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Research Article

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Separation of Superoxide Dismutase from Pig Blood and its Activity Protection

Shuangli Xiong*, Anlin Li and Xiaolei Yao

College of Life Science, Southwest University of Science and Technology, Mianyang 621010, China

*Corresponding Author E-mail: xiongshuangli@swust.edu.cn

ABSTRACT

Separation and purification of superoxide dismutase from pig blood and its activity protective agents including metal ions and carbohydrates were explored. The results exhibited that specific activity of SOD was 6594.55U/mg after it was purified by Sephdex G-100. There were three kinds of metal ions in terms of effects on the activity of SOD. Na^+, K^+ and Zn^{2+} could promote activity of SOD with the increase of their concentrations all the time. Ba^{2+}、Ca^{2+}、Cu^{2+} could also enhance activity of SOD, but it attenuated with the increase of their concentrations. Mg^{2+}、Fe^{2+}、Al^{3+} raised activity of SOD at lower low concentration but decreased it at higher concentration. All carbohydrates could slow down the reducing rate of relative enzyme activity and had stabilizing effects on SOD except for D-fructose and sucrose with higher concentration (0.1mol/L) and D-mannose with lower concentration (0.1mol/L). D-fructose (0.5mol/L), D-galactose (0.2mol/L), D-arabinose (0.2mol/L) and D-glucose (0.2mol/L) could greatly promote SOD activity all the time. These results suggested that some metal ions and carbohydrates can be added to functional food or other products contained in SOD as active components for stabilizing or promoting the activity of SOD.

Keyword *: superoxide dismutase, metal ions; carbohydrate; enzyme activity.*

INTRODUCTION

Superoxide dismutase (SOD), a metal acid protease existed extensively in animal and plant and microorganism, is able to catalyze superoxide dismutation reaction and has many important bioactivities such as antioxidation, antiradiation, anticancer and anti-ageing etc[1-3]. For finding resources enriched in SOD, many researches were mainly focused on the extration of SOD from a multitude of biological resources including animal and plant and microorganism. However, SOD has a short half-life. It is easily inactivated in aqueous solution. These properties limited its numerrous application in food and cosmetics and drugs. Recently, some researches were involved in activity protection of SOD for further promoting availability and availability[4]. As a kind of metal enzyme, metal ion may play important roles in maintaining structure and activity of SOD. In addition, carbohydrates have certain stabilizing capability for activity of enzyme in dryness or solution state[5]. Thus, elucidation for relation between metal/carbohydrate and SOD activity may contribute to choose appropriate metal ion or carbohydrates as additive. The reasonable formulation of SOD and its protective agent or synergistic agent including metal ion and carbohydrate will again greatly enhance the application value of SOD in food and cosmetics and drugs. Therefore, this work mainly investigated the extraction, separation and purification of SOD from pig blood. On this base, we also explored the effects and regularity of various metal ions and carbohydrates on activity of SOD in solution, which would provide theoretical and technological basis for development and application of SOD in the future.

MATERIALS AND METHODS

Materials

Pig blood was purchased from slaughterhouse (Qingyi town, Mianyang City, Sichuan Province), Coomassie brilliant blue R-250 (Shanghai Chemical Reagent Co., China), superoxide dismutase standard

(BR, 3500-7000u/mg, Shanghai Jissui Biological Technology co., China); L-rhamnose, D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, D-fructose, sucrose (Sigma Chemical Co., ST. Louis, MO, USA);). All other chemical reagents were of analytical grade.

Separation and purification of SOD from pig blood

Fresh pig blood, added in 0.4% of sodium citrate, was centrifuged with 4000×g for 10 min and collected precipitate to obtain hemacyte. It was washed with 0.9% of sodium chloride before it was magnetically stirred for 10 min to gain hemolysate. Hemolysate was again heated at 90 ºC in thermostatic water bath for 25 min with constant mechanical stirring. The suspension was centrifuged with 4500×g for 15 min to obtain supernatant which was added of mixture of chloroform and alcohol with ratio of 3 to 5. The mixture was immediately stirred and centrifuged with 4500×g for 15 min to obtain supernatant which was again precipitated with isovolumic acetone and left to stand for 5 h at 4 ºC. The precipitate was centrifuged with 4500×g for 20 min to obtain SOD. It was further purified by Sephadex G-100 column chromatography and freeze-dried for use.

