

Colorimetric Assay for Lysozyme Using *Micrococcus luteus* Labeled with a Blue Dye, Remazol Brilliant Blue R, as a Substrate

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Micrococcus luteus (*M. lysodeikticus*) labeled with Remazol brilliant blue R (blue ML) was prepared as a novel substrate for the colorimetric assay of lysozyme. The treatment of the labeled substrate with lysozyme resulted in the release of soluble blue products which can be easily measured spectrophotometrically at 600 nm. The blue color was most efficiently released at pH 7 and ionic strength of 0.2 on incubation with hen lysozyme at 40 °C. A new colorimetric method for the assay of lysozyme using this substrate was developed. The assay system gave a linear dose-response curve, and as little as 0.1 µg of human lysozyme (1 µg/ml, 100 µl) can be detected. The present method is more convenient and reproducible than the conventional lysozyme assay with bacterial cells. Application of the system to the determination of lysozyme in human serum is described.

Keywords lysozyme; colorimetric assay; human serum; *Micrococcus luteus*; Remazol brilliant blue R

Human lysozyme, which belongs to c-lysozyme, is widely distributed in many tissues and secretions.¹⁾ One of the biological functions of lysozyme is believed to be self-defense against infection by bacteria. It is known that any deviation of the lysozyme concentration from the normal level in serum or urine is related to diseases. For example, a high level of lysozyme was observed in the urine of patients with monocytic leukemia.²⁾ In a malignant cell of the human carcinoma cell line, a high level of lysozyme is also secreted.³⁾ These observations indicate that the measurement of the concentration of lysozyme in serum or urine is useful for the diagnosis or screening of diseases.⁴⁻⁶⁾

Clinically, lysozyme activity is most frequently determined by a bacteriolytic activity using *M. luteus* (*M. lysodeikticus*, ML) as a substrate; that is, by turbidimetry with a spectrophotometer (turbidimetric assay),⁷⁾ or by the area of the lytic zone on an agar plate-gel with the suspended bacterial cells embedded in it (lysoplate assay).⁸⁾ The turbidimetric assay has a high sensitivity,⁹⁾ but great care is needed because it is remarkably influenced by various environmental factors such as ionic strength, sucrose, detergent and so on.^{10,11)} Furthermore, this method is laborious and not suitable for the screening of many samples. Although the lysoplate assay is suitable for screening many samples, it is less quantitative and requires a long incubation time (18 h), and special apparatus and techniques.¹²⁾ In order to overcome these problems caused by using ML as a substrate, we modified *M. luteus* cells with a reactive blue dye, Remazol brilliant blue R (RBB-R), as a new substrate, and developed a colorimetric method for the measurement of lysozyme using this labeled substrate (blue ML). The method is simple, sensitive and accurate enough for screening many samples.

Experimental

Materials *M. luteus* (*M. lysodeikticus*, ML) and bovine serum albumin were obtained from Sigma. RBB-R, a reactive blue dye, was the product of Aldrich. Human and hen lysozymes were from Green Cross (Osaka, Japan) and Seikagaku Kogyo (Tokyo, Japan), respectively. Glycol chitin, a soluble substrate of lysozyme, was prepared as described elsewhere.¹³⁾ Other chemicals used were of reagent grade materials obtained from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan).

RBB-R-Labeled *M. luteus* (Blue ML) Labeling of ML with RBB-R was carried out in a manner similar to the method of synthesis of

RBB-R-labeled starch, a substrate of α -amylase.¹⁴⁾ Briefly, a solution of 200 mg of RBB-R in 20 ml of distilled water was added to a suspension of 300 mg of ML cells in 20 ml of distilled water at 50 °C with stirring. During the following 30 min, 4 g of sodium sulfate was added to the mixture in several portions. A solution of 200 mg of trisodium phosphate in 2 ml of distilled water was then added, and the mixture was stirred for an additional 30 min at 50 °C. The reaction mixture was centrifuged (2600 rpm, 10 min), and the supernatant was discarded. The pellet of the labeled cells was suspended in 20 ml of 0.05 M KH_2PO_4 -NaOH buffer (0.05 M phosphate buffer, pH 7.0) with a vortex mixer, centrifuged, and the supernatant was discarded. In the same manner, the cells were washed with the same buffer repeatedly until the supernatant became colorless. Finally, the cells were washed with distilled water and then dried by lyophilization to give about 200 mg of RBB-R-labeled ML (blue ML), which was stored in a refrigerator until use.

