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Activity and stability of recombinant human superoxide dismutase in buffer solutions and hypothermic perfusates.

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Abstract

The stability of recombinant human superoxide dismutase (r-hSOD) in buffer solutions was studied in solutions at various pH and temperatures. Additionally, we studied the effects of incubation with proteases, serum and two types of hypothermic perfusates. R-hSOD was stable in the pH range of 6-11 and at temperatures up to 80 degrees C for 30 min. R-hSOD activity was not affected by incubation with trypsin, aminopeptidase M or serum for 2 h. R-hSOD activity determined at various temperatures (4-37 degrees C) did not vary remarkably. R-hSOD in hypothermic perfusates was stable at 4-37 degrees C for 24 h.

KEYWORDS: recombinant human superoxide dismutase, activity, stability, hypothermic perfusate

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Activity and Stability of Recombinant Human Superoxide Dismutase in Buffer Solutions and Hypothermic Perfusates

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The stability of recombinant human superoxide dismutase (r-hSOD) in buffer solutions was studied in solutions at various pH and temperatures. Additionally, we studied the effects of incubation with proteases, serum and two types of hypothermic perfusates. R-hSOD was stable in the pH range of 6-11 and at temperatures up to 80℃ for 30 min. R-hSOD activity was not affected by incubation with trypsin, aminopeptidase M or serum for 2 h. R-hSOD activity determined at various temperatures (4-37℃) did not vary remarkably. R-hSOD in hypothermic perfusates was stable at 4-37℃ for 24 h.

Key words: **recombinant human superoxide dismutase, activity, stability, hypothermic perfusate**

The superoxide anion (O_2^-) has been implicated in the pathogenesis of tissue injury consequent to ischemia/reperfusion in several different organs including the heart (1) and kidney (2). Superoxide dismutase (EC 1.15.1.1.)(SOD), a copper- and zinc-containing metalloenzyme specific for O_2^-, has been used successfully to protect these organs from structural damage during the reoxygenation of ischemic tissues (3,4). To examine the effect of SOD on free radical-induced myocardial injury during tissue preservation and transplantation, perfusion was performed for several hours at low temperature (5). However, it is not known whether SOD is active at low temperatures nor whether it is stable in hypothermic perfusates for a longer time. In this study, we report the activity and stability of recombinant human superoxide dismutase (r-hSOD) in several solutions of various pH, and at various temperatures and incubation times.

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Materials and Methods

Chemicals. Xanthine oxidase was purchased from Boehringer Mannheim Yamanouchi Co., Tokyo, Japan. Cytochrome C (Type III) and sodium xanthine were obtained from Sigma Chemical Co., St. Louis, Mo, USA. Artificial blood Fluosol-DA was kindly supplied by Green Cross Corporation,

Osaka, Japan. TPCK-treated trypsin and aminopeptidase M were purchased from Worthington Biochemical Corporation, New Jersey, USA and Pierce Chemical Co., Rockford, IL, USA, respectively.

Recombinant human superoxide dismutase was cloned and expressed in *Escherichia coli* (6), and was highly purified in our laboratory. The specific activity of the purified and lyophilyzed sample used in this experiment was 3,600 U/mg protein. Protein concentration was determined by the method of Lowry *et al.*(7) with bovine serum albumin as a standard. All other chemicals were of analytical grade and were obtained from commercial sources.

Component of perfusates. The components of the two types of perfusates (8) used are illustrated in Table 1.

