ammonium sulfate precipitation, Sephadex G 75 gel filtration chromatography and QAE Sephadex ion exchange chromatography. The specific activity of the purified enzyme was 9191 U/mg. The purified enzyme was analyzed and partially characterized. SOD was localized in polyacrylamide gel by activity staining, based on the reduction of nitroblue tetrazolium (NBT) by superoxide. The enzyme molecular weight determined by gel chromatography is 37 kD. According to SDS PAGE it is composed of two subunits of equal size, joined by noncovalent interactions. The isoelectric point, assessed by isoelectric focusing is 5.3. The optimum pH for enzyme activity was in the range of 8 to 10. The optimum temperature for SOD activity was 60 ºC. After one hour of incubation at 40, 50 and 60 ºC the SOD activity increases, but at 80 ºC, the SOD is denaturated. After 24 hours of incubation at 25 ºC SOD activity only slightly decreases.

Keywords: superoxide dismutase, *Thermothrix* sp., isolation, purification, characterization.

INTRODUCTION

Thermophiles are a group of organisms characterized by their ability to live and flourish in unusually harsh conditions of high temperatures. Intrinsically stable and active at high temperatures, enzymes, products of thermophiles, offer major biotechnological advantages over enzymes of mesophilic or phychrophilic origin. Thermostable enzymes are easier to purify by heat treatment. Their thermostability is associated with a higher resistance to chemical denaturants and performing enzymatic reactions at high temperatures allows higher substrate concentrations, lower viscosity, fewer risks of microbial contamination and, often, higher reaction rates.

SODs are a class of metalloproteins which catalyze the dismutation of superoxide radicals ($O_2^{\cdot -}$) to oxygen and hydrogen peroxide.[2]

Serbian Chemical Society active member.

$$2O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2$$

SOD provides a vital defense mechanism against the formation of deleterious oxygen species. The presence of SOD in all aerobic organisms protects cells against oxidative stress.[3]

Four common forms of the enzyme are known, differing in the metal ion cofactor in the active site.[4] Copper and zinc containing SODs have been found in the cytosol of eucaryotes, in chloroplasts, and in the periplasm of some prokaryotes. Fe-SOD is present in both aerobic and anaerobic bacteria, Archaea, and plants, whereas Mn-SOD is present in bacteria, Archaea, mitochondria and chloroplasts. Dismutases of the Fe- and Mn- type are closely related in sequence and structural homology.[5] A novel type of cytosolic SOD containing nickel as a cofactor has recently been discovered in several *Streptomyces* species.[6]

This paper reports the isolation and partial characterization of a Mn-SOD from the thermophilic bacteria *Thermothrix* sp.

EXPERIMENTAL

Bacterial strain and growth conditions

The thermophilic bacteria used in this work were isolated from thermal spa water in Serbia, purified and characterized.[7] The bacteria were grown in 100 ml of nutrition broth (1.5 % peptone, 0.5 % meat extract, 0.5 % NaCl, 0.03 % K_2HPO_4, pH 7.2) in a 1000 ml Erlenmeyer flask, during 20 h at 55 ºC, and with 120 rpm agitation in a laboratory shaker. The cells were collected by centrifugation (15 min, 3000 rpm, 5 ºC, Beckman J2-21 centrifuge), and washed with 0.067 M phosphate buffer, pH 7.8.

Purification procedure

The pelleted cells were suspended in 3 ml of 0.05 M phosphate buffer, pH 7.8 containing 1 mM EDTA and ruptured with lysozyme (10 mg ml^{-1}, 20 min, 24 ºC). The suspension was centrifuged and the resulting clear supernatant was used.

The cell-free extract was treated with $(NH_4)_2SO_4$ in two steps. First, solid $(NH_4)_2SO_4$ was added to the extract to 50 % saturation in an ice bath, then the mixture was stirred for 15 min, and left at 4 ºC for 60 min. The precipitate was removed by centrifugation. In the second step, the supernatant was treated with solid $(NH_4)_2SO_4$ to 80 % saturation, stirred for 15 min, and left at 4 ºC for 60 min. The precipitate with SOD activity was centrifuged at 4000 rpm for 30 min (Hettich, Universal 30 RF centrifuge) and then dissolved in a minimal volume of phosphate buffer saline (PBS), pH 7.2 and dialyzed at 4 ºC against the same buffer.

Gel chromatography on Sephadex G75 was carried out in PBS pH 7.2 on a column of dimensions 26 × 550 mm. The fraction with SOD activity was eluted with 55 ml PBS and dialyzed against 0.01 M phosphate buffer pH 7.2 over night.