Human Serum About 5 ml of blood was drawn from each of 10 healthy adult men (22–27 years old), colleagues in our laboratory, and kept in test tubes at room temperature for about 5 h for coagulation. The serum was separated by centrifugation, and used immediately for the assay of lysozyme.

Absorbance Measurements A Hitachi 150-20 spectrophotometer equipped with a water circulated cell holder was used. For the assay of lysozyme using a suspension of blue ML as a substrate, the absorbance was measured in a 1 ml black quartz cell (0.3 × 1.0 × 4.5 cm). In other cases, the absorbance (or turbidity) was measured in a conventional quartz cell (1.0 × 1.0 × 4.5 cm).

Lysis of Blue ML by Lysozyme Dry blue ML (16 mg) was suspended in 10 ml of an appropriate buffer and centrifuged once. The pellet was resuspended in 10 ml of the same buffer, mixed well with a vortex mixer, and divided into 700 µl portions in 1.5 ml-micro test tubes (Eppendorf). After preincubation at 40 °C, 100 µl of sample containing lysozyme was added to each tube, and the mixture was briefly mixed with a vortex mixer, then incubated at 40 °C. The reaction was stopped by the addition of 50 µl of 1 N NaOH, which raised the pH to about 10.5. The mixture was then centrifuged (15000 rpm, 5 min), and the absorbance of the supernatant at 600 nm, the wavelength close to the absorbance peak (592 nm) of RBB-R, was measured.

Lytic Activity The lytic activities of human and hen lysozymes using ML as a substrate were determined turbidimetrically at 450 nm, pH 7.0 and 30 °C. To a suspension of ML (initial turbidity at 450 nm being about 1.2 absorbance units) in 2 ml of 0.05 M phosphate buffer, pH 7.0, was added 100 µl of a solution of lysozyme (about 10 µg/ml) in the same buffer. The initial rate of the turbidity decrease was monitored at 450 nm. The activity of lysozyme determined by the initial rate was expressed as the percentage of that of hen lysozyme.

Activity against Glycol Chitin The activity of lysozyme against glycol chitin was determined in 0.1 M sodium acetate buffer at pH 5.5 and 40 °C according to the method described elsewhere.¹³⁾

Turbidimetric Assay of Lysozyme in Human Serum The method was the same as that for determination of the lytic activity of lysozyme, except that freshly obtained human serum (100 µl) was used. The concentration of lysozyme in serum was determined from the standard curve obtained

with known concentrations of human lysozyme solutions.

Results

Characteristics of Lysozyme Action on Blue ML Upon incubation of blue ML with the lysozyme sample, the absorbance of the supernatant at 600 nm increased linearly to about 2.5, then leveled off with an increase in either the concentration of lysozyme or the incubation time (data not shown). These results indicate that lysozyme caused the lysis of blue ML and released soluble blue products from, probably, the cell wall labeled with RBB-R.

In view of the application of this reaction to the assay of lysozyme, the following parameters were examined.

Incubation Time Preferable absorbance range of 0.05—1.5 was obtained by incubation for 30 min or 4 h with a sample of 1—70 $\mu\text{g/ml}$ or 0.2—8 $\mu\text{g/ml}$, respectively, of hen lysozyme.

Preparation of the Suspension of Blue ML In order to obtain uniform suspension of blue ML reproducibly, the addition of 0.5 M sucrose in the buffer proved to be helpful. However, it was reported that lysozyme activity was repressed by sucrose in the turbidimetric assay using ML.¹¹⁾ Therefore, we examined the effect of sucrose on the lysozyme activity against blue ML. As shown in Fig. 1, lysozyme activity was not affected by the addition of 0.5 M sucrose in our assay system.

Mixing Mixing or shaking of the reaction mixture during the incubation did not appreciably affect the release of the blue color.

Effect of Other Protein As a high concentration of serum albumin was contained in serum (3.5—5.5% in human serum), the influence of bovine serum albumin on activity was examined. However, the release of the blue color was not influenced by 0—7% of bovine serum albumin in a sample (data not shown).

