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A New Lysozyme Assay Based on Fluorescence Polarization or Fluorescence Intensity Utilizing a Fluorescent Peptidoglycan Substrate[1]

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A new sensitive, rapid and simple method for lysozyme assay is described which is based on either fluorescence polarization or fluorescence intensity using fluorescein-labeled peptidoglycan as a substrate. The peptidoglycan was obtained from *Micrococcus lysodeikticus* after extensive digestion with Pronase and washing with Triton X-100 followed by various solvents. Subsequently, it was labeled with fluorescein isothiocyanate (FITC) at the amino group of the peptide. When the FITC-labeled substrate was subjected to lysozyme digestion, an increase of fluorescence intensity or a decrease of fluorescence polarization value (*P* value) was apparent in five minutes at a lysozyme concentration as low as 0.1 or 0.01 μg/ml, respectively. The effect of other hydrolytic enzymes including α-mannosidase, proteases and RNase on the *P* value was found to be negligible. The measured values represented the specificity and dose of lysozyme added. Apparent V_{max} and K_m values for two different lysozymes, chicken egg white and human, could be determined by this method.

Fluorescence polarization has a wide range of applications with its high sensitivity and simplicity, if an adequate instrument is available. For instance, it has been applied for many proteins and peptides (*2–4*). I have also demonstrated, (i) the antigen-antibody reaction of antitumor protein antibiotic neocarzinostatin or of angiotensin I and their antibodies at about 5 pmol/ml (*5*, *6*) and (ii) trypsin or plasminogen activator activity at 0.05 μg/ml (*7*, *8*). As reported recently, the decrease of molecular weight of fluorochrome labeled proteins during proteolysis was reflected by a decrease in the fluorescence polarization value (*P* value) (*7*, *9*). That is, Perrin's equation 1 is applicable for typical proteins with this fluorochrome (fluorescein);

$$\frac{1}{P}=\frac{1}{P_0}+\left(\frac{1}{P_0}-\frac{1}{3}\right)\times\frac{R\cdot T\cdot\tau}{V\cdot\eta} \qquad (1)$$

in which P is the measured fluorescence P value and V is a molecular volume of the fluorescing molecule which is allowed to change either by antigen-antibody complex formation or hydrolytic

[1] A preliminary account of this work was presented at the 52nd Meeting of the Japanese Biochemical Society, October 1979, Tokyo (*1*). A part of this investigation was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

Abbreviations: FITC, fluorescein isothiocyanate; *P* value, fluorescence polarization value; PBS, 0.01 M phosphate buffered 0.15 M NaCl, pH 7.1. Lysozyme: unless otherwise specified, it is from chicken egg white.

degradation. Other parameters are assumed to be constant; namely, P_0, R, T, τ, and η, representing a fluorescence polarization constant for a given fluorochrome and which can be obtained at $\tau/\eta = 0$, the gas constant, temperature of the measurement, the fluorescence relaxation time, and the viscosity of the solvent, respectively.

The advantages of the fluorescence polarization method are as follows: (a) high sensitivity, (b) any natural protein of limited availability can be used as a substrate for any unique proteases, (c) simple operation (no separation step), and (d) no radioactive or other elaborate reagent is required. In this communication, an application of this method to the assay of lysozyme is described.

MATERIALS AND METHODS

Instruments—The fluorescence spectropolarimeter, Model MAC-2 Type HR-1 (Japan Immuno Industries Co., Ltd., Takasaki, Japan), used for the measurements, had three innovative features: (i) It will print out the P value as shown in Eq. 2 every 82 s.