The determination of the molecular weight by gel filtration was carried out on Superose 12 HR 10/30 column equilibrated with 0.05 M phosphate buffer pH 7.2, and calibrated with the following molecular weight standards: immunoglobulin G (160 000 D), human serum albumin (67 000 D), β-lactoglobulin (35 000 D), cytochrome c (12 400 D), vitamin B_{12} (1 355 D), cytidine (246 D).

Ion exchange chromatography on QAE Sephadex was carried out in a 20 × 250 mm column, equilibrated with 0.01 M phosphate buffer pH 7.2. The column was washed with 0.1 M phosphate buffer pH 7.2 and the SOD was eluted with a concentration gradient of phosphate buffer pH 7.2 (0.2 – 0.3 M).

Protein concentration

Proteins were determined by the method of Bradford,[8] using bovine serum albumin as the standard.

Superoxide dismutase assay

SOD was assayed by the method of Winterbourn *et al*. based on the ability of SOD to inhibit the reduction of NBT by superoxide.[9] One unit was defined as the amount of enzyme causing one half of the maximum inhibition of NBT reduction.[9]

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli[11] using a 10 % separating gel, 4 % stacking gel, but without sodium dodecyl sulfate and β-mercaptoethanol. The protein bands were localized with Coomassie brilliant blue R250. The SOD was localized *in situ* by activity staining according to Beauchamp and Fridovich.[10] The glycoproteins were stained by specific staining with Schiff reagent.[12]

SDS PAGE was performed using a 10 % separating gel, 4 % stacking gel, with or without β-mercaptoethanol. The samples were heated for 5 min at 100 ºC in capped vials with 1 % (w/v) SDS in the presence or absence of β-mercaptoethanol. The standards used to make a plot of log molecular weight *versus* mobility of the protein band were: phosphorylase B (94 000 D), bovine serum albumin (67 000 D), ovalbumin (43 000 D), trypsin inhibitor (20 100 D) and α-lactalbumin (14 400 D). The protein bands were localized with Coomassie brilliant blue R250.

Isoelectric focusing was performed on a Phast system (Pharmacia), 500 Vh. The pH gradient was 3.0–9.0, and the standards used to make a plot of pH *versus* mobility of the protein band were: trypsinogen (9.30); lentil lectin, basic (8.65); lentil lectin, middle (8.45); lentil lectin, acidic (8.15); myoglobin, basic band (7.35); myoglobin, acidic band (6.85); carbonic anhydrase B, human (6.55); carbonic anhydrase B, bovine (5.85); β-lactoglobulin A (5.20); soybean trypsin inhibitor (4.55); amyloglucosidase (3.50).

RESULTS AND DISCUSSION

Purification of SOD

The results of a typical purification experiment are summarized in Table I. After each purification step, the protein content and the enzyme activity were determined.

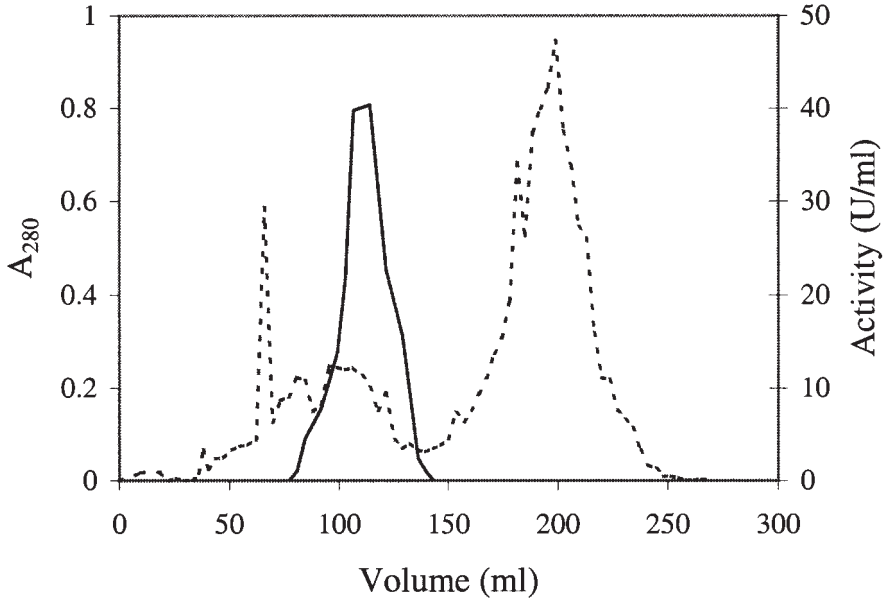

Fig. 1. Gel chromatography on a Sephadex G 75 column; protein concentration (---), superoxide dismutase activity (—).