Effects of Ionic Strength The effect of ionic strength was examined at pH 7.0 and 40 °C. As shown in Fig. 2, the blue color was most efficiently released at an ionic strength of around 0.2. On the other hand, the lytic activity of lysozyme by the turbidimetric assay showed a more remarkable ionic strength dependence, with an optimum at a low ionic strength of 0.07.

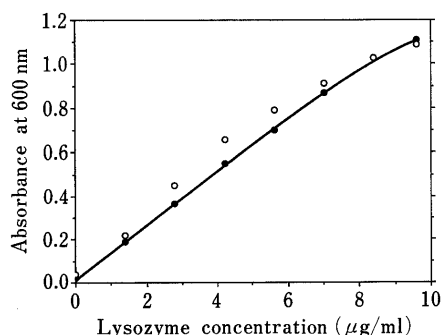


Fig. 1. Dose Dependence of the Release of Blue Color from Blue ML Catalyzed by Hen Lysozyme in the Presence (●) or Absence (○) of 0.5 M Sucrose at pH 7, Ionic Strength of 0.2 and 40 °C

To a preincubated suspension of blue ML (1 ml, 1.6 mg/ml) in 0.05 M phosphate buffer with or without 0.5 M sucrose, ionic strength of 0.2 and pH 7.0, was added 1 ml of the solution of hen lysozyme (0—10 $\mu\text{g/ml}$) in the same buffer, and the mixture was incubated at 40 °C for 30 min. After the reaction was terminated by adding 50 μl of 1 N NaOH and the sample was centrifuged, the absorbance of the supernatant at 600 nm was measured.

Effect of pH The pH-dependence of the activity of hen lysozyme against blue ML was investigated at 40 °C and the ionic strength of 0.2. As shown in Fig. 3, the optimum pH was about 7, and the activity decreased precipitously on either side of that pH.

Standard Conditions for the Assay of Lysozyme Based on the above initial experiments, we developed a new colorimetric method for the assay of lysozyme by use of blue ML as a substrate. The standard conditions employed here were as follows.

The buffer used for the suspension of blue ML was 0.05 M phosphate buffer (pH 7.0) containing 0.5 M sucrose with the ionic strength of 0.2 which was adjusted by NaCl. Lyophilized blue ML was washed with the buffer once and resuspended in the same buffer at a concentration of 1.6 mg/ml. The suspension was divided into 700 μl portions in micro test tubes. After preincubation at 40 °C for 10 min, 100 μl of a sample containing lysozyme was added. The mixture was then mixed briefly and incubated at 40 °C for an appropriate period, depending on the concentration of lysozyme in the sample. After stopping the reaction by

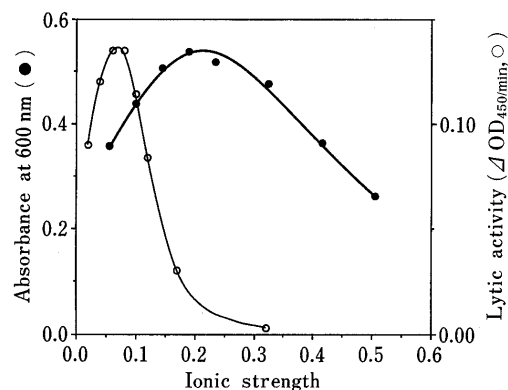


Fig. 2. Ionic Strength Dependence of the Release of the Blue Color from Blue ML and of Lytic Activity against ML, Both Catalyzed by Hen Lysozyme

To a preincubated suspension of blue ML (500 μl , 1.6 mg/ml) in 0.03 M phosphate buffer containing 0.5 M sucrose and an appropriate concentration of NaCl, pH 7.0, was added 500 μl of the solution of hen lysozyme (4.5 $\mu\text{g/ml}$) in the same buffer, and the mixture was incubated at 40 °C for 30 min. After centrifugation, the absorbance of the supernatant at 600 nm was measured. Lytic activity was measured at 30 °C and pH 7.0 in 0.01 M phosphate buffer containing various concentrations of NaCl. 50 μl of lysozyme solution (40 $\mu\text{g/ml}$) was added to 2 ml of the suspension of ML (0.25 mg/ml), quickly mixed, and the absorbance at 450 nm was monitored. The ionic strength of each buffer was adjusted to a given value by NaCl.