$$P=\frac{IA-IB}{IA+IB} \qquad (2)$$

where IA and IB are the fluorescence intensities of polarized light components parallel and perpendicular to the vector of the primary polarizer. Furthermore, 100 readings were integrated by a microcomputer in order to obtain a P value. (ii) A single photomultiplier tube is used, which enables the determination of fluorescence intensities of both parallel and perpendicular components generated by a single rotating polarizer. Thus the intensity is a function of cosine against the time of rotation (1,800 rpm). (iii) Use of three cavity filters (Ditric Optics, Hudson, Mass.) with near monochromatic performance exhibiting a high transmission (50%) and little depolarization of the emitting beam. By a modification of the instrument the fluorescence intensity is also printed out every 82 s at the time of print-out of the P value. The fluorescence intensity can be detected at a concentration of 0.5 pmol/ml of free fluorescein at or above pH 7. The instrument is operated at the optimal conditions for fluorescein chromophore which has maximum excitation and emission spectra at about 490 and 520 nm, respectively. A refrigerated water circulator, Model RTE-8 (Neslab Inst. Inc., Portsmouth, N.H.), was used to control the temperature at the operation constant of $30° \pm 0.01°C$.

Biochemicals—Chicken egg-white lysozyme [EC 3.2.1.17] was obtained either from Seikagaku Kogyo Co., Tokyo, or Sigma Chemical Co., St. Louis, Mo. Human urinary lysozyme was a generous gift from Dr. Sakiyama, Protein Research Institute, Osaka University, purified as described (*10*). α-Mannosidase [EC 3.2.1.24], purified from a marine gastropod (*Turbo cornutus*), was obtained from Seikagaku Kogyo Co., Ltd., Tokyo. DNase I [EC 3.1.21.1], RNase A [EC 3.1.27.5], trypsin [EC 3.4.21.4], and chymotrypsin [EC 3.4.21.1] were obtained from Sigma Chemical Co. Pronase was obtained from Kaken Chemical Co., Tokyo. Glycolchitin was a gift from Drs. Imoto and Hayashi, Kyushu University, Fukuoka. FITC with a purity of more than 90% was obtained from Dojin Chemical Co., Ltd., Kumamoto. All other reagents or solvents were from commercial sources and used without purification.

Preparation of Peptidoglycan—Dried cells of *Micrococcus lysodeikticus*, 130 mg, obtained from Sigma Chemical Co. or Seikagaku Kogyo Co., Ltd., were suspended in 10 ml of 0.01 M phosphate buffered 0.15 M NaCl (PBS) and allowed to stand for 1 to 3 h. Thirteen mg of Pronase, having 750 tyrosine units/mg, was added to the cell suspension and the mixture allowed to incubate for a period of 4 to 6 h at 37°C. The cells were centrifuged down at about 3,000 rpm for 20 min, and then the cells were resuspended in 10 ml of 1% Triton X-100 followed by incubation at 37°C for 18 h. The Triton X-100 solution was removed from the cells by centrifugation at 2,800 rpm for 30 min. The Pronase (10 mg) digestion and Triton X-100 treatment at 1% were repeated once again respectively. Then the cells were washed with PBS, twice each with 50% ethanol, and then with hot phenol at about 40°C followed by washing with ethanol and acetone respectively. During this process, proteins, nucleic acids, and lipids were removed. The insoluble peptidoglycan thus obtained was dried *in vacuo*. The yield was 24 mg.

Labeling with Fluorescein Isothiocyanate (FITC)—The peptidoglycan, 24 mg, was reacted with 11 mg of FITC in a test tube containing

2.5 ml of 0.5 M Na bicarbonate-carbonate buffer, pH 9.3, for 4 h at 37°C under reciprocal shaking at 2.0 Hz (*11*). Then the reaction mixture was washed twice each with carbonate buffer, distilled water, acetone and ethanol at about 2,800 rpm. About 21 mg of the reaction product was obtained after drying *in vacuo*, which contained 0.158 μmol fluorescein/mg.

Amino Acid Analysis—In order to confirm the known amino acid composition of the peptidoglycan (*8*, *9*), about 1 mg of the product was hydrolyzed with 0.2 ml of 6 M HCl at 110°C *in vacuo* for 18 h. After removal of HCl, the hydrolysate was subjected to amino acid analysis.

Assay of Lysozyme Activity by Fluorescence Polarization and Fluorescence Intensity—Lysozyme freshly dissolved in PBS was added in 50 μl aliquots to 2 ml of PBS containing the labeled substrate (about 50 pmol fluorescein equivalent) in cuvettes, and the reaction after mixing was monitored by both the *P* value and the fluorescence intensity every 82 s.