Table 1 Composition of perfusates A and B

Components		Quantities A	Quantities B
Na^+	(mEq/l)	10	10
K^+	(mEq/l)	110	110
Mg^+	(mEq/l)	8	8
Cl^-	(mEq/l)	10	10
HCO_3^-	(mEq/l)	10	10
HPO_4^-	(mEq/l)	85	85
SO_4^{2-}	(mEq/l)	8	8
Xylocaine	(mmol/l)	1	1
Glucose	(g/l)	30	30
Fluosol-DA	(ml/l)	—	400
Albumin (25%)	(ml/l)	—	50
pH at 37℃		7.3	7.3
pO_2	(mmHg)	580	510
Osmolarity	(mOsm)	350	410

Assay for SOD activity. The assay using cytochrome C followed the original method of McCord and Fridovich (9). Briefly, under the standard assay conditions cytochrome C (10^{-5}M) reduction was measured after 30 sec of incubation at 25℃ with 5×10^{-5}M xanthine oxidase (XOD) in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. Inhibition of the reduction of cytochrome C by 50% was defined as 1 unit of SOD activity. Absorption at 550 nm was recorded at 30-min intervals with a JASCO UVIDEC-610C spectrophotometer. All of the activity and stability data obtained in this study were expressed as the averages of two experiments.

Examination of pH and thermal stability of r-hSOD and effect of proteases or serum on r-hSOD. For the study of the pH stability, purified r-hSOD was dissolved in pure water at a concentration of 920 U/ml. The solution was mixed with an equal volume of 20 mM buffer solution of different pH containing 0.2 mM EDTA. After the mixture was incubated at 37℃ for 60 min, the remaining activity was measured as described above. The buffer solutions used were sodium acetate (pH 3.5-5), potassium phosphate (pH 6-8) and sodium carbonate (pH 9-11).

Thermal stability of r-hSOD was examined as follows. The purified r-hSOD was dissolved in 10 mM potassium phosphate (PB) containing 0.1 mM EDTA (pH 7.0 or 7.4) at a concentration of 460 U/ml. The solution was incubated at various temperatures for the time indicated below.

Effect of the treatment of r-hSOD (500 U/ml) with trypsin(10ug/ml), aminopeptidase M (1 mg/ml) or human serum was investigated. After the incubation at 37℃ for the time indicated below, the remaining activity was determined as above.

Results

pH stability. The effect of pH on the stability of r-hSOD was studied by incubating with either 0.01 M acetate buffer (pH 3.5-5), 0.01 M potassium phosphate buffer (pH 6-8) or 0.01 M carbonate buffer (pH 9-11) containing 0.1 mM EDTA at 37℃ for 60 min. The remaining activity was then determined and expressed as per cent of activity under the standard conditions. As shown in Fig. 1, r-hSOD was stable in the pH range of 6-11.

Thermal stability. R-hSOD in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA was treated for 30 min at various temperatures, and the remaining activity was determined. The results showed that r-hSOD was stable up to 80℃ as shown in Fig. 2. The time course of the activity change (at pH 7.4) at 72℃ was also inves-

tigated for 2 h. R-hSOD was shown to be stable during the heat treatment.

Effect of incubation with proteases or human serum on r-hSOD activity. Effect of trypsin, aminopeptidase M and human serum on r-hSOD was investigated. A r-hSOD solution (500 U/ml) was incubated with trypsin (10 μg/ml) in 0.1 M Tris-chloride (pH 8.7) or aminopeptidase M (1 mg/ml) in 0.1 M sodium phosphate buffer (pH 7.0) at 37°C. The lyophilized r-hSOD (500 U) was dissolved in 1 ml of serum and incubated as above. As shown in Table 2, r-hSOD was highly resistant to these treatments, and enzymic activity was not lost. The stability of r-hSOD to treatment with trypsin

