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The Study of Superoxide dismutase (SOD) and SOD-mimic Compounds in *Panax ginseng* C.A.Meyer

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ABSTRACT

Panax ginseng C.A.Meyer, 1 to 5 years old were electrophored and were stained for SOD activity. The result indicated a total of 13 distinct form of the enzyme and the pattern of achromatic bands were not different according to ages. Nine of the enzyme activities were eliminated with cyanide or peroxide treatment and were resistant to treatment of chloroform plus ethanol. It suggested that they may be cuprozinc containing SOD, whereas four were cyanide or peroxide resistant and were eliminated with cholroform plus ethanol treatment. They may be manganese containing SOD. Ginseng roots, 1 to 5 years old were analyzed for their SOD measurement of SOD activities of all extracts, the significant difference of SOD activities were not shown according to ages. All ginseng extracts had the total SOD activities of about 700-800 unit/g of fresh weight. Therefore, the SOD activities from SOD-mimic compounds were higher than one from SOD. The ratio between the SOD activity from SOD-mimic compounds and one from true SOD was approximately 2:1 to 3:1.

Key words : SOD, SOD-mimic compounds, NBT assay, isozyme, *Panax ginseng* C. A. Meyer, SOD activity.

Introduction

Reactive Oxygen Species(ROS: $^1O_2$:singlet oxygen, $O_2^{\cdot}$ - : superoxide anion radical, · OH :hydroxyl radical, H2O2: hydrogen peroxide) generally mean the highly reactive oxygen molecules or oxygen radicals generated from the triplet-state oxygen by excitation or reduction. The superoxide anion radical($O_2^{\cdot}$ -) has been known to be very harmful to the cellular components as a precursor of the more reactive oxygen species such as hydrogen peroxide and the hydroxyl radical. These are too reactive to be well tolerated within living system.

ROS including superoxide anion radicals may be triggered inflammation(Niwa et al., 1982; Niwa et al., 1983), DNA strand scission(Brawn and Fridovich, 1981; Chincolo et al., 1991; Lesko et al., 1980; Sagnipanti and Kraemer, 1989), lipid peroxidation(Hasegawa et al., 1992; Kellog and Fridovich, 1975; Miller et al., 1993), mutagenesis(Weitzmanm and Stossel, 1981), carcinogenesis(Bryninck et al., 1978; Moddy and Hassan, 1982; Nordenson and Beckman, 1976), tissue injury by circulatory disturbance and aging(Hasegawa et al., 1992; Halliwell and Gutteridge, 1984) in a biological system. Recently, superoxide dismutase (SOD) was adopted to prevent or rescue the various diseases that may be caused by ROS. The administration of SOD has been shown to improve the diseased state. But SOD cannot be absorbed into gastrointestinal tract because of its higher molecular weight above 30,000.

From this point of view, ginseng (*Panax ginseng* C. A. Meyer) is good for the searching of SOD-mimic compounds. Ginseng has been known to be an elixir in oriental medicine for a long time and Korean ginseng , especially, has been most widely used because of its eminent efficacy.

Therefore, the objectives of this reserch were to identify SOD isozymes and the multiforms in ginseng root, and to compare the total activity of SOD in ginseng roots according to ages and the activities of true SOD and SOD-mimic compounds in ginseng.

Materials and Methods

To identify multiple forms and isotype of SOD, nondenaturing PAGE was performed on 12% acrylamide gel according to the method of Davis(1964). SOD activity was detected in gels by the photochemical ρ-nitro blue tetrazolium (NBT) stain following the methods of Beauchamo and Fridovitch(Bonner, 1965). For the identification of ioszyme (Beauchamo and Fridovich, 1973), 5 mM H_2O_2 and 1 mM KCN were added to crude extract and electrophoresed. And for identification of CuZnSOD isozyme, chloroform and ethanol treatment were performed (Weisiger and Fridovich, 1973). All extracts were assayed for SOD activity photochemically, using the assay system (Table1).