RESULTS

Preparation of FITC-Labeled Peptidoglycan—From 130 mg of the dried bacterial cells, about 24 mg of peptidoglycan was obtained. After reaction with FITC and purification about 21 mg of FITC-labeled peptidoglycan was recovered. The amino acid analysis indicated that the peptidoglycan contained nearly the theoretical amount of amino acids from the pentapeptide of the peptidoglycan of *M. lysodeikticus*; L-Ala-D-γ-Glu(α-Gly)-L-Lys-D-Ala (*12*, *13*), as shown in Table I. The absorption spectra of the labeled and unlabeled peptidoglycan in PBS are shown in Fig. 1, indicating the presence of FITC-chromophore in the former. The *P* values were found, as was the case with the labeled proteins (*2*, *10*), to be independent of the concentration of the peptidoglycan (Fig. 2). *P* values are, however, more dependent on rotational Brownian motion or the molecular size, which became low as a consequence of lysozyme digestion as discussed below.

Specificity of Measured Activity—The labeled peptidoglycan resisted digestion with Pronase, trypsin, chymotrypsin, RNase (data not shown), DNase and α-mannosidase at concentrations of 2 to 5 μg/ml or 5 mU/ml (mannosidase) as judged by *P* values. When it was digested with lysozyme,

TABLE I. Amino acid composition of peptidoglycan substrate. The values in parentheses are the relative amounts in moles. No correction was made for decomposition for the value of Ser which was no more than 10%.

Amino acids	Relative amount found (mol)	Calc.
Asp	0.13 (0)	0
Thr	0.08 (0)	0
Ser	0.39 (0.4)	0
Glu	0.96 (1)	1
Pro	0.01 (0)	0
Gly	1.00 (1)	1
Ala	1.71 (1.7)	2
Cys	0.00 (0)	0
Val	0.12 (0)	0
Met	0.05 (0)	0
Ile	0.09 (0)	0
Leu	0.29 (0)	0
Tyr	0.01 (0)	0
Phe	0.05 (0)	0
Lys	0.97 (1)	1
NH_3	1.10 (1)	0
His	0.14 (0)	0
Arg	0.12 (0)	0

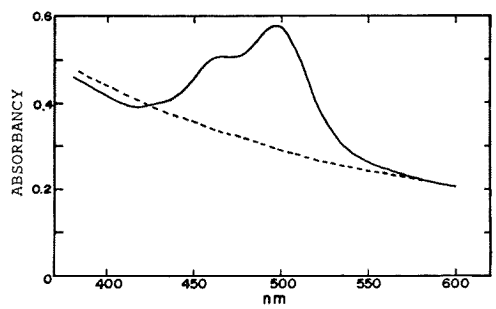

Fig. 1. Absorption spectra of peptidoglycan of *Micrococcus lysodeikticus* and its fluorescein conjugated derivative. About 0.06 mg of the peptidoglycan preparations were suspended in 2 ml of 0.01 M phosphate buffered 0.15 M NaCl solution, pH 7.1. Dashed line, peptidoglycan; solid line, FITC-conjugated peptidoglycan.

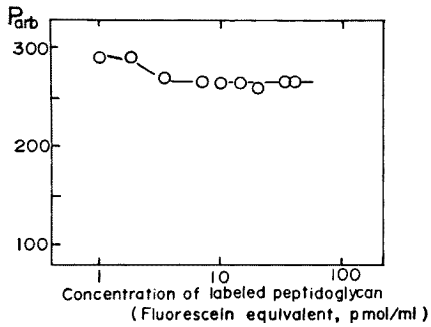

Fig. 2. Fluorescence polarization values of the fluorescein labeled peptidoglycan at various concentrations. *P* values shown are arbitrary. Measurements were made at 30±0.01°C in 2 ml of 0.01 M phosphate buffered 0.15 M saline, pH 7.1.

