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Endotoxin assay by bioluminescence using mutant firefly luciferase

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ABSTRACT

The Limulus reaction is an application of the defense mechanism of horseshoe crab for endotoxin detection. Endotoxin is a component of the cell wall in the outer membrane of gram-negative bacteria, and causes fever or shock when it enters the human blood stream. For endotoxin detection, gel formation or turbidity of the coagulation factor chromogen or fluorescence-modified peptide is used. However, these conventional methods have problems with regard to their measurement time or sensitivity. We recently obtained a mutant firefly luciferase that has a luminescence intensity over 10-fold higher than that of the wild type. Therefore, we developed a new endotoxin detection method that combines the Limulus reaction and bioluminescence using mutant luciferase. The new method detects 0.0005 EU/ml of endotoxin within 15 min.

Endotoxin (lipopolysaccharide) is a component of the cell wall in the outer membrane of gram-negative bacteria and can be fatal to humans when it enters the bloodstream. Limulus amebocyte lysate (LAL)[1] is a coagulation system that is induced by endotoxin [1–3]. There are several endotoxin detection methods employing the so-called Limulus reaction using LAL. Methods using turbidimetric and chromogenic end-points as well as turbidimetric and chromogenic kinetic methods are commonly used for endotoxin detection [4–7]. However, the two end-point methods have problems with their sensitivities (detection limit is about 0.01–0.1 EU/ml) and the kinetic methods have a problem with the measurement time (over 60 min) [8].

Bioluminescence detection has a high signal-to-noise ratio compared with other optical detection methods, such as fluorescence and chromogenic detection methods. We previously constructed a genetically modified North American firefly (*Photinus pyralis*) luciferase that generates a luminescence intensity at least 10-fold greater than that the wild-type luciferase [9]. Using this mutant luciferase, it was possible to detect a single *Escherichia coli* cell [10]. In this study, we report that a bioluminescence test using mutant firefly luciferase combined with the Limulus reaction provides rapid and highly sensitive endotoxin detection (Fig. 1).

Materials and methods

Preparation of endotoxin and LAL reagent

A lipopolysaccharide prepared from *E. coli* UKT-B (a product of Wako Pure Chemical Industries, Tokyo, Japan) was used as a standard endotoxin. A standard endotoxin solution was prepared using autoclaved distilled water (121 °C, 90 min) and diluted for calibration. Limulus ES Single Test Wako (Wako Pure Chemical Industries) was used as the LAL reagent. All other materials were of the highest purity commercially available. All experimental instruments and solvents were autoclaved at 121 °C for 90 min.

End-point chromogenic method

tert-Butoxycarbonyl-Leu-Gly-Arg-*p*-nitroanilide (peptidyl-pNA) was used as a chromogenic substrate (Seikagaku Biobusiness, Tokyo, Japan). The endotoxin end-point chromogenic assay was performed with LAL (QCL-1000; Lonza Group Ltd., Basel, Switzerland) according to the manufacturer's instructions. The reaction mixture containing 50 μl each of endotoxin and LAL was incubated at 37 °C for 10 min. Then 100 μl of peptidyl-pNA solution was added and the mixture was incubated at 37 °C. After incubation for 5 min, 100 μl of 25% v/v acetic acid was added to terminate the reaction and the liberated pNA was measured at 405 nm using a DU 800 UV/visible spectrophotometer (Beckman Coulter Inc., Fullerton, CA).

Preparation of luciferase FM

We obtained a mutant North American firefly (*P. pyralis*) luciferase that generates luminescence intensity more than 10-fold high-

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[1] *Abbreviations used*: LAL, Limulus amebocyte lysate; peptidyl-Luc, benzoyl-Leu-Gly-Arg-aminoluciferin; peptidyl-pNA, *tert*-butoxycarbonyl-Leu-Gly-Arg-*p*-nitroanilide.

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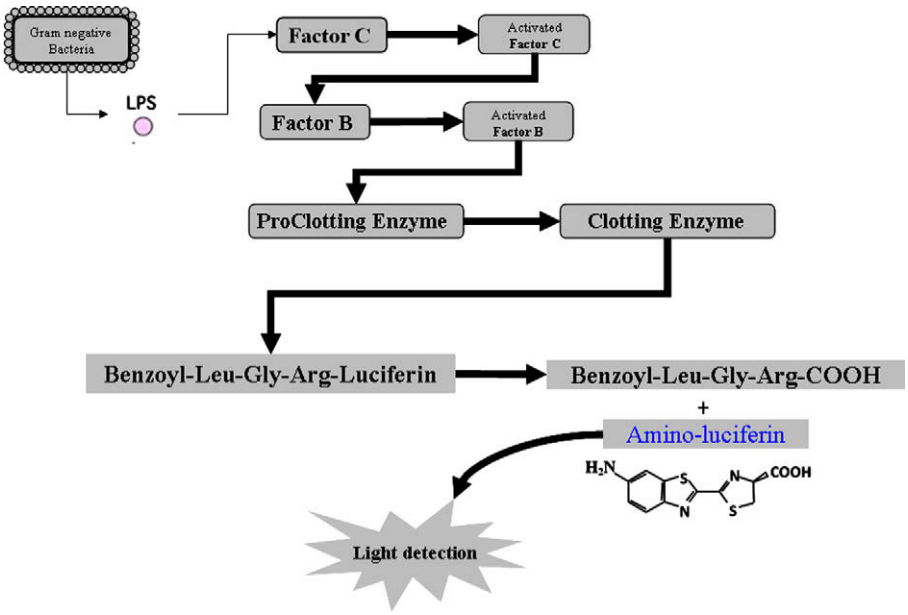

