Extremophiles (2005) 9:1–6
DOI 10.1007/s00792-004-0413-4

ORIGINAL PAPER

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# A thermostable manganese-containing superoxide dismutase from the thermophilic fungus *Thermomyces lanuginosus*

Received: 6 April 2004 / Accepted: 30 June 2004 / Published online: 30 July 2004


**Abstract** A thermostable superoxide dismutase (SOD) from a *Thermomyces lanuginosus* strain (P134) was purified to homogeneity by fractional ammonium sulfate precipitation, ion-exchange chromatography on DEAE-Sepharose, Phenyl-Sepharose hydrophobic interaction chromatography, and gel filtration on Sephacryl S-100. The molecular mass of a single band of the enzyme was estimated to be 22.4 kDa, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Using gel filtration on Sephacryl S-100, the molecular mass was estimated to be 89.1 kDa, indicating that this enzyme was composed of four identical subunits of 22.4 kDa each. The SOD was found to be inhibited by $NaN_3$, but not by KCN or $H_2O_2$, suggesting that the SOD in *T. lanuginosus* was of the manganese superoxide dismutase type. The SOD exhibited maximal activity at pH 7.5. The optimum temperature for the activity was 55°C. It was thermostable at 50 and 60°C and retained 55% activity after 60 min at 70°C. The half-life of the SOD at 80°C was approximately 28 min and even retained 20% activity after 20 min at 90°C.

**Keywords** Manganese superoxide dismutase · Purification · *Thermomyces lanuginosus* · Thermophilic fungi

## Introduction

Superoxide dismutase [(SOD) EC 1.15.1.1] is a metalloenzyme that catalyzes the dismutation of superoxide radicals to $H_2O_2$ and $O_2$, and it protects oxygen-metabolizing cells against the harmful effects of superoxide free radicals. There are three types of SODs that are distinguished according to their catalytic metal cofactor: copper, manganese, and iron (Hassan 1989; Fridovich 1995). SODs have recently found applications in gene therapy, used for cardiovascular diseases as well as in the pharmaceutical and cosmetic industries. SODs are currently of great interest as potential therapeutic treatments for oxidative damage. It has been shown that SODs might be beneficial in the treatment of postischemic reperfusion injury, rheumatoid and osteoarthritis, brain trauma, and influenza-induced lung pneumonitis. SODs have been proposed to be clinically useful for a wide variety of applications, including prevention of oncogenesis, tumor promotion, tumor invasiveness, and reduction of the cytotoxic and cardiotoxic effects of anticancer drugs (Angelova et al. 2001; Zhong et al. 1997; Mates and Sanchez-Jimenez 2000; Trotti 1997).

In industry, a major requirement for commercial SODs is thermal stability, because thermal denaturation is a common cause of enzyme inactivation. Thermostable SODs are potentially very useful due to their high stability. In recent years, there has been increasing interest in SODs of thermophiles, which were expected to produce thermostable SODs. It has been reported that SODs were isolated from hyperthermophiles of the genera *Sulfolobus*, *Aquifex*, *Pyrophilus*, *Pyrobaculum*, and *Aeropyrum* (Lim et al. 1997; Yamano and Maruyama 1999; Ursby et al. 1999; Knapp et al. 1999; Yamano et al. 1999; Whittaker and Whittaker 2000; Amo et al. 2003).