Identification of purity and activity of SOD

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Xiaona [6] using an electrophoretic apparatus (Powerpac Universal fundamental power supply, Mini-PROTEAN Tetra Electrophoresis System, Bio-rad Co., USA). Electrophoresis of the SOD was conducted at 20 mA and 210 V. The concentration of separating gel and stacking gel were 10% and 4% respectively. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 for 1h destained with decolorization solution for 2 h.

Activity staining technique was adopted for indentifying activity of SOD. The staining solution was mixtures of 0.2% NBT and N,N,N',N'-Tetramethylethylenediamine and 0.01% of riboflavin using deionized water as solvent. The gel was kept in staining solution for 30 min and away from sunlight after PAGE electrophoresis (without SDS) was finished. It was then illuminated with fluorescent lamp (20W) for several minutes. The SOD part was colorless and the other area without SOD was bluish violet.

Determination of protein content and activity of SOD

Protein assay adopted kaumas brilliant blue method with bovine serum albumin as standard [7]. The activity of SOD was determinate by the adjacent benzene three phenolic autoxidation method [7]. One activity unit was defined as enzyme amount needed when inhibiting rate per minute for pyrogallol autoxidation was up to 50% in 1mL reaction liquid.

Fourier transform infrared (FT-IR) spectroscopy of SOD

The spectrum of SOD was recorded on a Nicolet Nexus FT-IR spectrometer (Nicolet Instrument Co., USA) in the frequency range of 4000-400 cm^{-1}. The resolution ratio was $4cm^{-1}$. The number of scans was fifty. Amide Ⅰ zone was analyzed by second derivative and deconvolution and curvefitting for exploring secondary structure of SOD.

Effects of metal ion on SOD activity

1mLofNaCl、KCl、$BaCl_2 \cdot 2H_2O$、$CaCl_2 \cdot 2H_2O$、$CuCl_2 \cdot 2H_2O$、$Mg(CH_3COO)_2 \cdot 4H_2O$、$Zn(CH_3COO)_2 \cdot 2H_2O$、$FeSO_4 \cdot 7H_2O$、$AlCl_3 \cdot 6H_2O$ with different concentration (0.01、0.02、0.05、0.1、0.2、0.5、1 mmol/L) were added to 4 mL of purified SOD solution respectively and incubated for 5 h at 25ºC. The activity of mixtures were assayed using pyrogallol autoxidation method [7].

Effects of carbohydrate on SOD activity

1mL of L-rhamnose, D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, D-fructose, sucrose with different concentration (0.1、0.2、0.5、1 mol/L) were added to 4 mL of purified SOD solution respectively and incubated for 5 h at 25ºC. The activity of mixtures were assayed using pyrogallol autoxidation method. Relative enzyme activities of SOD mixed in metal ions or carbohydrates were calculated using deionized water instead of metal ions or carbohydrates as blank control.

RESULTS AND DISCUSSION

Separation and purification of SOD

The change of total activity, total protein, specific activity and activity recovery of SOD during extraction and purification process were listed in table 1. Specific activity of SOD was increased to 6594.55U/mg

from 2840.41U/mg. The activity recovery of SOD was 62.15, purification multiple was 39.14, which indicated the SOD has higher purity.

Table 1 Extration and purification of SOD from pig blood

Separation procedure	Total activity（×10^{4}U）	Total protein（mg）	Specific activity（U/mg）	purification multiple	Recovery rate（%）
1	11.81	704.06	167.74	1.00	100
2	10.13	250.01	405.04	2.42	85.77
3	8.85	31.16	2840.41	16.93	74.94
4	7.34	11.13	6594.55	39.14	62.15

Note: 1-thermal denaturation treatment (90 ºC in thermostatic water bath for 25 min), 2- Treatment of Chloroform- alcohol, 3- Actone precipitation, 4- SephadexG-100 purificaiton.