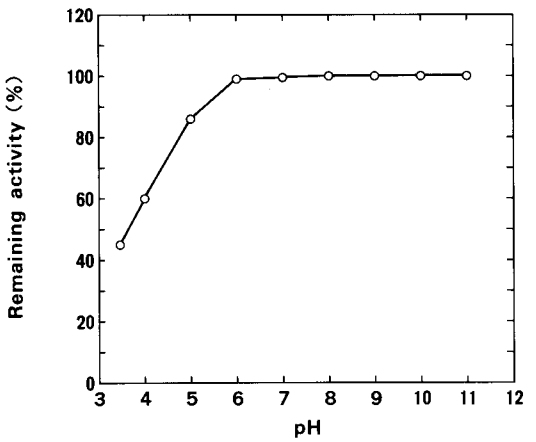

Fig. 1 pH stability of recombinant human superoxide dismutase (r-hSOD). R-hSOD activity was measured by the method described in Materials and Methods. An aqueous solution (460 U/ml) of r-hSOD solution was incubated with either 0.01 M acetate buffer (pH 4-5), 0.01 M potassium phosphate buffer (pH 6-8) or 0.01 M carbonate buffer (pH 9-11) containing 0.1 mM EDTA at 37°C for 60 min, and the remaining activity was then determined. The enzyme activity under the standard conditions was defined as 100%. Percentages of remaining activity are the average of two experiments.

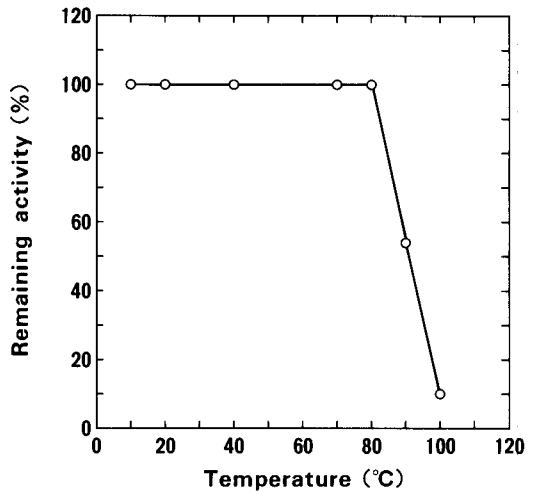

Fig. 2 Thermal stability of recombinant human superoxide dismutase (r-hSOD) in potassium phosphate buffer (pH 7.0). R-hSOD was treated in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA for 30 min at various temperatures, and the remaining activity was determined. Percentages of remaining activity are the average of two experiments.

Table 2 Stability of recombinant human superoxide dismutase (r-hSOD) incubated with trypsin, aminopeptidase M or serum[a]

Incubation with	Remaining activity (%)[b]					
	Incubation time (min)					
	0	30	60	90	120	180
Trypsin	100.0	100.5	99.8	101.5	100.7	98.5
Aminopepdase M	100.0	104.3	102.8	100.4	102.9	100.2
Serum	100.0	99.6	98.7	101.7	100.1	103.2

a: A solution of r-hSOD (500 U/ml) was incubated with trypsin (10 μg/ml) in 0.1 M Tris-chloride (pH 8.7) or aminopeptidase (1 mg/ml) in 0.1 M sodium phosphate buffer (pH 7.0) at 37°C. The lyophilized r-hSOD (500 U) was dissolved in 1 ml of human serum, and incubated at 37°C. Aliquots were removed at intervals and assayed for SOD activity by the method described under Materials and Methods.

b: Average of two experiments.

was coincident with that of bovine SOD (10).

Activity of r-hSOD in saline and perfusates at various temperatures. The activity of r-hSOD in saline and two perfusates at various temperatures was investigated. In this experiment, lyophilized r-hSOD (17,780 U/ml) was dissolved in one ml of each solution and assayed for activity at various temperatures. A control run without r-hSOD was also carried out at each temperature. The enzyme activity under the standard conditions (25℃, saline) was defined as 100%. As shown in Table 3, r-hSOD activity in saline and two perfusates at various temperatures was not changed remarkably even at 4 and 15℃.