SODs are found in fungi. From known reports, SODs have been isolated only from mesophilic fungi, and SODs from thermophilic fungi have not been reported (Holdom et al. 1995, 1996; Hamilton and Holdom 1997; Jacobson et al. 1994; Tesfa-Selase and Hay 1995; Misra and Fridovich 1972; Ravindranath and Fridovich 1975; Rapp et al. 1973; Lavelle and Michelson 1975; Fang et al. 2002; Angelova et al. 2001; Kazanina and Selezneva 1992; Miszalski et al. 1998; Jacob et al. 2001; Diez

Communicated by K. Horikoshi

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et al. 1998). *Thermomyces lanuginosus* Tsikl is a thermophilic fungus with a high maximum temperature for growth (Brock 1978). The fungus has been shown to produce a thermostable amylolytic enzyme (Jensen et al. 1988; Li et al. 1998), proteases (Li et al. 1997), lipoxygenase (Li et al. 2001), and lipase (Liu et al. 1973), but there are no published reports on SOD in *T. lanuginosus*. In this study, we report the purification and characterization of a thermostable manganese superoxide dismutase (MnSOD) in *T. lanuginosus* P134 isolated from China.

## Materials and methods

### Growth conditions

*Thermomyces lanuginosus* P134 was isolated from compost collected in Beijing on YPS agar (Cooney and Emerson 1964). *T. lanuginosus* P134 was grown in shake cultures at 50°C in 20 250-ml flasks with 50 ml liquid medium (Li et al. 1998). After incubation for 8 days, mycelium was harvested by filtration through quantitative filter paper and washed exhaustively with distilled water. The mycelia were then frozen immediately in liquid nitrogen and stored at −80°C before use.

### Preparation of the crude enzyme

A preparation procedure was carried out at 4°C. Harvested mycelium was macerated with a mortar and pestle, and SOD was extracted in phosphate buffer (30 ml, 100 mM phosphate buffer, pH 7.0). The slurry was centrifuged at 10,000 *g* for 30 min at 4°C, and the supernatant was used for the purification of SOD.

### SOD purification

1. All procedures of the SOD purification were carried out at 4°C. The following buffers were used: buffer A, 50 mM Tris-HCl (pH 8.0); buffer B, buffer A containing 50% saturation ammonium sulfate.
2. Fractional ammonium sulfate precipitation: solid ammonium sulfate was added to the supernatant to 50% saturation. After 12 h, the precipitate was centrifuged (10,000 *g*, 30 min), and ammonium sulfate was added to the supernatant to 65% saturation. After 12 h, the precipitate from subsequent centrifugation (10,000 *g*, 30 min) was dissolved in buffer A and dialyzed overnight against three changes of the same buffer.
3. The dialyzed sample was put on a DEAE-Sepharose column equilibrated with buffer A. After the column was washed with five column volumes of buffer A, a 240-ml linear gradient of NaCl (0–0.3 M in buffer A) was applied. Fractions with SOD activity were pooled.
4. Phenyl-Sepharose hydrophobic interaction chromatography: the sample from the DEAE-Sepharose column with 50% saturation ammonium sulfate added was applied to a Phenyl-Sepharose column previously equilibrated with buffer B. After the column was washed with five column volumes of buffer B, SOD was eluted with a 120-ml linear gradient of ammonium sulfate from 50–0% saturation. Fractions with SOD activity were pooled and concentrated to 2 ml, using Centricon-30 Microconcentrators according to the manufacturer's instructions.
5. Gel filtration on Sephacryl S-100: 2 ml of the concentrated sample from Phenyl-Sepharose column was applied to a Sephacryl S-100 gel filtration column previously equilibrated with buffer A. SOD was eluted with buffer A. Fractions with SOD activity were pooled and concentrated for determination of purity and properties.

### Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify protein purity of the enzyme under denaturing conditions, as described by Laemmli (1970). PAGE was performed according to Laemmli (1970). Zones of protein were localized by staining with 0.2% Coomassie Brilliant Blue R250. Zones of SOD activity were stained with nitroblue tetrazolium (Beauchamp and Fridovich 1971). To identify the type of SOD, duplicate gels were incubated with 10 mM KCN, 10 mM $NaN_3$, or 10 mM $H_2O_2$ during activity staining to inactivate CuZnSOD, MnSOD, or FeSOD, respectively (Asada et al. 1975; Britton et al. 1978).