Purity and activity identification of SOD

As observed in Fig.1A, The SOD purified by Sephadex-G100 showed one protein band which was the same as SOD band (data not shown). The molecular weight was about 31kDa. Riboflavin can be easily reduced in the presence of oxides. The reduced riboflavin was rapidly oxidized to generate superoxide anion free radical which can reduce NBT to form blue methyl hydrazone. SOD can scavenge superoxide anion free radical and inhibit the formation of methyl hydrazone. Thus, bright spot was occurred after gel was stained nitroblue tetrazolium, which indicated that the SOD was active. As shown in Fig.1B, bright spot was occurred in sample band (31kDa) after gel was stained nitroblue tetrazolium (Fig.1B), which again indicated that the SOD was Cu, Zn-SOD with electrophoretic purity.

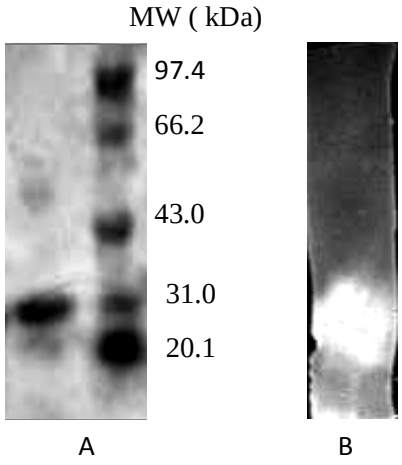

Fig.1 SDS-PAGE of SOD
gel were stained with coomassie brilliant blue R-250 (A) or nitroblue tetrazolium(B).

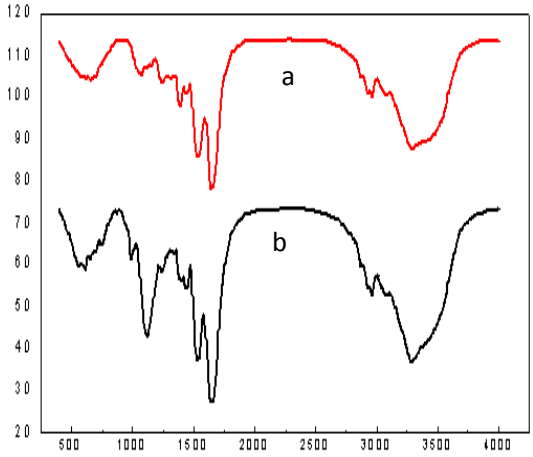

Fig.2 The IR spectra of SOD

a- Standard Cu, Zn-SOD from bovine blood
b- SOD from pigblood

IR spectrum assay of SOD

The FT-IR spectrum of SOD was shown in Fig.2. The SOD displayed a broad stretching intense peak at around 3400 cm^{-1} characteristic of the O-H and N-H stretching vibration, and a weak signal band of C-H stretching vibrations at around 2980 cm^{-1}. The peak peak at around 1650 cm^{-1} was ascribed to C-O stretching vibrations of amide I band. The absorbance at 1534cm^{-1} was owing to C-H deformation vibration of the amide Ⅱ band. Absorption band over the range of 1237-1126cm^{-1} was due to the C-N and N-H bending vibrations of amide Ⅲ zone. These were similar to those of Cu, Zn-SOD from bovine blood. Secondary structure of SOD was mainly β-pleated sheet by computer-aided analysis (second derivative and deconvolution and curvefitting method). The absorbance peak in the range of 1640-1660cm^{-1} was the token of random coil and α-helix, and that in 1665-1680cm^{-1} was characteristic absorption of β-turn, which was consistent with the reports in the literature[8, 9].

