ENZYME IMMUNOASSAY FOR MANGANESE-SUPEROXIDE DISMUTASE IN SERUM AND URINE¹⁾

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An enzyme immunoassay has been developed to measure human manganese-superoxide dismutase (Mn-SOD) in serum and urine. The assay can be done in less than 5 h, is highly sensitive (detecting limit, 0.1 ng of Mn-SOD) and simple. There is no interference from factors in serum or urine under assay conditions, and the method is specific for human Mn-SOD.

Serum Mn-SOD concentrations were markedly increased in patients with liver diseases, but not in renal diseases. On the other hand, urinary Mn-SOD levels were elevated in a few patients with nephrotic syndrome and lung cancer, but were decreased in patients with hypertension. Furthermore, investigations were conducted on the clinical course of serum Mn-SOD levels in a case of alcoholic hepatitis, and on correlations between serum Mn-SOD and the conventional liver function tests.

The localization of Mn-SOD in liver was also explored using immunofluorescent staining. The fluorescence was intense in the degenerated portions of liver tissue from a patient with drug-induced hepatitis.

Keywords—enzyme immunoassay for manganese-superoxide dismutase; manganese-superoxide dismutase in serum and urine; manganese-superoxide dismutase in liver; liver diseases; oxygen toxicity

INTRODUCTION

Superoxide dismutases (SODs) are currently of interest in biochemistry, oncology, gerontology and immunology, because they perform the essential defence against oxygen toxicity.²⁾ SODs have been isolated from numerous sources. Several types of SOD have been described and their physical, chemical and catalytic properties have been explored.^{3,4)} Human cells contain two forms of SOD with different immunological specificities.^{5,6)} One is a copper and zinc containing SOD (Cu,Zn-SOD) found mainly in the cytoplasm of all cells. The other is a manganese containing SOD (Mn-SOD) principally present in the mitochondria.

An elaboration of sensitive, specific and simple assay for SODs is required in order to comprehend the mechanisms implicated in a wide

variety of problems connected with the metabolism of molecular oxygen. Various enzymatic assays for SODs have been outlined,⁶⁾ but they have numerous disadvantages including the lack of sensitivity required in many studies. This is especially true in the analysis of SODs in serum and urine. Moreover, the distinction between Cu,Zn-SOD and Mn-SOD, based, for example, on the difference of sensitivity by cyanide, is not absolute.⁷⁾ Immunological techniques can contribute to the solution of these problems. Radioimmunoassays have already been reported on bovine, rat and human SODs.^{8–13)} However, the assays have several disadvantages due to the use of radioisotopes, such as high capital costs of radioisotope counting equipment and the relatively high costs for each result when conducted in small batches due to the uneconomic use

of reagents.

We previously reported a sensitive and specific enzyme immunoassay for human Cu,Zn-SOD in serum and urine. It was then suggested that the assay could be applied to diagnosing renal diseases.¹⁴⁾

This paper described an enzyme immunoassay for human Mn-SOD and localization of Mn-SOD in the liver which was examined by immunofluorescent staining.

MATERIALS AND METHODS

Materials — β -Galactosidase from *Escherichia coli* (about 30 units/mg, lactose as substrate, at 25°C) was purchased from Boehringer Mannheim GmbH (West Germany). *m*-Maleimido-benzoyl-*N*-hydroxysuccinimide ester (MBS) was obtained from Pierce Chemical Co. (U.S.A.) and 4-methylumbelliferyl- β -D-galactose (4MUG) was from Koch-Light Laboratories Ltd. (England). Polystyrene balls (3.2 mm in diameter) were purchased from Ichiko Co. (Japan). All other chemicals used were of an analytical grade.

Determination of Creatinine — First morning urine samples were collected from healthy subjects and from patients with various diseases. The creatinine in urine was determined by the method of Bonsness and Taussky.¹⁵⁾

Purification of Mn-SOD — Mn-SOD from human liver was prepared according to the method described previously.¹⁶⁾ After purification, the specific activity of the enzyme was 3000–3400 McCord and Fridovich units/mg and the enzyme was proved to be homogeneous by an analysis on polyacrylamide gel electrophoresis.