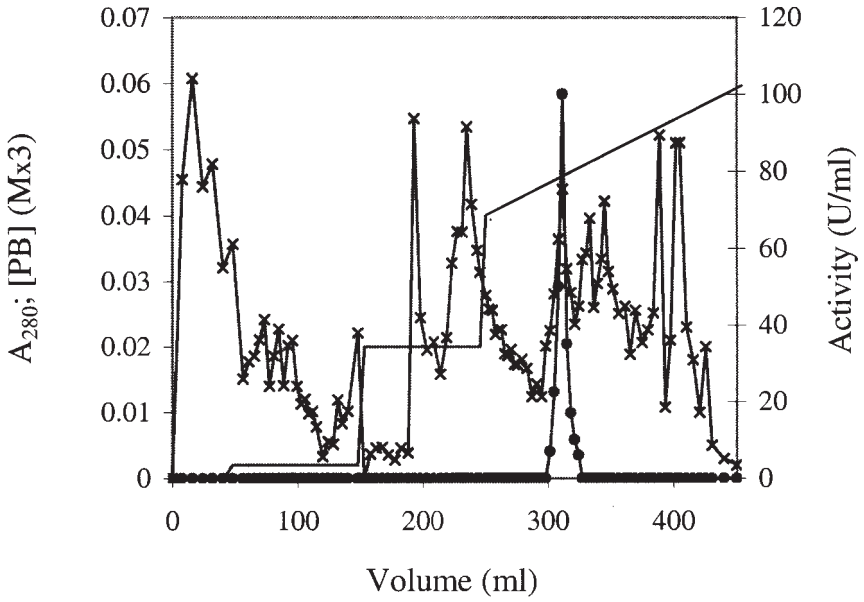

Fig. 2. Ion-exchange chromatography on a QAE Sephadex column; protein concentration (×-×-×), superoxide dismutase activity (–●–), concentration of eluting buffer (—).

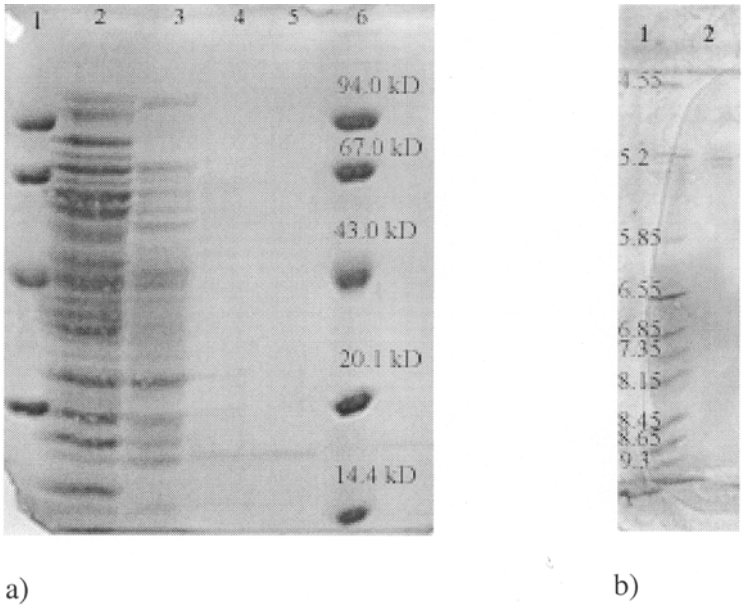

Fig. 3. a) SDS PAGE in 4/10 PAA gel; 1 – protein markers, 2 – *Thermothrix* sp. cell-free extract, 3 – pellet after ammonium sulfate precipitation at 80 % saturation, 4 – fractions with SOD activity after gel chromatography, 5 – fractions with SOD activity after ion-exchange chromatography, 6 – protein markers b) IEF on a Phast system. pH gradient 3.0–9.0; 1 – pI markers, 2 – purified SOD.

According to our results, ammonium sulphate precipitation to 50 % saturation eliminated 50 % of the ballast protein, without loss in SOD activity, and the second precipitation step from 50 to 80 % ammonium sulfate saturation resulted in a SOD preparation of higher specific activity. After gel chromatography on Sephadex G 75 column, 90 % of the ballast protein was discarded with a loss of SOD activity of about 57 %. Results of this chromatographic procedure are shown in Fig. 1. The SOD was eluted from a QAE Sephadex colum with a concentration gradient of phosphate buffer pH 7.2 (0.2 – 0.3 M). Figure 2 presents the results of the ion exchange chromatography. The SOD fraction showed only one band of SDS PAGE after QAE Sephadex chromatography. It can be concluded that the SOD was purified to homogeneity after two-step ammonium sulphate precipitation, gel chromatography, and ion exchange chromatography. The total purification achieved by the procedure outlined in Table I was 105 fold over the first soluble extract, with a yield of 15 %. The specific activity of the purified enzyme was 9191 U/mg (in literature 2500–10000 U/mg[14,16]). The degree of purification of the SOD in every purification step was examined by SDS PAGE as shown in Fig. 3a.