Effects of metal ion on SOD activity

As shown in Fig. 3. There were three kinds of metal ions in terms of effects on the activity of SOD. Na^+, K^+ and Zn^{2+} could promote activity of SOD with the increase of their concentrationsall the time (Fig.1 A). Although Ba^{2+}、Ca^{2+}、Cu^{2+} could enhance activity of SOD, it attenuated with the increase of their concentrations (Fig.1 B), Mg^{2+}、Fe^{2+}、Al^{3+} raised activity of SOD at lower low concentration but decreased it at higher concentration (Fig.1 C). Among Na^+, K^+ and Zn^{2+} three metal ions, K^+ had the highest promoting capability for activity of SOD. The relative enzyme activity was up to 132.17% when its concentration was 0.05 mmol/L. Activity of SOD did not greatly increased when the concentration of K^+ was over 0.05 mmol/L. Na^+ could not sharply promote activity of SOD when its concentration was less than 0.5 mmol/L. However, after concentration of Na^+ was more than 0.5 mmol/L, activity of SOD was rapidly promoted. The activation of these three for SOD activity was probably ascribed to the coordination between them and enzyme active center [10]. Despite a downward trend (Ba^{2+}、Ca^{2+}、Cu^{2+}), relative enzyme activity of SOD was also over 100%. When the concentrations of Ba^{2+}、Ca^{2+}、Cu^{2+} were 0.01、0.05、0.02mmol/L, the promoting effects for SOD activity achieved maximum, they were131.28%、148.39%、148.46%, respectively.

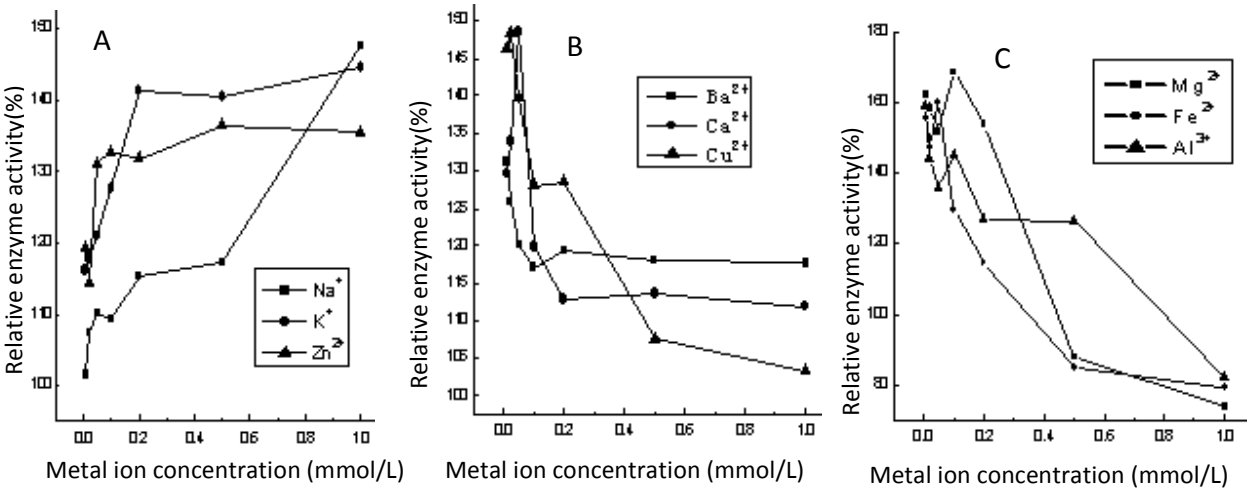

Fig.3 Effect of metal ion on the activity of SOD

For Mg^{2+}、Fe^{2+}、Al^{3+}, all of them could increase the relative enzyme activity and over 100% when the concentration of them were less than 0.2mmol/L although the activating ability depressed with the enhancement of their concentrations (0-0.2 mmol/L) (Fig.1 C), which make known that they increase the SOD activity at lower concentration may be due to the coordination between exogenous metal ions with endogenous metal ions in SOD active center but contrary to higher concentration [10].