Stability of r-hSOD in perfusates. The stability of r-hSOD in two types (A and B) of perfusates described in Table 1 was determined at various temperatures. Lyophilized r-hSOD (17,780 U) was dissolved in one ml of each perfusate, followed by incubation at various temperatures. Aliquots were removed at intervals and assayed for activity. Initial activity was defined as 100 %. As shown in Table 4, the stability of r-

Table 3 Activity of recombinant human superoxide dismutase (r-hSOD) dissolved in physiological saline and two perfusates (A and B) at various temperatures[a]

Temperature (°C)	Activity of r-hSOD (U/ml)[b] in		
	Saline	Perfusate A	Perfusate B
4	17,160 (96.4)	17,380 (97.8)	17,150 (96.5)
15	16,650 (93.5)	16,840 (94.6)	16,120 (90.6)
25	17,800 (100.0)	17,780 (99.9)	17,780 (99.9)
37	17,750 (99.7)	17,710 (99.5)	17,780 (99.9)

a: Lyophilized r-hSOD (17,780 U) was dissolved in one ml of each solution and assayed for activity at various temperatures. A control without r-hSOD was also carried out at each temperature. The enzyme activity under the standard conditions (25°C, saline) was defined as 100%. Percentage was given in parentheses. Components of perfusates A and B are given in Table 1.

b: Average of two experiments.

Table 4 Stability of recombinant human superoxide dismutase (r-hSOD) in two perfusates (A and B)[a]

Temperature (°C)	Perfusates	Remaining activity (%)[b]					
		Incubation time (h)					
		0	3	6	12	24	48
4	A	100.0	96.7	99.8	99.8	100.0	–[c]
	B	100.0	102.8	102.8	102.8	98.0	–
15	A	100.0	103.2	100.0	111.5	111.5	–
	B	100.0	104.5	104.5	107.6	104.5	–
25	A	100.0	100.0	92.4	100.0	92.2	100.0
	B	100.0	100.0	100.0	100.0	92.4	100.0
37	A	100.0	100.0	–	106.0	96.8	–
	B	100.0	100.0	–	100.0	100.0	–

a: Lyophilized r-hSOD (17,780 U) was dissolved in one ml of each perfusate, followed by incubation at various temperatures. Aliquots were removed at intervals and assayed for activity by the method described under Materials and Methods. Components of perfusates A and B are given in Table 1.

b: Average of two experiments

c: Not assayed

hSOD in these perfusates was very good at 4, 15, 25 and 37℃ for up to 24 h. In one experiment, in which r-hSOD was incubated at 25℃, it was stable for 48 h.

Discussion

R-hSOD was stable over a pH range of 6 to 11 and thermostable up to 80℃ in buffer solution. This high stability of r-hSOD was thought to be due to the intra-subunit disulfide bond and the metal binding structure coordinated by Cu and Zn (11). To determine whether r-hSOD would be effective in preventing free radical-induced myocardial injury during heart preservation and transplantation, it is necessary to examine whether r-hSOD activity and stability change under conditions approximating those in the clinical situation. Customarily, all hearts receive a continuous perfusion with oxygenated modified Collins' solution (Table 1. A), to which Fluosol-DA and albumin are added, at 4℃ (Table 1. B). The activity of r-hSOD was not affected by incubation with protease and serum at 37℃ for 3 h, and in two types of hypothermic perfusates at 4-37℃ for 24 h. We concluded from these results that the stability of r-hSOD was similar to the native enzyme, SOD, which is known to be stable (12). The physicochemical properties of r-hSOD are coincident with the native enzyme, the N-terminal alanine of which is acetylated (13). Two types of SOD have been identified in eukaryotic cells. One type contains Cu/Zn and is localized in the cytosol, whereas the other contains Mn and is found in the matrix of mitochondria (14). SOD (Cu/Zn) is a metalloenzyme containing 2 Cu and 2 Zn per dimer (15). Zn^{2+} plays a structural role and enhances stability whereas Cu^{2+} is directly involved in the catalytic cycle (16). These metal ions also protect r-hSOD against attack by proteases and shifts in pH or temperature. For example, apo-r-hSOD, from which Cu and Zn were removed was easily digested with trypsin. In this study, we observed that the catalyzing ability of r-hSOD was stable at 4-37℃ for at least 24 h. Although further study is needed to examine the stability of r-hSOD *in vivo*, the present results may suggest the possibility of perfusion experiments to examine the effect of r-hSOD on free radical-induced myocardial injury.

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