Extracts were diluted 30 times for SOD assays(Beauchamo and Fridovich, 1971; Constantine et al., 1977). An assay for superoxide dismutase was based on the photochemical generation of $O_2^{\cdot}$ - and on the use of nitroblue tetrazolium (NBT) as the indicating scavenger. The photochemical procedure was chosen as being independent of other enzymes and proteins and, therefore, more reliable in the case of crude extracts than enzymic assay systems(McCord and Fridovich, 1969). The apparatus devised for exposing the tubes to light was composed of a rotating test tube holder immersed in water in a cylindrical glass container thermostated at 25C°. A circular fluorescent lamp was attached on the outside wall of the water bath and the entire assembly was fitted in a box lined with aluminum foil. The reaction was initiated and terminated by turning the light on and off. There was no detectable amount of the reaction occurring under room light during preparation of the solutions and spectrophotometric measurements. The initial rate of the reaction was determined as increase of absorbance at 560nm. Under the described conditions, the increase of absorbance in absence of SOD was 0.09unit/5 min and was linear up to 15min. In the presence of SOD, the reaction was inhibited and the amount of inhibition was used to quantitate the enzyme. Each extract was assayed twice and the results varied less than 0.005 absorbance unit/5min. In order to differentiate between true SOD and SOD-mimic compounds in NBT assay system, it is necessary to measure O_2.- dismutation by comparing dialyzed and undialyzed crude extracts, and extracts in the presence and absence of cyanide (KCN, about 5×10^{-5}mM/l). The gel stained for SOD activity was analyzed by densitometer (Digital Densitowl DMV-33C, Tokyo Japan) at 560nm. The density range was 1.0. The water-soluble protein content of all crude SOD extracts was determined by the method of Bicinchoninic acid(BCA). Bovine serum albumin was used as a standard.

Table 1. Reaction mixture for the assay of SOD activity

Rsaction Mixture*	Concentration
riboflavin	1.3 M
methionine	13mM
p-nitro blue tetrazolium	63 M
sodium carbonate	0.05M (pH 10.2)
crude SOD	appropriate volume
distilled water	added to the final volume of 3ml

*.The photochemical assay system is more reliable than the xanthine/xanthine oxidae system for the determination of SOD activity in crude extracts(15).

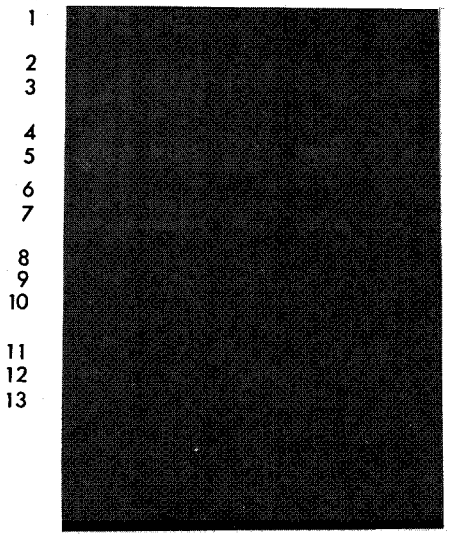

Figure 1. Native PAGE of *Panax ginseng*. The dark gel was stained for SOD activity. SOD bands were numbered in order of incresing relative mobility. Lane 1, the crude extract of one-year-old ginseng; Lane 2, the crude extract of two-year-old ginseng; Lane 3, the crude extract of three-year-old ginseng; Lane 4, the crude extract of four-year-old ginseng; Lane 5, the crude extract of five-year-old ginseng;

Results

Multiple Forms of SOD

The activity stain, which permits visualization of SOD, has made the existence of multiple forms of this enzyme all too apparent. Figure 1 illustrates the results obtained with ginseng extracts. Achromatic bands 1, 2, 3, 4, 5, and 6 were apparent, but bands of activity beyond 6 were very faint and were represented by the shaded band. The pattern of achromatic bands were not different according to the number of years, but there are apparent difference in the size of bands in each lane.