Effects of carbohydrates on SOD activity

Generally, carbohydrates not only have protective effect on activity but also stabilize enzyme in solution. The different protective effects on SOD activities were observed for different tested carbohydrates with various concentrations and times (Fig.4). All carbohydrates could slow down the reducing rate of relative enzyme activity and had stabilizing effects on SOD except for D-fructose and sucrose with higher concentration (0.1mol/L) and D-mannose with lower concentration (0.1mol/L). The groups treated by D-fructose of sucrose with lower concentration (0.1-0.5 mol/L) had stronger relative enzyme activity than that treated by deionized water all the time, which exhibited that they processed good protection capability for SOD activity at lower concentration. The SOD activity sharply decelerated after their concentrations were more than 1 mol/L, which were similar to that treated by deionized water. For D-mannose group, the relative enzyme activities were less than that of blank group despite higher relative enzyme activity at 0.1 mol/L. After the concentration of it was over 0.5 mol/L, the relative enzyme activities much higher than that of blank group, which were in accordance with that of D-xylose and

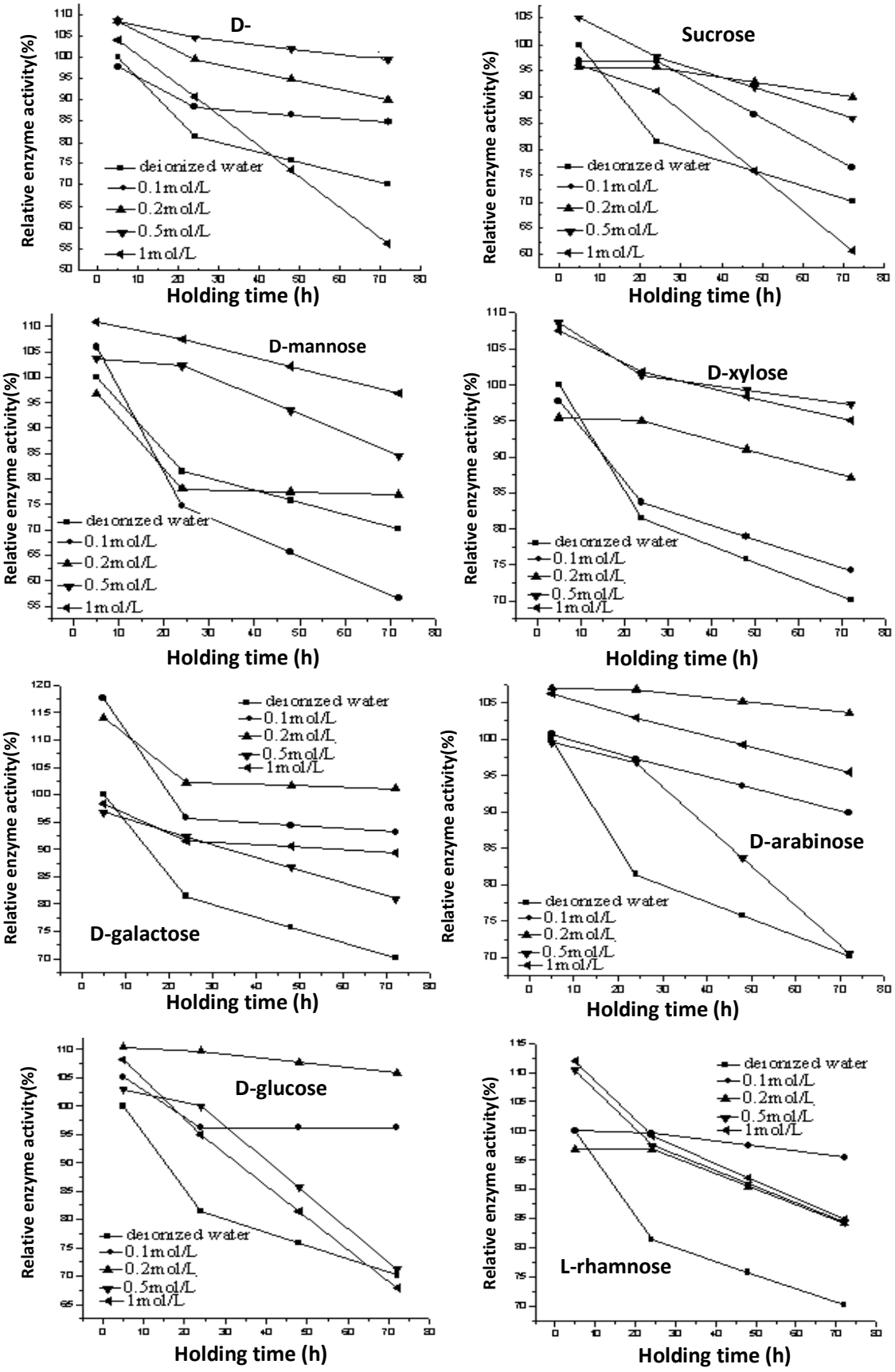

Fig.4 Effects of carbohydrates on the activity of SOD

indicated that both of them had stronger stabilizing ability for SOD activity at higher concentration. D-galactose and D-arabinose showed similar protective capacity for SOD activity. When time was less than 10 h, they can remarkably promoted SOD activity at lower concentrations (0.1 mol/L and 0.2 mol/L) but slightly retarded SOD activity at higher concentrations (0.5 mol/L and 1 mol/L). The stabilizing capability of them for SOD activity at lower concentration were also better than that treated by higher concentration all the time which was again stronger than that of blank group. Among the tested carbohydrates, only D-glucose always improved SOD activity all the time at various concentrations. At 0.2mol/L of concentration, it exhibited the best protective effects for SOD activity which were more than 105 all the time. At 0.2mol/L of concentration, SOD activity decreased sharply with the extension of time and showed the lowest relative enzyme activity. Interestingly, L-rhamnose (0.1mol/L) exhibited stably protective effects for SOD activity all the time and the relative enzyme activity was over 97.5%. It was stronger than that of blank and the other concentration.