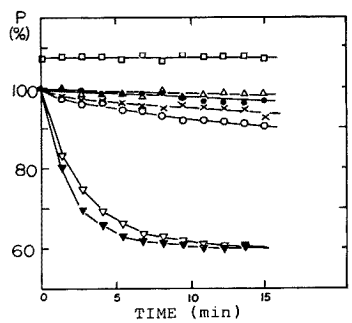

Fig. 3. Specificity of hydrolytic activity of peptidoglycan as measured by *P* value and various hydrolytic enzymes. □, α-mannosidase at 5 mU/ml; △, DNase at 2 μg/ml; ●, control (no enzyme); ×, trypsin at 5 μg/ml; ○, Pronase at 5 μg/ml; ▽ and ▼, lysozyme at 2 and 4 μg/ml, respectively. The temperature and the buffer are the same as in Fig. 2.

however, a decrease of *P* value was apparent (Fig. 3) and this progressed with time.

Dose Response of Lysozyme Activity—Using various dose-levels of the lysozyme a proportional dose-response to the amount of lysozyme was observed both by fluorescence intensity (Fig. 4) and by fluorescence polarization (Fig. 5). The present substrate was found to exhibit a significant change both in fluorescence intensity and *P* value when the lysozyme content was more than 0.1 μg/ml and 0.01 μg/ml, respectively.[2] When the change of *P* value was plotted against the dose of lysozyme, a linear correlation was observed

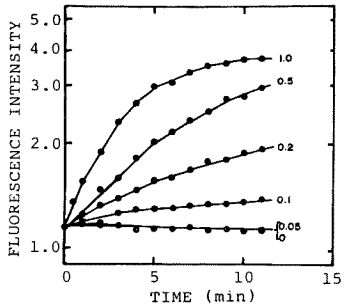

Fig. 4. Increase of fluorescence intensity during hydrolysis of the peptidoglycan by lysozyme at various concentrations. Numbers in the figure indicate concentrations of lysozyme in μg/ml. The conditions are the same as in Fig. 2.

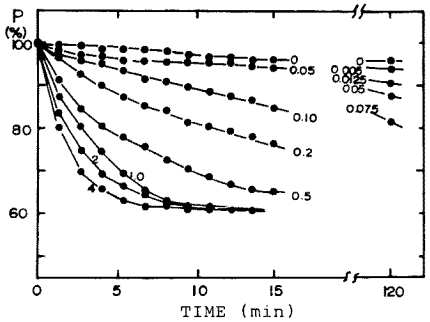

Fig. 5. Decrease of *P* value during the hydrolysis of the peptidoglycan by lysozyme at various concentrations. Numbers in the figure indicate the concentrations of lysozyme in μg/ml. The conditions are the same as in Fig. 2.

above 0.01 μg/ml (Fig. 6). On the contrary, FITC-labeled glycolchitin was found to be much less sensitive to lysozyme (data not shown) even at 10 μg/ml.

Determination of V_{max} and K_m—Both chicken and human lysozyme at a concentration of 0.05 μg/ml, respectively, were used to hydrolyze the labeled peptidoglycan in PBS (pH 7.1) at 30.0°C

[2] Theoretically, the sensitivity of the fluorescence intensity measurement is higher than that of the *P* value. For the present measurements, however, *P* values were obtained after integration of 100 readings in 50 s. Thus the ratio of signal/noise as well as the sensitivity for *P* values are higher than the fluorescence intensity measurement at one point reading.

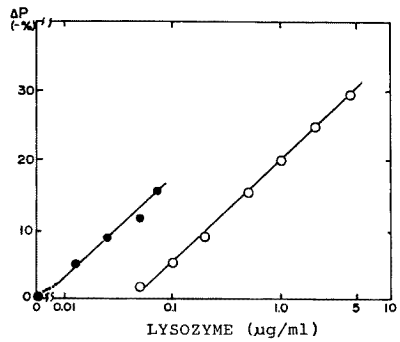

Fig. 6. The relationship between the amount of lysozyme and change of P value. The assay conditions are the same as in Fig. 2. Open and solid circles indicate the changes of P value in 2.5 min and 2 h, respectively.

