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Immunoassay based on a polyclonal antibody for the quantification of *Porphyromonas gingivalis* SOD : a preliminary report

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Summary

Superoxide dismutase (SOD) as a free radical scavenger in *Porphyromonas gingivalis* is well documented. The aim of this work was to develop an enzyme-linked immunosorbent assay using recombinant *P. gingivalis* SOD as an antigen. The sandwich complex was detected using a secondary antibody conjugated to β-D-galactosidase. Under optimum conditions, the sensitivity and the limit of detection were determined from 25pg to 500pg. In future, the application will be extended to the expression of SOD from *P. gingivalis* under various growing conditions.

Introduction

The black-pigmented oral anaerobe *Porphyromonas gingivalis* has been increasingly implicated as an etiologic agent of a severe form of periodontal disease[1,2]. Since *P. gingivalis* can be established and maintained in a periodontal pocket, an enzyme which may contribute to the virulence of this organism is superoxide dismutase (SOD ; EC 1.15.1.1.). SOD protects the organism from oxidative damage by catalyzing the conversion of the superoxide anion to hydrogen peroxide and molecular oxygen ; along with catalase and peroxidase, it belongs to a specific cellular system that has evolved for cellular protection against oxidative stress. Interestingly, catalase and peroxidase activity could not be detected in *P. gingivalis*[3].

P. gingivalis SOD (*P. g.* SOD) is a family of metalloenzymes which can use both iron and manganese as cofactors, and the manganese-containing SOD (Mn-SOD) is more stable in the presence of H_2O_2 than iron-containing SOD (Fe-SOD)[4]. Furthermore, Mn-SOD was demonstrated to be required for the survival of the bacteria in the presence of air[5]. SOD of anaerobically grown *P. gingivalis* had iron as a cofactor, although SOD from aerobically grown *P. gingivalis* had manganese. The mechanism of this phenomenon is not clear.

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The purpose of this study was to develop a new enzyme-linked immunosorbent assay (ELISA) for the quantification of *P.g.* SOD protein. In many cases of SOD purification, the total activity of the crude extract was lower than in the next purification procedure. This may be due to the production of superoxide anions by minute membrane fragments still present in crude extracts and minimizing real SOD activity by artifacts[6,7]. ELISA is expected to estimate the true content of expressed SOD instead of estimating SOD activities in crude extract.

Materials and Methods

Materials

β-D-galactosidase was purchased from Roche Diagnostics (Mannheim, Germany) ; *N,N'-o*-phenylenediamialeimide, 4-methylumbelliferyl-β-D-galactoside, bovine serum albumin (BSA) and 4-methylumbelliferone were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) ; polystyrene beads (3mm in diameter) were from General Science Co. (Tokyo, Japan) ; Sephadex G-25, Protein A Sepharose 4 Fast Flow, and Sepharose 6B were purchased from GE Healthcare (Uppsala, Sweden). All other reagents were of the highest purity commercially available.

Antiserum production

Antigenically pure recombinant *P. g.* SOD was prepared as described previously[4]. Antibody against SOD was raised in male rabbits (2kg) by injecting the antigen intracutaneously as a mixture with Freund's complete adjuvant four times (total amount of SOD is 2.0mg) at 2-week intervals. Antisera were harvested 30 days after the last injection and stored at 4℃.

Preparations of immunoglobulins from anti-P. g. SOD sera

Anti-*P. g.* SOD IgG fraction was isolated from antisera by Protein A Sepharose 4 column (1ml) pre-equilibrated with phosphate-buffered saline (PBS : pH7.2 / 0.0182M Na_2HPO_4 / 0.0028M KH_2PO_4 / 0.15M NaCl). IgG fraction was eluted with 0.1M glycine-HCl buffer, pH2.5. The pH of the protein fraction was adjusted to 5.0 with 0.1M acetate, dialyzed against 0.05mM sodium phosphate buffer, pH7.0, and then concentrated to a small volume.