As a whole, some carbohydrates (D-galactose, D-arabinose, D-glucose and L-rhamnose) had stronger protective capability at lower concentration (0.1mol/L and 0.2 mol/L). some carbohydrates (D-mannose and D-xylose) had stronger ability at higher concentration (1mol/L). For all carbohydrates, 0.2 mol/L of concentration is a relatively good. In all measured carbohydrates, D-fructose (0.5mol/L), D-galactose (0.2mol/L), D-arabinose (0.2mol/L) and D-glucose (0.2mol/L) could promote SOD activity and the relative enzyme activities were higher than 100% all the time. D-glucose (0.2mol/L) showed the highest protective capability for SOD activity because the relative enzyme activity showed slow decrease tendency and was more than 105%. These protective may be due to the change of surface tension of water in SOD solution [11]. Carbohydrates have many hydroxyl groups which can form hydrogen bond with SOD. This will increase conformational stability of SOD. Another factor may be the change of the micro environment around the SOD molecules which lessen the collision chances between the SOD molecules and promote stability of SOD [12].

CONCLUSIONS

The present study examined separation of SOD from pig blood, the effects of metal ion and carbohydrates on its activity. Different metal ions and carbohydrates had different Influence law for its activity protection. All the measured metal ions had stronger protective ability for SOD activity at a certain concentration. Na^+, K^+ and Zn^{2+} , Ba^{2+}、Ca^{2+}、Cu^{2+} could enhance activity of SOD. Mg^{2+}、Fe^{2+}、Al^{3+} raised activity of SOD at lower low concentration but decreased it at higher concentration. All carbohydrates could stabilize activity of SOD except for D-fructose and sucrose with higher concentration (0.1mol/L) and D-mannose with lower concentration (0.1mol/L). Both concentration and time had a great influence on the activity of SOD. It can be seen that numerous metal ions and carbohydrates had activation of stabilization capability for SOD activity. They can be added to functional food or other products contained in SOD as active components. These data suggested that the type and additive amount need to be taken into account for use of metal ion and carbohydrate.

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