TABLE I. Purification of SOD from *Thermothrix* sp.

	Volume/ml	Total protein mg	Total activity U	Specific activity U/mg	Yield/%	Purification (fold)
Cell-free exract	17.5	53	4132	87		
50 % AS s.[a]	18.2	27	4224	155	102	1.8
80 % AS p.[b]	5.7	27	2723	99	66	1.1
Dialysis	8.5	26	2311	88	56	1.0
Sephadex G 75	50.6	2.5	992	392	24	4.5
QAE Sephadex	20.7	0.07	624	**9191**	**15**	**105.4**

[a]ammonium sulfate supernatant, [b]ammonium sulfate pellet

Characterization of SOD

Molecular weight. The molecular weight of the SOD was determined by gel filtration on a Superose 12 HR 10/30 column equilibrated with 0.05 M phosphate buffer pH 7.2. By this method, the molecular weight of the purified SOD was determined to be 37 kD. The subunit molecular weight was determined by SDS PAGE. In the presence or absence of β-mercaptoethanol, the SOD showed only one band. The molecular weight in the absence and presence of β-mercaptoethanol was found to be 18.6 kD and 17.7 kD, respectively. It can be concluded that the enzyme was composed of two subunits of equal size, and that these subunits are not joined by interchain disulfide bonds. All known Mn-SODs (bacterial and mitochondrial) are either homodimers of homotetramers with subunit molecular weights of about 20 kD.[13]

Specific staining for oligosaccharide-containing polypeptides indicated that the SOD from *Thermothrix* sp. is a glycoprotein. The only known SOD that is a glycoprotein is mammalian extracellular Cu/Zn SOD.[14]

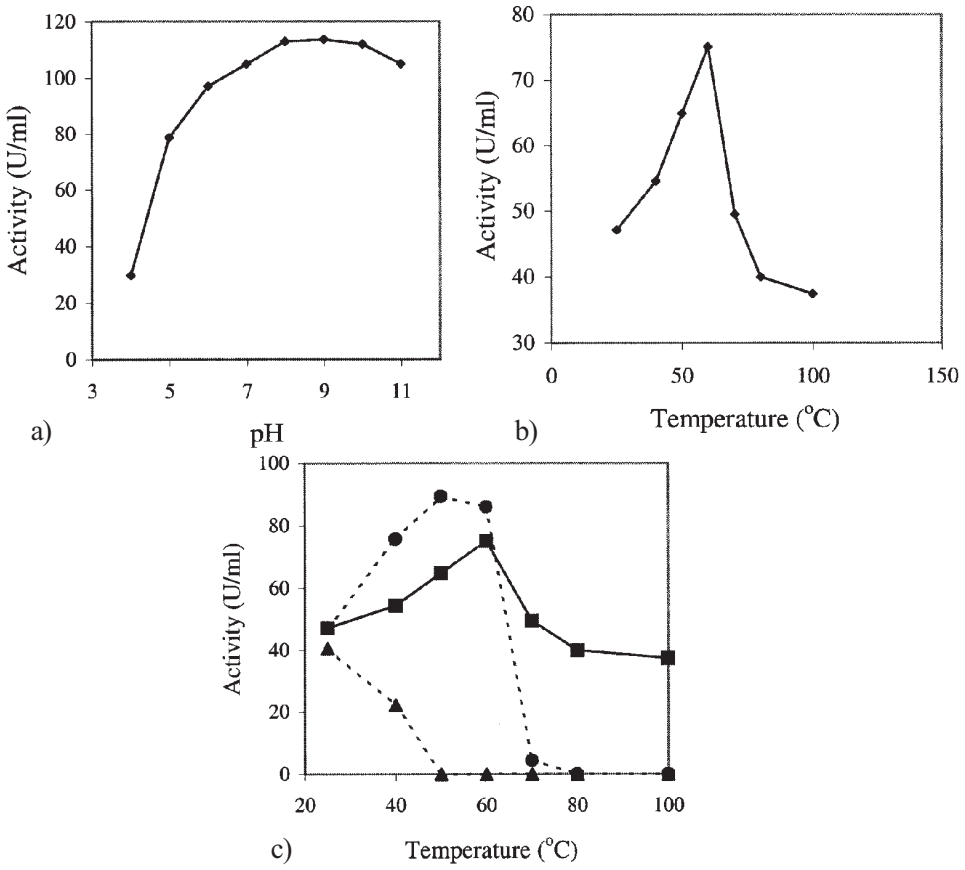

Fig. 4. a) Effect of pH on the activity of the purified SOD. b) Effect of temperature on the activity of the purified SOD. c) Effect of temperature on the stability of the SOD purified, after 15 min (–■–), 60 min (–●–) and 24 h (–▲–) incubation.