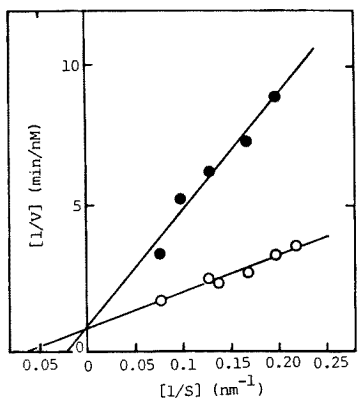

Fig. 7. A comparison of Lineweaver-Burk plots of human and chicken lysozymes as determined by the fluorescence polarization method. Substrate: FITC-labeled peptidoglycan. Lysozyme concentrations of human (○) and chicken egg white (●) were 0.05 μg/ml, respectively. Initial reaction velocity was determined based on the change in P value for the initial 2 min.

in a cuvette placed in the instrument. The substrate concentration ranged from about 4 to 80 nM, fluorescein equivalent. The initial reaction velocity was determined by quantitation of the initial reaction product based on the fluorescence intensity (amount) of a resulting product having a decreased P value (P') and by multiplication of P' (representing an altered molecular volume of the product) according to Eq. 1. The results obtained are expressed as a Lineweaver-Burk plot in Fig. 7. From these data, apparent K_m values of chicken and human lysozyme were found to be 66.9 and 16.7 nM, and V_{max} were 1.61 and 1.42 nM/min, respectively. Note that the concentration of the substrate is based on that of fluorescein fluorochrome, all the values thus obtained express merely apparent concentration.

DISCUSSION

The method described in this report is based on the change in P value or Brownian motion resulting from a change in molecular weight of a fluorescent peptidoglycan. The assay is based on the ability of lysozyme to hydrolyze a bond between *N*-acetyl muramic acid and *N*-acetyl glucosamine linked by a β-1,4 configuration in the peptidoglycan. As a consequence, more accelerated rotational Brownian motion of this polymer will result. Peptidoglycans are present specifically in the cell wall of bacteria, while lysozyme is an unique enzyme universally found in humoral fluids in animals and avian egg-whites which is involved in nonspecific defense against bacterial infection. A rapid and sensitive quantitation of lysozyme activity in serum, urine or other biological fluids can aid the evaluation of a host animal's reactivity against infection, or that of renal function, or diagnosis of leukemia (*14*, *15*). More recently, quantitation of lysozyme activity has become important for the assessment of immunological (or monocytic) reactivity in the course of treatment with immunopotentiators along with interferon and activation of phagocytic macrophages (*16*, *17*).

Lysozyme activity has previously been determined by bacteriolytic activity, or most frequently by turbidometry with a spectrophotometer, or by the area of the lytic zone around a cup or disk containing a lysozyme specimen on an agar plate-gel with the suspended bacteria, *M. lysodeikticus*, embedded in it. Another method, with low sensitivity and used less frequently, is based on the measurement of the viscosity of a solution containing glycolchitin substrate. A more recent development is the use of radioimmunoassay or enzyme immunoassay. A fluorometric assay utilizing a synthetic 4-methylumbelliferyl derivative of *N*-acetylglucosamine oligomer has been done (*18*) and it had a considerable advantage for the kinetic analysis although the method requires

more than 20 μg/ml of lysozyme (*19*). Very recently, a semiautomatic kinetic method has been described which is based on the classic turbidometry although with considerable rapidity (*20*). All of these methods are either time consuming or less sensitive or employ a relatively insensitive substrate. For example, the diffusion assay on an agar plate takes more than a day, and it requires at least 1 μg/ml to be reliable. When compared with these methods, the present method is advantageous for its simplicity, sensitivity and rapidity. That is, this assay is comparable in sensitivity to the radioisotope assay but much simpler to carry out. For instance, one only need to add an aliquot of sample containing lysozyme to a cuvette (in which there is 2 ml of labeled peptidoglycan substrate) and the instrument will print out the *P* values or the fluorescence intensity. No separation procedure involving centrifugation or filtration, or prolonged incubation is needed. The sensitivity is sufficiently high to determine lysozyme activity in a single drop of a urine specimen from a healthy man or a patient with kidney disease within 5 min (data not shown).