Fig. 1. The principle of detection of endotoxin by bioluminescence.

er than that of wild-type luciferase [9]. This mutant luciferase named luciferase FM was used for endotoxin detection in this study. The enzyme was dissolved in TMAT buffer (50 mM Tris–HCl buffer, pH 8.0, containing 1 mM magnesium acetate and 100 g/l trehalose) for the LAL assay.

Bioluminescence method

Luciferin-modified peptide benzoyl-Leu-Gly-Arg-aminoluciferin (peptidyl-Luc) was designed with reference to the cleavage point in coagulogen and was synthesized at our request by ABD Bioquest Inc. (Sunnyvale, CA). It was used as the substrate for endotoxin detection by luminescence [11]. Peptidyl-Luc was dissolved in dimethyl sulfoxide and diluted with TMAT buffer, and its volume adjusted to obtain a concentration of 75 μM. A reaction mixture containing 50 μl each of endotoxin and LAL was incubated at 37 °C for 10 min. Then, 50 μl peptidyl-Luc was added and the mixture was incubated at 37 °C. After incubation for 5 min, 50 μl each of 10 μM ATP and 250 μg/ml luciferase FM were added and mixed immediately, and luminescence intensity was measured using a Lumitester C-110 (Kikkoman Corporation, Chiba, Japan).

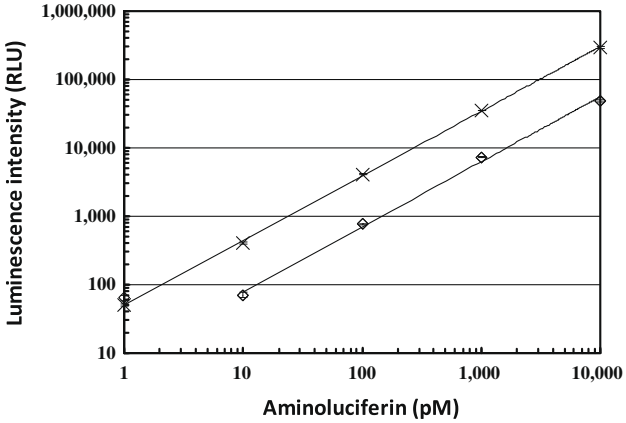

Fig. 2. Comparison of the luminescence intensities of luciferase FM and wild-type luciferase with D-aminoluciferin concentration. First, 50 μl of D-aminoluciferin at 0.05 pM to 50 nM is mixed with 100 μl TMAT buffer. Then, 50 μl of 10 μM ATP and 50 μl of 250 μg/ml luciferase FM or wild-type luciferase are added and mixed immediately, and luminescence intensity is measured using a Lumitester C-110. Symbols: ×, luciferase FM; Δ, wild-type luciferase. The data are shown as averages ± standard deviations ($n = 5$).

Results

Comparison of luciferase FM with wild-type luciferase

The D-aminoluciferin detection test results are shown in Fig. 2. The symbols represent the mean of five replicates for each measurement. Wild-type luciferase was unable to detect less than 10 pM D-aminoluciferin. However, luciferase FM detected D-aminoluciferin at 1 pM, and a linear correlation between luminescence and D-aminoluciferin concentration was observed. The relative luminescence intensity of luciferase FM at 1 pM D-aminoluciferin was 41 ± 4 relative luminescence units (RLU). Luciferase FM thus showed better performance in detecting D-aminoluciferin for endotoxin detection.

Optimization of the concentration of LAL in the luciferase FM-bioluminescence assay system

When we measured the luminescence intensity of luciferase FM to D-aminoluciferin on adding the amount of LAL specified by the manufacturer, we found that the presence of LAL inhibited the bioluminescence reaction. Under this condition, the luminescence intensity in the presence of LAL was decreased to 1/10 of the level measured in its absence (Fig. 3). Moreover, decrease of the amount of LAL in the luciferase FM-bioluminescence assay was required to achieve high sensitivity for detecting endotoxin. Therefore, we sought to optimize the amount of LAL for endotoxin detection by bioluminescence. Reaction mixtures containing 50 μl of 0.05 EU/ml endotoxin and various concentrations of LAL (0–0.48 mg/ml) were incubated at 37 °C for 10 min. Then, 50 μl of peptidyl-Luc substrate was added and the mixture was incubated at 37 °C. After incubation for 5 min, 50 μl each of 10 μM ATP and 250 μg/ml luciferase FM were added and mixed immediately, and luminescence intensity was measured. Fig. 4 shows the relationship between luminescence intensity and amount of LAL. Luminescence intensity at 0.2 mg/ml LAL was almost twice as high as that at 0.48 mg/ml LAL. The optimum concentration of LAL for endotoxin detection