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A Novel Isoenzyme of CuZn-superoxide Dismutase from *Nicotiana tobacum*

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Abstract: An isoenzyme of CuZn–superoxide dismutase, denoted as CuZnSODⅢ, has been separated and purified from *Nicotiana Tobacum* (tobacco) leaves to apparent homogeneity. Its molecular mass is 22976.6Da. It is composed of one subunit, which is consisted of 187 amine acid residues and contains 1 copper and 0.5 zinc atom. The activation energy of the thermal denaturation process has been obtained as about 143.5kJmol^{-1}. Meanwhile, some properties of spectra were investigated.

Keywords: Superoxide dismutase, tobacco, purification, molecular properties.

Superoxide dismutases (SODS; EC 1.15.1.1) are a class of metalloenzymes that can catalyze the dismutation of the toxic superoxide radical, to protect the cell from oxidative damage. In plants, environmental adversity often leads to the increased generation of reactive oxygen species such as O_2^- and H_2O_2, therefore, SODs have proposed to be very important during different stress conditions. Tobacco SODs can be resolved into five electrophoretic forms by gel electrophoresis: one mitochondrial MnSOD, one chloroplast FeSOD, and three CuZnSODs[1], in which one of CuZnSODs and the chloroplast FeSOD has been separated and purified[1,2]. In this work , we report the one of three CuZnSODs isoenzymes named CuZnSODIII in tobacco leaves. As known, this is the first report on the purification of this isoenzyme from tobacco leaves.

Experimental

The fresh tobacco leaves (*Nicotiana Tobacum*) were harvested directly from field. DEAE-52 and Sephadex G-75 were purchased from Pharmacia. Molecular weight markers for gel electrophoresis were obtained from Sigma.

Washed tobacco leaves (1 kg) were homogenized with 500 mL of a medium containing 50 mmol·L^{-1} potassium phosphate (pH 7.6, buffer A). The resulting homogenate was suspended in 1000 mL of buffer A for 24 h(4℃). Then sonicated for 1 h, and then filtered through four layers of cheesecloth. Solid ammonium sulfate was added

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to 55% saturation. The solution was stirred for 12 h (4℃). After centrifuged at 13,000 g for 10 min, the precipitate was dissolved in 200 mL buffer A. The solution was added 0.25 vol. of ethanol and 0.15 vol. of chloroform. The slurry stirred for 1 h at room temperature, the precipitate and the excess of chloroform were removed by centrifugation at 10,000 g for 8 min and 3 vol. of cold acetone was added under stirring. The precipitate from subsequent centrifugation at 6,000 g for 15 min was dissolved in 120 mL of 20 $mmol·L^{-1}$ potassium phosphate (pH 7.6 buffer B). The solution was concentrated by Amicon PM 10 membrane to less than 20 mL.

The concentrated enzyme solution was loaded onto a DEAE-52 column (2.6 × 60 cm) equilibrated with buffer B. After eluted with the same buffer for 5 h at 0.25 $mL·min^{-1}$, fractions of 5 mL volume were collected. The active fraction were pooled and concentrated by PEG (4℃) to 10 mL with Amicon PM 10 membrane. Dialyzed in 10 $mmol·L^{-1}$ phosphate buffer (buffer D, pH 7.6) for 24 h, and then applied to the Sephadex G-75 column equilibrated with buffer D, the column was eluted with 500 mL buffer D at a flow rate of 0.25 $mL·min^{-1}$. Fractions, containing SOD activity were pooled, concentrated and dialyzed against deionized water, stored at -20℃ before used.

Results and Discussion

Purification of the isoenzyme fractions

As a result of the first chromatography (DEAE-52 column) separation (**Figure 1**), it can be observed that three bands with cyanide-sensitive superoxide dismutase activity were completely separated. They are three CuZnSODs as isoenzyme, *i.e*., CuZnSOD Ⅰ, CuZnSOD Ⅱ and CuZnSOD Ⅲ. On the next chromatography(G-75 column) CuZn -SODⅢ has been purified to apparent homogeneity and shown as a peak with upwards and downwards labels in **Figure 2**. For the native PAGE and SDS-PAGE, it also presented a single band.

The main problem in the purification of SODs is the interference of polyphenols and their oxidation products in the plant tissues. In the fresh tobacco leaves, high percentage of polyphenols makes SODs purify more difficultly. Thus, selecting optimal conditions of the enzyme extraction is very important. In this work, the optimum saturation of ammonium sulfate (55%) was chosen, while the volume of acetone was reached 3 times (v:v). If the color of crude enzyme solution was very dark, a procedure of DEAE-52 solid extraction was necessary.

Molecular properties of CuZnSOD Ⅲ

On SDS-PAGE (**Figure 3**), there is only one band of ~23000(±500) Da. The relative molecular mass determination of CuZnSODⅢ gives a value of 22976.6 Da by MALDI -TOF-MS (LDI1700-MALDI-TOF-MS，Biomolecular,USA) in **Figure 3**. There is a primary peak that can be assigned as 22976.6, corresponding to the ($M+H^+$) peak, while, 11462.8 Da corresponding to the ($M+2H^+$) peak. From these results, it suggested that CuZnSODⅢ is a monomer of 22976.6 Da.