Preparation of Anti-human Mn-SOD Antibody — One tenth milligram of purified Mn-SOD which was emulsified in complete Freund's adjuvant was injected subcutaneously into each of ten guinea pigs. The guinea pigs received three further injections (0.1 mg of the enzyme protein) at 2–3 weeks intervals and were bled 10 d after the last injection. The crude IgG fraction was prepared from antisera by fractionation with 33% saturated ammonium sulfate, followed by passage through a DEAE-cellulose column.¹⁷⁾ In order to

use this in enzyme immunoassay, the IgG was further purified through adsorption onto a Protein A-Sepharose CL-4B column equilibrated with 0.1 M sodium phosphate buffer (pH 8.0), and elution with 1 M acetic acid. The eluate was dialyzed against distilled water and lyophilized. The purified antibody was used for immobilization on a polystyrene ball and for preparation of an antibody labeled β -galactosidase.

Preparation of Anti-human Mn-SOD Antibody-coated Polystyrene Balls — Anti-human Mn-SOD Antibody was immobilized non-covalently on polystyrene balls as follows. Polystyrene balls were first washed with 10% detergent (Scat 20-X, Nakarai Chemicals Co., Japan). These balls were then immersed in the 0.1 mg/ml antibody solution which was dissolved in 0.25 M sodium phosphate buffer (pH 7.5) at 30°C for 2 h and left to stand at 4°C overnight. They were finally washed with 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM MgCl₂, 0.1% bovine serum albumin and 0.1% NaN₃ (buffer A) and stored in buffer A at 4°C until use.

Preparation of the Antibody- β -galactosidase Conjugate — Antibody- β -galactosidase conjugate was prepared using MBS as a crosslinking reagent according to the method of O'Sullivan *et al.*¹⁸⁾

Standard Enzyme Immunoassay — A piece of antibody-coated polystyrene ball was incubated in a glass test tube (10×75 mm) with various amounts of each standard or sample (5 μ l of serum or 20 μ l of urine) in 0.2 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl, 1 mM MgCl₂, 0.05% Tween 20, 1% bovine serum albumin and 0.1% NaN₃ (buffer T). After incubation with shaking at 37°C for 1 h, the reaction solution was aspirated and discarded. Then each ball was washed twice with 1.5 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl and 0.05% Tween 20. The balls were incubated at 37°C for 3 h with antibody- β -galactosidase conjugate (2 mU of β -galactosidase) in 0.2 ml of buffer T and again washed as above. The balls were then transferred to another glass test tube (15×105 mm) by

inverting it. Each ball was preincubated in 0.2 ml of buffer A at 30°C for 5 min, and then the enzyme reaction was started by addition of 0.1 ml of 0.3 mM 4MUG. After incubation with shaking at 30°C for 20 min, 2.5 ml of 0.1 M glycine-NaOH buffer (pH 10.3) was added and the amount of 4-methylumbelliferone formed was measured by fluorometry using a Shimadzu RF-510 spectrofluorometer. Wavelengths used for excitation and emission analyses were 360 nm and 450 nm, respectively. One unit of β -galactosidase activity is defined as that which hydrolyzed one μ mol of 4MUG per min under the conditions described above.

RESULTS

1. Specificity of Anti-human Mn-SOD Antibody

The specificity of the antibody was examined by Ouchterlony immunodiffusion and immunoelectrophoresis, as shown in Fig. 1. A completely fused precipitation line was observed between the antibody and purified Mn-SOD or homogenates of human liver, kidney and heart. Similarly, no cross reaction was found when the antibody was tested against purified human Cu,Zn-SOD. From these results, the antibody was specific for human Mn-SOD.

2. Calibration Curve of Mn-SOD

The calibration curve for human Mn-SOD is

FIG. 1. Ouchterlony Immunodiffusion (A) and Immunoelectrophoresis (B) of Human Mn-SOD against Anti-human Mn-SOD Antibody

Well 1: purified Mn-SOD; well 2: human liver homogenate; well 3: human kidney homogenate; well 4: human heart homogenate; well 5: purified Cu,Zn-SOD; well a: anti-human Mn-SOD antibody. Immunoelectrophoresis was performed at 4°C and 10 V/cm with 1% agarose gel in 30 mM Veronal buffer (pH 8.6) for 60 min.

shown in Fig. 2. This enzyme immunoassay could detect from 0.1 to 50 ng of Mn-SOD protein, have sensitivity enough to determine both serum and urinary Mn-SOD levels.

FIG. 2. Calibration Curve for Human Mn-SOD by the Enzyme Immunoassay

3. Precision of the Assay

Within-day assay precision was examined by 10 replicate measurements of Mn-SOD concentration in three separate sera and urines. Within-day coefficients of variation (C.V.) were 7.4–14.1% in serum and 6.7–10.7% in urine, as shown in Table I. Between-days assay precision was examined by 7 consecutive analysis on different days of Mn-SOD concentration in three sera and urine. Between-days precisions showed C.V. of 7.7–17.8% in serum, and 6.4–19.1% in urine (Table II).