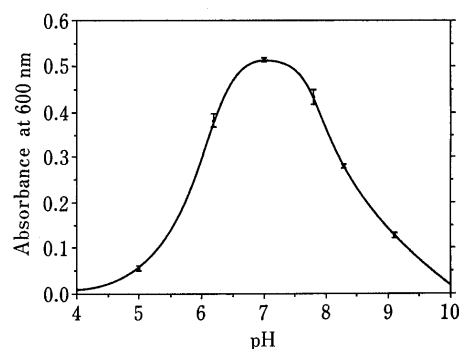


Fig. 3. pH-Dependence of the Release of the Blue Color from Blue ML at an Ionic Strength of 0.2 and 40 °C

The buffers used were 0.02 M acetate buffer (pH 4—5), 0.015 M phosphate buffer (pH 6.2—7.8) and 0.02 M Tris buffer (pH 8.2—10). The ionic strength was adjusted to 0.2 by NaCl. Other conditions are the same as described in Fig. 2.

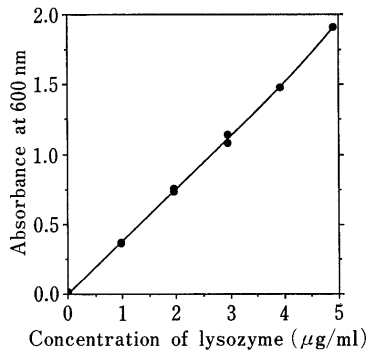


Fig. 4. Standard Curve for the Assay of Human Lysozyme at pH 7.0, Ionic Strength of 0.2 and 40 °C

TABLE I. Levels of Lysozyme in Human Sera from 10 Adults

Substrate	Concentration of lysozyme (µg/ml)									
	A	B	C	D	E	F	G	H	I	J
Blue ML	1.5	1.5	1.2	1.4	1.4	0.9	1.5	0.7	1.1	1.4
ML	1.3	1.3	1.1	1.2	1.3	0.8	1.4	0.8	1.0	1.3

TABLE II. Activities of Hen and Human Lysozymes against Blue ML, ML and Glycol Chitin (GC)^{a)}

Lysozyme	Activity (%) against		
	Blue ML	ML	GC
Hen	100	100	100
Human	250	310	103

a) Details are given in the text.

adding 50 µl of 1 N NaOH, the mixture was centrifuged and the absorbance of the supernatant at 600 nm was measured. The concentration of lysozyme in the sample was determined from the standard curve prepared by authentic lysozyme solutions.

Assay of Lysozyme in Human Serum The new method developed here was applied for the determination of the concentration of lysozyme in human serum. Since the concentration of lysozyme in human serum was reported to be in a µg/ml-range,¹⁾ a 4 h-incubation was employed. In Fig. 4, a standard curve prepared by authentic human lysozyme is shown. A linear correlation between the release of the blue color and the concentration of human lysozyme was observed. Using this standard curve, the concentrations of lysozyme in sera obtained from 10 healthy adult men were determined. The results are shown in Table I. For comparison, the results obtained from turbidimetric assay are also indicated in Table I. Both results were in good agreement with each other, and the concentrations of lysozyme in the sera employed here were found to be in the range of 0.7 to 1.5 µg/ml.

Comparison of the Activities of Hen and Human Lysozymes against Blue ML, ML and Glycol Chitin The activities of hen and human lysozymes against blue ML at pH 7.0, ionic strength of 0.2 and 40 °C were determined and compared with their lytic activities at pH 7.0, ionic strength of 0.1 and 30 °C as well as with glycol chitin activities at pH 5.5

and 40 °C. The results are summarized in Table II. Human lysozyme showed about three times higher lytic activity than hen lysozyme against ML, but only similar activity against glycol chitin, as had already been reported.¹⁵⁾ The activity of human lysozyme against blue ML was 250% that of hen lysozyme. Thus, human lysozyme clearly possesses higher activity against blue ML as well as ML than hen lysozyme, which indicates that blue ML is a substantially similar substrate to ML as compared to glycol chitin.