P values have been shown to reflect the changes of molecular size, provided that there is no change in spectral properties, as has previously been shown with proteases (*7–9*). Therefore, the hydrolytic degradation process of peptidoglycan by lysozyme is accompanied by a decrease in the *P* value. As a consequence, a similar result to the protease assay was obtained in the assay of lysozyme, as shown in Figs. 3, 5, and 6.

Based on the decreased *P* value for the reaction product, the two enzymological parameters K_m and V_{max} were obtained from the initial rate of reaction (less than 10 min) (Fig. 7). The results indicated a higher activity and lower K_m value for human lysozyme than that of chicken egg-white when *M. lysodeikticus* peptidoglycan was used as the substrate. A similar finding, 2–3 times higher activity of the former, has been reported previously although it was not based on the initial velocity (*21*).

In addition to the *P* value, the increase in the fluorescence intensity during hydrolysis of fluorochrome (fluorescamine) labeled proteins by proteases was also reported as a reliable quantitative measurement with high sensitivity (*22*). This phenomenon of an increase of fluorescence intensity was observed also in the hydrolysis of peptidoglycan by lysozyme (Fig. 4). The increased fluorescence intensity may be brought about by the decreased energy transfer (less quenching) as a result of degradation of the polymer which contains a number of fluorochromes perhaps fixed in a small area. In addition, the increased efficiency of excitation due to increased Brownian motion of the fluorochrome will facilitate fluorescence. Conversely, a decrease in fluorescence intensity is found with increasing molecular weight (decreased Brownian motion), as in antigen-antibody complex formation (*2, 23*).

Since the concentration of the labeled peptidoglycan used for the present assay is very low (about 5 μg/ml) it exhibits very little absorption at 490 or 520 nm ($A<0.026$). Therefore, the interference by light scattering of the suspended substrate is negligible. Furthermore, the three cavity filters (Ditric Optics Inc.) used for the fluorescence measurement (at 520 nm) exhibited excellent performance eliminating unwanted incident light or light scattering at an excitation wavelength of 490 nm. For instance, the 520 nm filter permits the passage of less than 1% of the light at 518 nm while 50% of the light at 520 nm is transmitted, and there will be virtually no transmission of light at 490 nm.

The muramyl peptide has the following sequence: *N*-acetylmuramic acid-L-Ala-D-Glu(α-Gly)-L-Lys-D-Ala in which terminal D-Ala is occasionally crosslinked to ε-NH_2 of L-Lys on another muramyl peptide. It is known, however, that about 60 to 80% of ε-NH_2 of lysine is free. Therefore, the fluorescein chromophore appears to be attached to ε-NH_2 of L-Lys in this case (*13, 24*). Since the peptidoglycan is a relatively homogeneous polymer compared with proteins and the spectral alterations (such as a shift in the absorption or emission spectra) resulting from the hydrolytic reaction with lysozyme are very small and undetectable (data not shown), the present results appear to reflect the intrinsic change associate only with the change in molecular weight due to degradation by lysozyme.

We have observed that minute contamination by saliva or a tear (less than 10 μl) with the present assay conditions exhibited a extremely high lysozyme activity, therefore, one must be aware of such possible contamination during measurement.

It was observed that repeated freezing and thawing or a long period of storage at 4°C in PBS of the labeled peptidoglycan yielded gradually lower *P* values. It is not clear, however, whether this is caused by contaminating hydrolytic enzymes which liberate free chromophore or by gradual decomposition.

The results described show that the fluorescence polarization method can be applied to the hydrolytic process of a polymer of *N*-acetylmuramic acid-*N*-acetylglucosamine in addition to proteins as previously reported. This methodology can be also utilized for enzymological studies. The hydrolysis of other biopolymers such as DNA, RNA, and amylose may also be determined utilizing similar fluorescence assay.

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