4. Recovery of Mn-SOD from Serum and Urine

Purified human Mn-SOD was added to a series of 5 sera and urines chosen to cover a range from 0.5 to 10 ng per tube of Mn-SOD concentration. The result is shown in Table III. The average recoveries from the serum and the urine were 95.2–100% and 95.6–99.2%, respectively.

5. Serum Mn-SOD Concentrations in Various Diseases

The normal range of serum Mn-SOD concentration was 84.5 ± 30.0 ng/ml (mean $\pm$ S.D.), as shown in Fig. 3. Most sera of patients with liver diseases appeared to have a high Mn-SOD concentration: liver cancer, 1803 ± 1509 ng/ml;

TABLE I. Within-day Assay Precision

Subjects	Mn-SOD concentration (mean $\pm$ S.D.)	C.V.(%)
Serum	ng/ml	
1	66.1 $\pm$ 4.86	7.4
2	545 $\pm$ 53.4	9.8
3	1011 $\pm$ 143	14.1
Urine	μ g/g of creatinine	
1	58.3 $\pm$ 6.24	10.7
2	148 $\pm$ 9.94	6.7
3	535 $\pm$ 53.7	10.0

TABLE II. Between-days Assay Precision^{a)}

Subjects	Mn-SOD concentration (mean $\pm$ S.D.)	C.V.(%)
Serum	ng/ml	
1	148 $\pm$ 24.6	16.5
2	234 $\pm$ 18.1	7.7
3	461 $\pm$ 82.0	17.8
Urine	μ g/g of creatinine	
1	74.8 $\pm$ 14.7	19.1
2	167 $\pm$ 10.6	6.4
3	520 $\pm$ 43.8	8.4

a) Each of serum and urine samples was assayed for 7 d by this enzyme immunoassay.

TABLE III. *Recovery of Mn-SOD Added to Human Serum and Urine*

Number of samples	Mn-SOD concentration (ng)		Recovery (%)
	Added	Recovered	
Serum			
5	1.0	1.00±0.19	100 ±19
5	5.0	4.76±0.62	95.2±13
Urine			
5	2.0	1.98±0.20	99.2±10
5	10.0	9.56±1.12	95.6±12

FIG. 3. *Mn-SOD Concentrations in Sera of Normal Subjects and Patients with Various Diseases*
Bars indicate the mean value of Mn-SOD concentrations. Figures in parenthesis show the number of samples.TABLE IV. *Correlation between Serum Mn-SOD Levels and Other Clinical Data*

Relation (Number of samples)	Correlation coefficient (<i>r</i>)
Mn-SOD-AST (126)	0.217
Mn-SOD-ALT (127)	0.301
Mn-SOD- γ -GT (123)	0.100
Mn-SOD-ALP (127)	0.427
Mn-SOD-LAP (125)	0.222
Mn-SOD-LDH (127)	0.427
Mn-SOD-Cu,Zn-SOD ^{a)} (126)	0.262

AST: aspartate aminotransferase; ALT: alanine aminotransferase; γ -GT; γ -glutamyltransferase; ALP: alkaline phosphatase; LAP: leucine aminopeptidase; LDH: lactate dehydrogenase.

a) Cu,Zn-SOD concentration was determined by the method reported previously.¹⁴⁾

alcoholic hepatitis, 579 ± 550 ng/ml; acute hepatitis, 556 ± 483 ng/ml; chronic hepatitis, 444 ± 354 ng/ml; fatty liver, 417 ± 332 ng/ml.
6. *Correlation between Serum Mn-SOD Concentrations and Other Clinical Data*

FIG. 4. *Clinical Course of Serum Mn-SOD and Other Clinical Data in a Patient with Alcoholic Hepatitis*
Mn-SOD: manganese-superoxide dismutase, ●—●; AST: aspartate aminotransferase, ▲---▲; ALT: alanine aminotransferase, ■---■; γ-GT: γ-glutamyltransferase, ○---○. γ-GT activity was determined using γ-glutamyl-p-nitroanilide as a substrate.