Isotype identification

In comparison with lane 1(non-treatment of inhibitor), lane 2-4 (Lane 2: 1mM KCN treatment, Lane 3: 5mM H_2O_2, Lane 4: 1mM KCN and 5mM H_2O_2 treatment) showed the achromatic bands 1, 2, 3 and 4 still appeared whereas the other achormatic bands, beyond 4 disappeared

Therefore, the achromatic bands 1, 2, 3, and 4 were KCN and H_2O_2 resistant SOD and the other achromatic band, beyond 4, were KCN and H_2O_2 sensitive SOD. In complementary experiment, crude extract with treatment of chloroform and ethanol was electrophoresed and stained for activity. As a result, the achromatic bands 1, 2, 3, and 4 that were resistant to H_2O_2 and KCN disappeared, whereas the other bands, beyond 4, that were sensitive to H_2O_2 and KCN still appeared .

Densitometry analysis of the gel.

The gel from native PAGE was traced by densitometry. The result showed negative peaks because of staining for activity. The negative peaks are numbered in order of increasing relative mobility, from 1 to 13.

Enzyme Quantification

Beauchamo and Fridovich(1971) defined 1 unit of SOD as the amount that inhibits the NBT photoreduction by 50% and quantitated the enzyme on basis of the percent inhibition it caused. Percent inhibition and SOD concentration were not linear. However, Asada et al. (1974), established a linear relationship between SOD concentration and the V/v ratio fitting the equation (1):

$$V/v=1+K'[SOD] \quad (1)$$

where V and v represent the rate of the assay reaction in absence and in presence of SOD, respectively. This linear relationship was also observed in the present study with crude extracts and the photochemical assay system.

If the SOD unit is redefined, the equation of Asada et al. (1974) allows convenient and accurate determination of SOD activity.

Equation 1 can be rewritten as (2):

$$K'[SOD]=(V/v)-1 \quad (2)$$

SOD activity comparison

In the measurement of SOD activities of all extracts, significant differences are not shown according to ages (Table 4). From one year to five years, all ginseng

Table 2. SOD Activity of ginseng crude extract according to ares.

Ages	SOD units/g fresh weight	SOD units/g dry weight
One-year-old	710.5	3871.9
Two-year-old	704.7	3685.4
Three-year-old	533.9	3529.0
Four-year-old	835.0	2822.3
Five-year-old	799.5	3149.9

Table 3. SOD activity of ginseng crude extract after dialysis[1] and cyanide[2] treatment

Ages	SOD units/g fresh weight	SOD units/g dry weight
One-year-old	235.5	1283.5
Two-year-old	249.9	1307.1
Three-year-old	238.3	1575.4
Four-year-old	201.3	680.4
Five-years-old	219.4	864.2

1;Exaustive dialysis against phosphate buffer
2;Cyanide (5×10^{-5}M) make peroxidase inactivate, not superoxide dismutase(63).

Table 4. SOD content of crude extract

Ages	Fresh weight(g/g dry weight)	Protein(mg/g fresh weight)	Units/mg protein
One-year-old	5.45	16.6	42.8
Two-year-old	5.23	17.1	41.2
Three-year-old	6.61	13.2	40.5
Four-year-old	3.38	17.1	48.8
Five-year-old	3.94	21.2	37.7

racts have about 700-800 units/g of raw weight, but eptionally three years ginseng show relatively low activity ible 2). The survey of true SOD activity of crude extracts s performed. The consequence bought out the dramatic rease of SOD activity, leading to almost the reduction 3/4 degree(Table 3). Compared with total SOD ivity (about 700-800unit/g of raw weight), true SOD ivities are 200-250 units/g of raw weight, that is ut 1/4 of total activity. The ratio between total SOD ivity and true SOD activity are approximately 3:1 to 4:1. d SOD-like compounds activity correspond to about 3/4 total activity (Fig. 2).

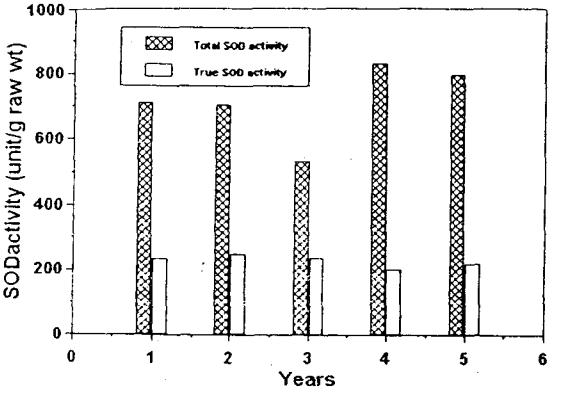

Figure 2. Comparison of total SOD activity and true SOD activity. The ratio between SOD activity by SOD-mimic compounds and SOD activity by true SOD were approximately 2:1 to 3:1.