Labeling of the antibody with β-D-galactosidase

Anti-P. g. SOD IgG was coupled with β-D-galactosidase using a bifunctional coupling reagent, *N,N'-o*-phenylenediamialeimide[8]. In brief, anti-*P.g.* SOD IgG (0.33mg in 0.1M sodium acetate buffer, pH5.0) was reduced with 10mM 2-mercaptoethylamine at 37℃ for 90min, and the resulting IgG was prepared by a Sephadex G-25 column, and was treated with excess amounts of *N,N'-o*-phenylenediamialeimide to introduce maleimide residues on anti-*P. g.* SOD IgG. Maleimide IgG was reacted with β-D-galactosidase to produce the IgG-enzyme conjugate. Anti-*P. g.* SOD IgG labeled with β-D-galactosidase was separated by a Sepharose 6B column (2.5 x 24.5cm), and stored at 4℃ in 0.01M sodium phosphate buffer, pH7.0 / 0.1M NaCl / 1mM $MgCl_2$ / 0.1% BSA (buffer A).

Immobilization of antibody on polystyrene beads as solid phase

Anti-*P. g.* SOD IgG were immobilized non-covalently on polystyrene beads as solid phase. Beads were immersed in a solution of IgG (100mg/ml in 0.05M sodium phosphate buffer, pH7.0, containing 0.1% NaN_3), kept at 4℃ overnight under gentle stirring, and washed with buffer A for at least 3 days

before used for immunoassay. The solid phase with immobilized antibody was stable at 4℃ for at least 6 months[9].

Sandwich enzyme immunoassay procedure

The assay system was essentially similar to that described previously[9]. Schematic representation of sandwich ELISA procedure is shown in Fig.1. Beads of the solid phase with immobilized antibody were incubated with various amounts of purified recombinant *P. g.* SOD at 37℃ with vigorous shaking in a final 150μl of 0.01M sodium phosphate buffer, pH7.0 / 0.3M NaCl / 1mM $MgCl_2$ / 0.5% gelatin / 0.1% BSA / 0.1% NaN_3 (buffer B). After 5h, the reaction medium was removed by aspiration, and the beads were washed twice with 1ml chilled buffer A in each tube. The beads were then incubated at 4℃ overnight under shaking with 1mU of β-D-galactosidase-conjugated anti-*P. g.* SOD IgG in 200μl buffer A, washed with buffer A, and bound enzyme protein was assayed with a fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside. Beads were incubated with 0.1mM substrate in a final 150μl of buffer A at 37℃ for 20min. The reaction was terminated by adding 750μl of 0.5M glycine-NaOH, pH10.3, and the fluorescence intensity of the 4-methylumbelliferon released was estimated against a control solution at 450nm with an excitation wavelength of 360nm.

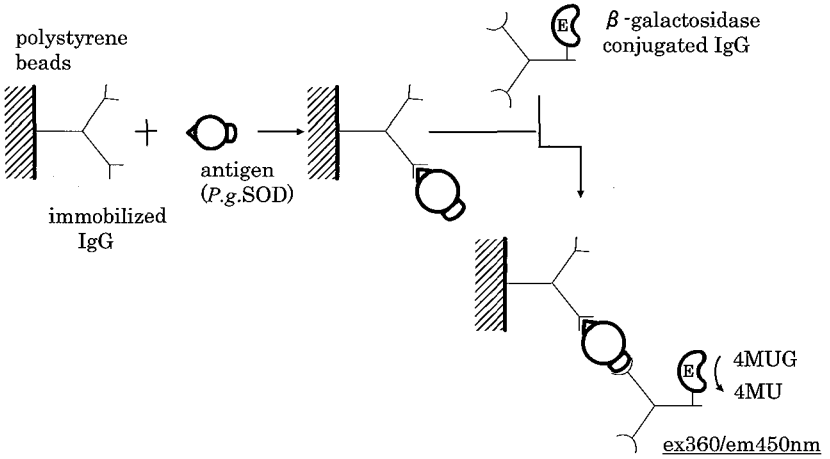

Figure 1 : The principles of the sandwich ELISA illustrated.
This procedure requires the antigen to have at least two binding site. Antigen (*P. g.* SOD) reacts with excess solid-phase immobilized antibody, and after incubation followed by washing, the bound antigen is reacted with excess β-D-galactosidase labeled antibody. After further washing, the bound β-D-galactosidase (sandwich typed complex) is assayed with a fluorogenic substrate, 4-methyl-umbelliferyl-β-D-galactoside (4MUG), and the fluorescence intensity of the 4-methyl-umbelliferone (4 MU) released was estimated at 450nm with an excitation wavelength of 360nm.