### Enzyme assays and determination of protein

The SOD activity of the crude enzyme was determined by using the method of Stewart and Bewley (1980). Protein was determined by the method of Lowry et al. (1951), with crystalline bovine serum albumin as the standard.

### Effect of pH and temperature on activity of the SOD

The influence of pH on SOD activity was determined in the presence of buffers of a wide pH range (pH 5–10). The buffers used were: 0.2 M $CH_3COOH$–$CH_3COONa$ (pH 5.0, 5.5, 6.0), 0.2 M $NaH_2PO_4$–$Na_2HPO_4$ (pH 6.0, 6.5, 7.0, 7.5), and 0.2 M Tris-HCl (pH 7.5, 8.0, 8.5, 9.0, 9.5, 10.0). Activity was estimated as a percentage of the maximum. Temperature optimum was determined at different temperatures (35–80°C), under standard conditions.

**Table 1** Purification of superoxide dismutase from *Thermomyces lanuginosus*

| Step | Total volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Crude extract | 230 | 257.3 | 16,802 | 65.3 | 100 | 1.0 |
| 50–65% $(NH_4)_2SO_4$ | 50 | 20.8 | 10,498 | 504.7 | 62.5 | 7.7 |
| DEAE-Sepharose | 60 | 8.2 | 8,493 | 1,035.7 | 50.5 | 15.9 |
| Phentyl-Sepharose | 35 | 3.4 | 4,204 | 1,236.5 | 25.0 | 18.9 |
| Sephacryl S-100 | 12 | 1.3 | 2,289 | 1,760.8 | 13.6 | 27.0 |

### Thermostability

Thermal stability of SOD was examined in the range of 50–90°C. The SOD was incubated in 0.2 M $NaH_2PO_4$–$Na_2HPO_4$ (pH 7.0) buffer, and samples were removed at fixed time intervals and allowed to cool on ice before residual activities were determined under standard conditions. Activity was measured as a percentage of the maximum.

### Enzyme inhibition studies

The SOD inhibitors KCN, $NaN_3$, or $H_2O_2$ were used to determine the effects on SOD activity. The enzyme was preincubated with each inhibitor for 1 h at room temperature. The SOD activity of the samples was determined under assay conditions. Control with distilled water was treated in the same way as the test samples. The buffer used was 0.2 M $NaH_2PO_4$–$Na_2HPO_4$ (pH 7.0).

## Results and discussion

A summary of the purification result is given in Table 1. The SOD was purified 27.0-fold, with a yield of 13.6% to a specific activity of 1,760.8 U/mg of protein. The zymogram from the PAGE showed a single clear activity band. The enzyme showed one protein band when run in PAGE. The mobility of the single protein band stained with Coomassie Brilliant Blue coincided with that of the single SOD activity band by activity staining (Fig. 1). These results indicated that the SOD had been purified to homogeneity by fractional ammonium sulfate precipitation, ion-exchange chromatography on DEAE-Sepharose, Phenyl-Sepharose hydrophobic interaction chromatography, and gel filtration chromatography on Sephacryl S-100.

Electrophoresis of the enzyme on SDS-PAGE gave a single band with a molecular mass of 22.4 kDa (Fig. 2). The molecular mass of the SOD was estimated to be approximately 89.1 kDa by gel filtration on Sephacryl S-100 (data not shown). The results showed that the SOD was composed of four identical subunits of 22.4 kDa each. The molecular mass of the enzyme falls within the 80,000–92,000 range reported for other tetrameric MnSODs (Hassan 1989; Bannister et al. 1987; Fridovich 1995; Zelko et al. 2002).