Discussion

Preliminary experiment confirmed that band area is proportional to the enzyme concentration in these studies (data not shown). Quantitative differences of the SOD bands were evident from the "band area". High concentrations of ginseng extracts did not produce additional bands. The same banding patterns were obtained under various extraction conditions such as pH 10 to 11, K-phosphate concentrations 0.1 to 0.4 M in the presence or absence of 0.1 mM EDTA. SOD apparently existed as a family of electrophoretically discrete forms in ginseng roots. These multiple forms were readily separated during electrophoresis on native polyacrylanide gel. The result indicated that thirteen discrete bands were shown after electrophoresis. It is not known whether these multiple forms are truly isozymes or whether they are the part of the broken SOD enzyme. Among these achromatic bands 1, 2, 3, and 4 were cyanide-resistant, chloroform-ethanol treatment sensitive SOD, which might be the multiple forms of manganese-containing SOD. The other bands could be eliminated with both 1 mM KCN and 5 mM H_2O_2, therefore, indicating that these bands were due to the multiple forms of cupro-zinc containing SOD. Cytosol contains both CuZnSOD and MnSOD but only MnSOD is found in the mitochondrial matrix (Girffin and Palmer, 1989). Generally, other data reported that isozyme of MnSOD type is only one (Constantine et al., 1977; Weitzmanm and Stossel, 1973). But in ginseng, electrophoretically distinct MnSOD enzymes existed at least three in the gel.

In the densitometry tracing of gels, peak area might express SOD quantity. The study of densitometry tracing of gel indicated that band areas 1, 2, 3 and 4, which contain MnSOD were larger than the rest of band areas, which contains CuZnSOD. Peak area might mean the enzymes quantity. Therefore, MnSOD was a abundant isozyme type in ginseng roots. Comparison with activities according to ages, beyond one' s expectations, the change of activity according to the number of years was not significant. The difference between total SOI activity and true SOD activity was very large. Thi result indicated that ginseng contains many SOD-mimi compounds and The SOD activity from SOD-mimi compounds was much large than the one of true SOD. Thes results are in conflict with the assertion by other author that the interference by SOD-mimic compounds were insignificant distinctly (Constantine et al.,1977). Dialysi and KCN treatment of crude SOD extracts indicated significan interference of small molecules (molecular weigh below 12,000) with the assay. Therefore, most of superoxid anion radical scavenging activity was derived from small molecular weight compounds.

적 요

고려인삼 1년근에서 5년근을 전기영동하였고 SOD 활성에 대한 염색을 수행하였다. 그 결과 총 1 개의 구분되어지는 무색의 밴드를 나타내었다. 그리 고 밴드의 패턴은 1년근에서 5년근이 모두 동일하였 다. 이들 효소 중의 9개는 시아나이드 처리나 과산화 수소 처리에 의해 밴드가 제거되었고 클로로포름과 에탄올처리에 대해서는 안정했다. 이들 효소들은 CuZnSOD효소들이다. 반면에 4개의 밴드는 시아나 이드처리나 과산화수소 처리에 의해 안정하고 클로 로포름과 에탄올 처리에 의해서는 제거되었다. 그러 므로 이들은 MnSOD효소들이다. 인삼 1년근에서 5 년근까지 SOD 활성의 비교를 광화학적 에세이 방법 에 의해 측정하였다. 이들 SOD 활성 측정의 결과에 서 년수에 따른 SOD 활성의 유의성있는 차이는 보 여지지 않았다. 모든 인삼 추출물의 총 SOD 활성은 대략 700-800 units/g of fresh weight 이었다. 그러나 SOD 효소에 의한 SOD 활성은 대략 200-250 units/g of fresh weight 이었다. 그러므로 SOD 활성 유사 물질 에 의한 활성이 SOD 효소에 의한 활성보다 높다 SOD 활성 유사 물질과 SOD 효소에 의한 활성의 비 는 대략 2:1에서 3:1이다.

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