Analytical methods

SOD activity was measured by inhibition of the xanthine/xanthine oxidase-induced reduction of cytochrome *c* at pH7.8 and slightly modified according to a previous reports[10,11].

Protein concentration was estimated by the method of Hartree[12] using crystalline BSA as the standard.

Results and Discussion

A standard curve in a log-log scale, prepared from data obtained by homologous ELISA using

polyclonal anti-*P. g.* SOD antibody is shown in Fig.2. *P. g.* SOD protein content versus bound β-D-galactosidase activity was linear between 25 to 500pg of purified recombinant *P. g.* SOD protein per tube.

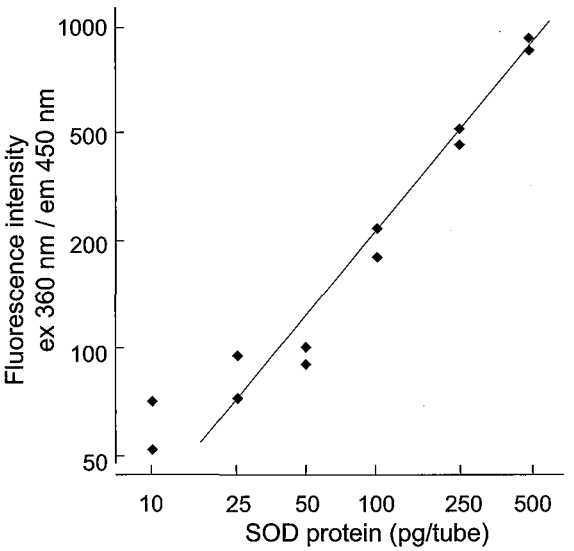

Figure 2 : Standard curve of purified *P. g.* SOD by the sandwich ELISA technique.
The fluorescence intensity of 4-methylumbelliferon released from a fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside, by the β-D-galactosidase conjugated anti-*P. g.* SOD IgG was estimated against standard purified *P. g.* SOD with solid phase (polystyrene beads) immobilized anti-*P. g.* SOD IgG.

In preliminary investigations, we evaluated the expression of recombinant wild and mutant type *P. g.* SOD in host *Escherichia coli* cells, which are SOD-deficient cells, QC 774[13]. Construction of the expression vector, and site-directed mutagenesis were carried out using the method described previously[11]. A leucine to tryptophan at amino acid position 72mutation (Leu72Trp) and tyrosine to phenylalanine at amino acid position 77mutation (Tyr77Phe) were tested. These wild and mutant DNA were sequenced to ensure the absence of spurious mutations (Hiraoka, unpublished results). Table 1 shows the outcome of ELISA of the SOD protein content in the crude extract of host cells. Mutant SODs had a lower concentration than wild SOD. In particular, Tyr77Phe mutant had a significantly low expression of SOD protein ; however, SOD activity was seemingly high and not concordant with the results of SOD protein. There is no simple explanation of this result at the present time, but there is a possibility of proteolysis after the expression of enzyme protein.

These results indicate that the developed ELISA technique can be used to evaluate the expression of SOD protein. The combined use of the present ELISA method and determination of enzyme activity is also effective.

In conclusion, the sandwich ELISA described here can be used to quantify *P. g.* SOD concentration in a crude extract of the cell. Evaluation of the expression of SOD from *P. gingivalis* under changing growing conditions is the next challenge toward understanding the physiological role of SOD. A modification of the present method is being tested for easier and more rapid measurement using a plate reader in our laboratory.

Table 1 : Comparison of expression value and activity of SOD from wild-and mutant-type *P. g.* SODs.
Values are given as the means ± S.D.