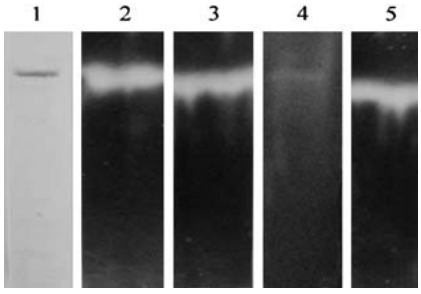


**Fig. 1** Detection of the superoxide dismutase (SOD) from *Thermomyces lanuginosus* by polyacrylamide gel electrophoresis (PAGE). *Lane 1* Protein staining, *lane 2* activity staining without inhibitors, *lane 3* activity staining with 10 mM KCN, *lane 4* activity staining with 10 mM $NaN_3$, *lane 5* activity staining with 10 mM $H_2O_2$

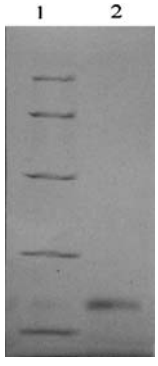


**Fig. 2** Sodium dodecyl sulfate (SDS)-PAGE pattern of the SOD from *T. lanuginosus*. *Lane 1* Standard proteins, *lane 2* the purified SOD

The SOD was found to be inhibited by $NaN_3$, but not by KCN or $H_2O_2$ (Fig. 1). Inhibition of the SOD activity by SOD inhibitors KCN, $NaN_3$, and $H_2O_2$ was 2.4, 1.3, and 96.7%, respectively. This result suggests that the SOD in *T. lanuginosus* was of the MnSOD type. MnSODs were previously reported in other fungi (Ravindranath and Fridovich 1975; Lavelle and Michelson 1975; Tesfa-Selase and Hay 1995; Fang et al. 2002; Miszalski et al. 1998; Jacob et al. 2001; Lamarre et al. 2001; Diez et al. 1998). This is the first report on SOD from thermophilic fungi.

*T. lanuginosus* MnSOD exhibited optimal activity from pH 7.0–8.5. Outside this range, activity was lost rapidly. Optimum pH of the enzyme was 7.5 (Fig. 3). Activity increased from 35°C to a maximum at 55°C,

with a rapid decrease in activity at a temperature above 70°C (Fig. 4). *T. lanuginosus* MnSOD is a thermostable SOD. Figure 5 shows the temperature stability of