Figure 1 DEAE-5 2 ion exchange chromatogram
I, Ⅱ,Ⅲ express three active fractions

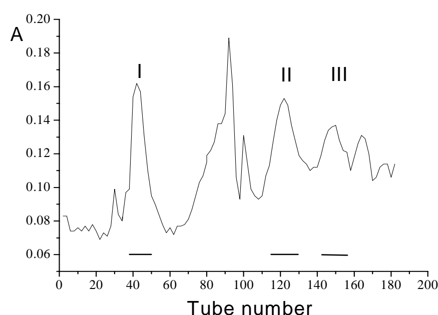


Figure 2 G-75 gel chromatogram
of the third active fraction

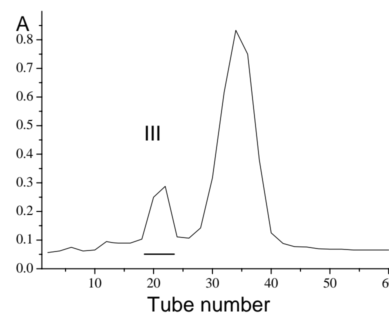


Figure 3 Matrix assisted laser desorption ionization mass spectrum and SDS–PAGE(12.5%) of CuZnSOD

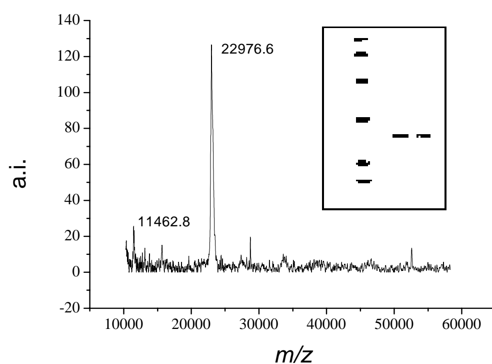
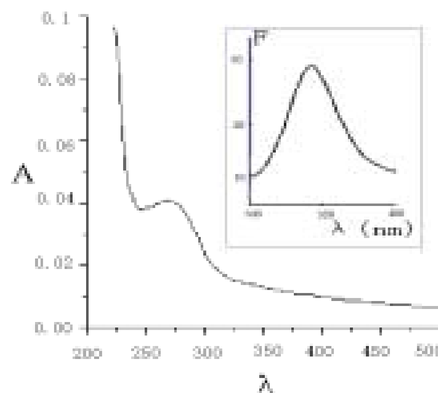


Figure 4 Absorption and Fluorescence spectrum of CuZnSODⅢ in deionized water, 0.5mg/m L



Amino acid composition of CuZnSODⅢ was unique, it consisted of only one subunit, made of 187 amino acid (assuming relative molecular mass of 23000, and giving to the nearest integer as residues per molecule), and it is different from the subunits from other SODs(consisted of two subunits)[3], involving about 150 amino acid. But it possesses similar properties to others, *e.g*. Gly(27), Glu(22) , Ser(20) and Asp(20) residues were relatively rich . Tyr(5), Met(1) and Trp(1) residues were poor. Numbers of other amino acids in one CuZnSODⅢ subunit are: Lys,10; His,12; Arg,2; Thr,11; Pro,10; Ala,13; Cys,1; Val,9; Ile,6; Leu,11; Phe,6.

The metal contents in CuZnSODⅢ were quantified by ICP-AES, each molecule contains 1.0 copper atom and 0.5 zinc per molecular of 22,976.6 Da subunit, no iron and manganese could be detected in this isoenzyme.

Spectroscopic properties

The UV-vis absorption spectra of different SODs are quite distinctive due to their metal

species in the active site, in which CuZnSOD exhibits an absorption band at 660-680 nm. Because of lacking Tyr and Trp residues, also CuZnSOD dose not present a maximum UV-absorption band at 280 nm and reveals an absorption peak at 260-270 nm. CuZnSOD Ⅲ exhibits an maximum absorption at 269 nm (**Figure 4**), and weak absorption at around 280 nm, indicating that contents of tyrosine and tryptophan residues were poor, this is consistent with the amino acid analysis.

Fluorescence spectra of CuZnSODⅢ have been investigated (**Figure 4**). The maximum excitations at 281 nm in deionized water, compared with free tryptophan emission spectrum, the maximum emission of CuZnSODⅢ was blue-shifted approxi -mately 20 nm (335 nm and 355 nm, respectively), indicating that the tryptophan residue was in the hydrophobic environment of CuZnSODⅢ.

EPR spectra can give some information of the metal type of SODs, for example, CuZnSOD exhibited an EPR spectrum with $g_{\perp}$=2.073 and $g_{/\!/}$=2.260 ; and FeSOD revealed three resonance lines around g=4.3, in addition, native MnSOD was EPR-silent. The EPR of CuZnSODⅢ reveals distinctive $g_{\perp}$values as 2.08. The $g_{/\!/}$ from free electron signal as the interaction of copper nucleus with electrons and its coordination with some atom involved in CuZnSODⅢ demonstrates that the coordination structure of copper ion was some what distorted from the standard geometric configurations, resulting in the hyperfine splitting constant $A_{/\!/}$ by 100. It indicated the character of Cu(Ⅱ)-complex.

Thermal Stability

The temperature dependence on the inactivation rate of CuZnSODⅢ was assayed and expressed as percentage of residual activity at different times after incubation at 40, 50, 60 and 70℃. The data obtained in these experiments show an apparent first-order inactivation kinetics , and the relative kinetic constant (K_{den}) was calculated. The Arrhenius plot of log K_{den} *versus* 1/T is a straight line with a computed regression line coefficient r =0.996. From the slope of this line we have calculated an activation energy of 143.5 kJ·mol^{-1} for the enzyme denaturation.

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