Isoelectric point. The isoelectric point, determined by isoelectric focusing on a Phast system (pI gradient was 3.0–9.0) was found to be 5.3, as is shown in Fig. 3b.

Metal inhibition. Inhibition reactions with purified enzyme in the presence of some SOD inhibitors (cyanide, hydrogen peroxide and sodium azide) indicated that this enzyme is Mn-SOD. These results confirmed results with SOD eluted from polyacrylamide gel.[8]

pH optimum. The SOD activity was determined over a wide pH range from 4 to 11. The SOD activity was the greatest in the pH range from 8–10 (Fig. 4a). It is clear that purified SOD is very active in alkaline solutions. For other Mn SODs, their activity decreases at pH values greater than 7.8.[15]

Temperature optimum and thermostability. The SOD activities were determined after preincubation of the enzyme for 30 min over a wide temperature range (25–100

ºC). The optimum temperature for the SOD activity was 60 ºC as is shown in Fig. 4b. The SODs from mesophilic bacteria have a temperature optimum at lower temperatures (for example, SOD from alkaliphilic *Bacillus* have a temperature optimum at about 35 ºC), close to their growing temperature.[16]

The influence of temperature on the SOD activity was determined after preincubation of the enzyme for different times (15 min, 1 h, and 24 h), at different temperatures (25, 40, 50, 60, 70, 80, and 100 ºC). After one hour incubation at 40, 50 and 60 ºC, the SOD activity was increased, but at 70 ºC it was decreased. After one hour incubation at 80 ºC, the SOD was denatured (the activity was lost). After 24 hours incubation at 25 ºC, the SOD activity was decreased by only 7 %, but at 40 ºC the loss was 40 %, and at 50 ºC no activity remained (Fig. 4c). SODs are thermostable enzymes, and after one hour incubation they are stable up to 50 ºC, as shown for *Desulfovibrio gigas*.[17] The activity of the SOD from the investigated bacteria was increased after one hour incubation at 40, 50 and 60 ºC.

CONCLUSION

SOD from the thermophilic bacteria *Thermothrix* sp. was purified to homogeneity and characterized for the first time. The enzyme was purified 105 times, with 15 % yield. It was estimated that SOD constituted 0.86 % of the protein of the crude soluble extract.

The results demonstrate that *Thermothrix* sp. isolated from thermal spa water in Serbia is a potential producer of thermostable Mn SOD and that its large scale cultivation for SOD production seems justified.

ИЗВОД

ПРЕЧИШЋАВАЊЕ И КАРАКТЕРИЗАЦИЈА СУПЕРОКСИД–ДИСМУТАЗЕ ИЗ ТЕРМОФИЛНИХ БАКТЕРИЈА *Thermothrix* sp.

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Супероксид–дисмутаза (SOD; EC 1.15.1.1.) је компонента антиоксидативног система која даје значајне резултате у третману оксидативних оштећења. Бактерије изоловане из термалних бањских вода Србије, детерминисане као *Thermothrix* sp., имају високу супероксид–дисмутазну активност. Супероксид–дисмутаза из ћелијског екстракта је пречишћена двостепеним таложењем амонијум–сулфатом, гел–хроматографијом на Sephadex G75 колони и јоноизмењивачком хроматографијом на QAE Sephadex колони. Специфична активност пречишћеног ензима је 9191 U/mg. Супероксид–дисмутаза је анализирана и окарактерисана. Молекулска маса пречишћеног ензима, одређена гел–хроматографијом, износи 37 kD. Ензим се састоји из две идентичне субјединице спојене нековалентним интеракцијама. Изоелектрична тачка, одређена изоелектричним фокусирањем је 5,3. Оптимална pH вредност деловања ензима представља опсег pH од 8 до 10. Максимална активност ензима је на 60 ºC. Инкубација на 40, 50 и 60 ºC (60 мин) повећава супероксид–дисмутазну активност, док се на 80 ºC ензим денатурише, док инкубација од 24 сата на собној температури незнатно смањује активност.

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