Discussion

Recently, a colorimetric assay of lysozyme using *p*-nitrophenyl penta-*N*-acetyl-β-chitopentanoside as a substrate has been reported.¹⁶⁾ In this method, the yellow color of *p*-nitrophenolate anion produced is monitored at 405 nm. Fluorometric assays of lysozyme using the bacterial cell wall or its fragments labeled with fluorescamine^{17,18)} or with fluorescein isothiocyanate,¹⁹⁾ or using a synthetic 4-methylumbelliferyl derivative of *N*-acetylglucosamine oligomer²⁰⁾ have also been developed recently. However, body fluids such as serum and urine are generally yellow and contain many fluorescent materials. Therefore, the method to measure yellow color or fluorescence may not be suitable for the purpose of the assay of lysozyme in body fluids.

But, body fluids are rarely blue. Thus, a method to detect blue color may be the base for the colorimetric assay of lysozyme in body fluids.

A reactive blue dye, RBB-R, has an absorption maximum at around 600 nm and its spectrum barely changes in a pH range from 2 to 11. Therefore, we modified *M. luteus* cells with RBB-R to prepare a new substrate of lysozyme which releases the blue color. Since RBB-R can modify the hydroxyl groups of sugars (mainly 6-hydroxyl group of hexose),^{14,21)} the sugar moieties of the peptidoglycan of the cell wall are supposed to be labeled with RBB-R in this modification. Thus, the soluble blue products released from the labeled cells (blue ML) by lysozyme are presumed to be due to RBB-R-labeled small fragments of the peptidoglycan resulting from the hydrolysis of the cell wall of blue ML.

By use of blue ML as a substrate, the new colorimetric method detecting the blue color (600 nm) for the determination of lysozyme was developed here. The method was simple and sufficiently sensitive to detect 1 µg/ml of human lysozyme by using 100 µl of a sample over a 4 h incubation period. Use of more sample and/or prolonged incubation may permit detection of lysozyme at less than 0.1 µg/ml. Although the method requires a relatively long incubation period when the concentration of lysozyme in a sample is as low as 1 µg/ml, it may not be inconvenient, because many samples can be treated at the same time.

Since the release of blue color can be directly detected by the eye, we examined a much simpler method for the screening of lysozyme in human sera. That is, 1 ml of human sera and a series of authentic human lysozyme solutions (1 to 2 µg/ml) were mixed with 1 ml of the suspension of blue ML (1.6 mg/ml) in 0.1 M phosphate buffer, pH 7.0, in glass test tubes, and the mixtures were kept at room temperature overnight. Under these conditions, the cells precipitated completely to leave clear supernatants. By comparing the blue color of the supernatants using the naked eye, the concentrations of lysozyme in human sera could be easily

estimated to be about 1 $\mu\text{g/ml}$ (ranging from 0.5 to 1.5 $\mu\text{g/ml}$ depending on the sample), which was in good agreement with the values indicated in Table I. Thus, we concluded that the eye-test is sufficient for the first screening of lysozyme in human serum.

The activity of lysozyme against ML is markedly affected by various environmental factors such as ionic strength¹⁰⁾ and sucrose.¹¹⁾ However, the activity of lysozyme against blue ML was relatively insensitive to the ionic strength (Fig. 2), and was not appreciably affected by sucrose (Fig. 1). The effect of sucrose on the lysis of ML by lysozyme is not the inhibition of lysozyme activity. In fact, lysozyme can hydrolyze cell wall components in the presence of sucrose to release cell wall fragments. Only the burst of the resulting naked protoplast due to osmotic pressure is prevented by sucrose.¹¹⁾ This may explain the insensitivity of lysozyme activity against blue ML to sucrose. The decreased sensitivity of the present method to environmental factors would be favorable to the assays of lysozyme in clinical samples.

As for the pH-activity profile, blue ML resembles ML with an optimum near pH 7 (Fig. 3), but not glycol chitin, which has an optimum near pH 5.^{22,23)} Furthermore, as for human lysozyme, blue ML resembles ML rather than glycol chitin because both blue ML and ML were effectively hydrolyzed by human lysozyme as compared with hen lysozyme (Table II). These results suggest that blue ML is a substrate similar to ML rather than to glycol chitin. Although there are some differences in the ionic strength-dependence and the activity of human lysozyme against blue ML and ML, these differences may be responsible for the dye introduced to the cell wall of ML.

In conclusion, blue ML prepared here is a good substrate of lysozyme, and the colorimetric assay system using it would be very useful for clinical application because it is simple, accurate and suitable for screening many samples at the

same time.

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