Considering the liver diseases collectively, there was no significant correlation between the Mn-SOD concentrations and values of the liver function tests. Moreover, serum levels of Mn-SOD were unrelated to serum Cu,Zn-SOD levels. These correlations are given in Table IV.
7. *The Clinical Course of a Patient with Alcoholic Hepatitis*

Figure 4 shows the serum Mn-SOD concentrations and activities of various serum enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ-glutamyltransferase (γ-GT) in a patient with alcoholic hepatitis after admission to hospital. The Mn-SOD levels were different from AST, ALT and γ-GT activities during admission, and remained high for about two weeks after admission.
8. *Urinary Mn-SOD Concentrations in Various Diseases*

Figure 5 shows the urinary Mn-SOD concentrations in healthy subjects and patients with various diseases. The normal range of urinary Mn-SOD concentration was 17.4 ± 8.26 μg/g of creatinine (mean ± S.D.). The urinary Mn-SOD concentration elevated in a few patients with nephrotic syndrome and lung cancer, but patients with hypertension had a low Mn-SOD concentration (1.47 ± 0.72 μg/g of creatinine).
9. *Localization of Mn-SOD in the Liver Tissue of a*

FIG. 5. *Mn-SOD Concentrations in Urines of Normal Subjects and Patients with Various Diseases*
Bars indicate the mean value of Mn-SOD concentrations. Figures in parenthesis show the number of samples.

Patient with Drug-induced Hepatitis

The immunofluorescent staining pattern in the liver tissue of a patient with drug-induced hepatitis (diphenylhydantoin) is shown in Fig. 6. The fluorescence was intense in the degenerated portion of liver, but the control sections were negative.

DISCUSSION

We described here an enzyme immunoassay for human Mn-SOD. It should also be noted that recent reports indicate that immunoassays are interfered by serum factors,¹⁹⁻²¹⁾ especially when solid phases are used as a separation technique. Kato *et al.*²²⁾ reported that gelatin suppressed the serum interference in the measurement of insulin using a sandwich enzyme immunoassay. Application of gelatin to the present method did not provide clear utilization though such as interference was avoided by using gelatin in the enzyme immunoassay for human Cu,Zn-SOD as previously reported.¹⁴⁾ In order to remove interfering factors from serum and urine in this assay, we attempted to use a buffer containing Tween 20 reported by Goldsmith.²³⁾ From the results of good recoveries, it was suggested that interfering

factors were almost completely removed through the use of the buffer containing Tween 20. However, the nature of the serum or urine factors that interfere with the assay, the inhibition mechanism of the immunoreaction and the role of Tween 20 in overcoming the interference are not clear at present.

Baret *et al.*¹¹⁾ reported that serum Mn-SOD levels increased in liver diseases such as hepatitis and cirrhosis, but they have not shown any detail about the relationship between Mn-SOD and liver diseases. In this study, serum Mn-SOD concentrations also elevated remarkably in many patients with liver diseases, but did not increase in renal diseases. The biochemical data in liver function tests did not show any significant correlation with the levels of Mn-SOD in sera from patients with liver diseases and no correlation was observed between Mn-SOD and Cu,Zn-SOD. In the case of alcoholic hepatitis, the changes of serum Mn-SOD concentration were different from those of AST, ALT and γ -GT activities during admission. It appears from these results that the appearance of Mn-SOD in liver diseases is initiated by a mechanism different from that of the enzymes which are generally used in liver function tests.

Immunofluorescent staining revealed a degenerated portion of liver tissue in a patient with drug-induced hepatitis. It seems possible that the increased levels of serum Mn-SOD in liver diseases is derived from liver tissue. Therefore, it may be thought that the measurement of serum Mn-SOD is useful in clarifying new pathological conditions of liver disease.

On the other hand, urinary Mn-SOD levels decreased in patients with nephrotic syndrome and lung cancer. In order to explain these changes of urinary Mn-SOD levels, it will be necessary to examine the time course of the enzyme and subject it to a biological test using tissue staining and this enzyme immunoassay.

We have already developed the enzyme immunoassay for human Cu,Zn-SOD and observed that Cu,Zn-SOD in serum and urine elevated markedly in renal diseases.¹⁴⁾ In this study,

FIG. 6. *Photomicrograph of Biopsy Specimen from the Liver of a Patient with Drug-induced Hepatitis*
The specimen was stained with fluorescein isothiocyanate labeled anti-human Mn-SOD antibody by direct immunofluorescent procedure.

it becomes clear that the levels of serum Mn-SOD were different from those of serum Cu,Zn-SOD in various diseases, especially in liver and renal diseases. It should stimulate interest to investigate the changes of both SODs in these pathological conditions. Furthermore, we expect that the measurement of both SODs by the enzyme immunoassay will be useful in the clinical evaluation of liver and renal diseases and we are carrying out further clinical investigations.

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