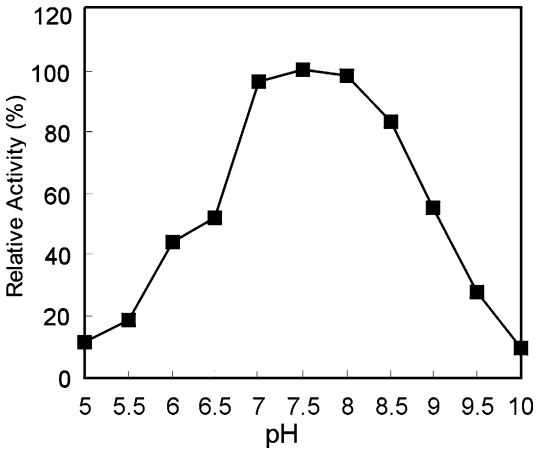


**Fig. 3** Effect of pH on the activity of the SOD from *T. lanuginosus*

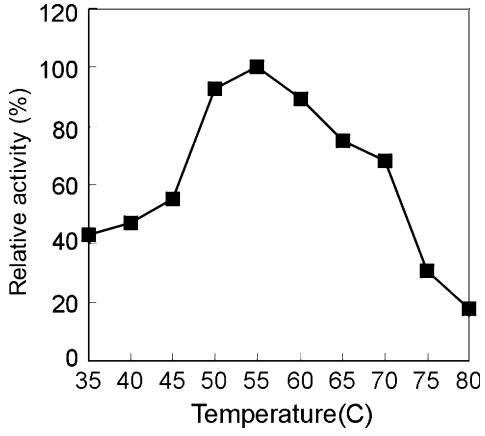


**Fig. 4** Effect of temperature on the activity of the SOD from *T. lanuginosus*

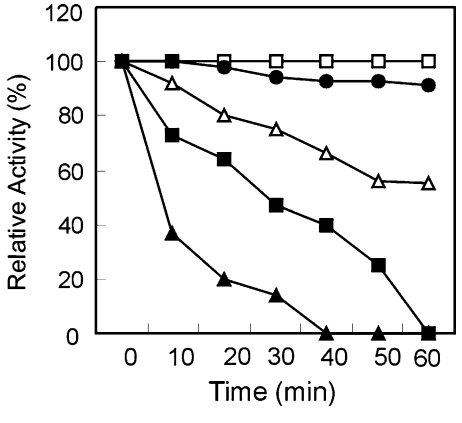


**Fig. 5** Kinetics of thermostability of the SOD from *T. lanuginosus*. *Open square* 50°C, *filled circle* 60°C, *open triangle* 70°C, *filled square* 80°C, *filled triangle* 90°C

*T. lanuginosus* MnSOD. The enzyme was thermostable at 50 and 60°C and retained 55% activity after 60 min at 70°C. The half-life of the SOD at 80°C was approximately 28 min and even retained 20% activity after 20 min at 90°C. For comparison of SOD in fungi, *T. lanuginosus* MnSOD is more thermostable than the CuZnSODs of *Aspergillus flavus*, *A. niger*, *A. nidulans*, and *A. terreus* (Holdom et al. 1996) and has a resemblance to *A. fumigatus* CuZnSOD in thermostability (Holdom et al. 1995). To our knowledge, the MnSOD from *T. lanuginosus* is the most thermostable SOD so far isolated from fungi, though not as much as those exhibited by hyperthermophilic archaeon such as *Sulfolobus acidocaldarius* (Knapp et al. 1999), *Aeropyrum pernix* (Yamano et al. 1999), and *S. solfataricus* (Ursby et al. 1999; Yamano and Maruyama 1999). *T. lanuginosus* was the most frequently isolated thermophilic fungus from compost, dung, and soil, and the eukaryote with a high maximum temperature for growth (Brock 1978). The resistance of *T. lanuginosus* SOD to heat inactivation has probably evolved in response to a high-temperature environment. It was also reported that *T. lanuginosus* produced thermostable alpha-amylase, glucoamylase, protease, and lipoxygenase (Jensen et al. 1988; Li et al. 1997, 1998, 2001). The thermostability characteristic of the SOD from *T. lanuginosus* makes the enzyme suitable for use in industry.

MnSOD has been characterized from a wide range of organisms including bacteria, fungi, plants, and animals (Bannister et al. 1987); in eukaryotic cells, it is found to be localized mainly in mitochondria (Halliwell and Gutteridge 2000). Mitochondria possess an electron-transporting system and, using this system, produce superoxide anions (Sanders et al. 2000). To protect oxygen-metabolizing cells against the toxic effects of superoxide anions, mitochondria prepare MnSOD, which dismutases superoxide anions (Nishikawa et al. 2000). In recent years, the biological functions of MnSOD have attracted increasing attention among researchers. It has been shown that MnSOD is involved in senescence, cell impairment, and carcinogenesis (Melov et al. 2000; Bostwick et al. 2000). In humans, the most promising role of MnSOD is associated with its inhibition of tumorigenicity. Many studies suggest that MnSOD may function as a general tumor suppressor gene (Zhong et al. 1997). It is believed that MnSOD will be applied to cancer therapy in the future. MnSOD produces $H_2O_2$ as a by-product of its catalytic reaction. In plants, at low concentrations $H_2O_2$ acts as a diffusible signaling molecule in signal transduction pathways that lead to the activation of gene expression (Grant and Loake 2000). The biological functions and the potential therapeutic effect of *T. lanuginosus* MnSOD are not clear. Therefore, further studies on the MnSOD of *T. lanuginosus* are necessary.

**Acknowledgements** This work was supported by grants from IFS (Sweden), 863 (China), and NFSC (China).

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