	SOD (μg) / total protein (mg)	expression ratio	SOD activity (U/mg)	activity ratio
Wild type	1.92 ± 0.54	1.0	2.45 ± 0.72	1.0
Leu72Trp mutant	1.58 ± 0.31	0.82	1.96 ± 0.22	0.80
Tyr77Phe mutant	0.021 ± 0.004	0.011	0.540 ± 0.033	0.22

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References

1) Slots J and Genco RJ (1984) Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease : virulence factors in colonization, survival, and tissue destruction. J Dent Res **63** : 412-21.
2) Mayrand D and Holt SC (1988) Biology of asaccharolytic black-pigmented *Bacteroides* species. Microbiol Rev **52** : 134-52.
3) Amano A, Tamagawa H, Takagaki M, Murakami Y, Shizukuishi S and Tsunemitsu A (1988) Relationship between enzyme activities involved in oxygen metabolism and oxygen tolerance in black-pigmented *Bacteroides.* J Dent Res **67** : 1196-9.
4) Yamakura F, Taedin RL, Petsko GA, Ringe D, Hiraoka BY, Nakayama K , Fujimura T , Taka H and Murayama K (1998) Inactivation and destruction of conserved Trp 159 of Fe-super oxide dismutase from *Porphyromonas gingivalis* by hydrogen peroxide. Eur J Biochem **253** : 49-56.
5) Nakayama K (1994) Rapid viability loss on exposure to air in a superoxide dismutase-deficient mutant of *Porphyromonas gingivalis.* J Bacteriol **176** : 1939-43.
6) Britton L, Malinowski DP and Fridovich I (1978) Superoxide dismutase and oxygen metabolism in *Streptococcus faecalis* and comparisons with other organisms. J Bacteriol **134** : 229-36.
7) Terech A and Vignais PM (1981) A manganese-containing superoxide dismutase from *Paracoccus denitrificans.* Biochim Biophys Acta **657** : 411-24.
8) Yoshitake S, Hamaguchi Y and Ishikawa E (1979) Efficient conjugation of rabbit Fab' with β-D -galactosidase from *Escherichia coli.* Scand J Immunol **10** : 81-6.
9) Mogi M, Harada M, Hiraoka BY, Fukasawa K, Komatsu M and Nagatsu T (1986) Sandwich enzyme immunoassay for dipeptidyl aminopeptidase Ⅳ in the serum of people with oral cancer. Archs Oral Biol **31** : 505-7.
10) McCord JM and Fridovich I (1968) The reduction of cytochrome c by milk xanthine oxidase. J Biol Chem **243** : 5753-60.
11) Hiraoka BY, Yamakura F, Sugio S and Nakayama K (2000) A change of the metal-specific activity of a cambialistic superoxide dismutase from *Porphyromonas gingivalis* by a double mutation of Gln-70 to Gly and Ala-142 to Gln. Biochem J **345** : 345-50.
12) Hartree EF (1972) Determination of protein : a modification of the Lowry method that gives a linear photometric response. Anal. Biochem **48** : 422-7.
13) Carlioz A and Touati D (1986) Isolation of superoxide dismutase mutants in *Escherichia coli* : is superoxide dismutase necessary for aerobic life ? EMBO J **5** : 623-30.

抄録：一抗体免疫法による *Porphyromonas. gingivalis* SOD の定量（プレリミナリーレポート）

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歯周病原菌 *Porphyromonas gingivalis* にとって，スーパーオキシドジスムターゼ（SOD）は酸化ストレスから菌体を保護するために不可欠の酵素である．本研究の目的は，酵素タンパク質の定量的酵素免疫測定法（ELISA法）を開発し，SODの発現量を正確に評価する事にある．

ウサギで *P. gingivalis* SOD（*P. g.* SOD）の抗体を作製し，精製IgGをポリスチレン・ビーズに結合させて捕獲抗体とした．また，別にIgGにβ–D–ガラクトシダーゼを結合させて酵素標識抗体とし，一抗体固層法によるサンドイッチ型定量的酵素免疫測定法（ELISA法）を確立した．測定可能なSOD量は，25～500pgであった．

確立したELISA法の応用を試みた．組換え体・野生型 *P. g.* SODと変異型酵素の発現量を菌の粗抽出液で比較したところ，変異型酵素で著しく発現量が減少していた．この現象は酵素活性の測定結果とは一致せず，粗抽出液中の酵素量の評価にELISA法が有効である事を示唆した．