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The use of lysozyme modified with fluorescein for the detection of Gram-positive bacteria

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ABSTRACT

Lysozyme (1,4-β-N-acetylmuramidase) is commonly applied in the food, medical, and pharmaceutical industries. In this study, we tested a novel application of fluorescein-modified lysozyme (using carboxyfluorescein with a triazine-based coupling reagent) as a new tool for the detection of Gram-positive soil bacteria. The results, obtained by cultivation methods, fluorescence analysis, and laser interferometry, showed that, after optimization, fluorescein-modified lysozyme could be used to evaluate the prevalence of Gram-positive bacteria essential in bioremediation of soils with low pH, such as those degraded by sulfur.

1. Introduction

Lysozyme, also known as 1,4-β-N-acetylmuramidase, is a bacteriolytic enzyme that hydrolyzes β-1,4-glycosidic linkages between N-acetylglucosamine and N-acetylmuramic acid in peptidoglycan (murein), which is a major component of bacterial cell walls (Xue et al. 2004). Another bacteriolytic mechanism exhibited by lysozyme consists of its ability to bind to nucleic acids in microorganisms, causing bacterial genetic material to mutate or disintegrate (Steinrauf et al. 1999). Due to its cationic properties, lysozyme has been found to bind to lipopolysaccharides (LPSs), which decreases its enzymatic activity and immunostimulatory activity. Lysozyme/LPS complexes inhibit the production of TNFα, thus lowering the risk of developing a strong inflammatory reaction caused by the presence of large amounts of endotoxins in the body (Ohno and Morrison, 1989). The antimicrobial action of lysozyme is associated with specific hydrolysis of 1,4 β-D linkages between N-acetylhexosamines in the peptidoglycan layer of the bacterial cell wall (Proctor and Cunningham 1988). Peptidoglycan is an essential and unique cross-linked cell-wall heteropolymer in bacteria, which makes Gram-positive bacteria sensitive to lysozyme action, while Gram-negative bacteria are rather resistant. This marginal activity of lysozyme against Gram-negative bacteria is related to the presence of LPSs (inhibiting its enzymatic activity) in their outer membrane (Hughey and Johnson 1987; Ohno and Morrison 1989; Visalsok et al. 2004). Due to its properties, lysozyme is commonly applied in the food, medical, and pharmaceutical industries (Liu et al. 2006; Schneider et al. 2010; Lasanta et al. 2010; Ishiguro et al. 2002; Haas et al. 2002).

In this study we present the lysozyme modification with fluorescein and its new application. The modification of lysozyme proceeded in an aqueous solution according to a procedure described elsewhere (Zając and Kamiński 2010, 2013) using a water-soluble coupling reagent, CMpSFAT/NMM/BET × $5H_2O$ (Fig. 1S; supplementary data). We observed that *Escherichia coli* cells did not interact with fluorescein-modified lysozyme by our method, in contrast to *Staphylococcus aureus* cells. Therefore, we decided to follow this observation and use lysozyme-FITC as a tool for the detection of Gram-positive bacteria in soils. The detailed objectives of this study were: (1) to find out if lysozyme-FITC might be used to detection of Gram-positive and Gram-negative bacteria, and (2) to optimize the method of soil bacteria detection by lysozyme-FITC.

2. Materials and methods

2.1. Materials

Escherichia coli ATCC 8739 and *S. aureus* ATCC 6538P were used as model Gram-negative and Gram-positive bacteria. Nuclepore membranes for laser interferometry with a pore diameter of 0.2 μm

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were purchased from the Joint Institute for Nuclear Research in Dubna, Russia.

The research material analyzed in this study consisted of soil samples collected from arable and non-arable (industrially degraded) regions in Poland. The former were collected from the Klonowskie Ridge, Holy Cross Mountains, located in central Poland (approximate location 50°53′N, 20°55′E) and the latter from a sulfur mine in the village of Grzybów, Poland (approximate location 50°32′03″N, 21°05′42″E). The sample of degraded soil was characterized by strong acidification (pH 2.41 in H_2O), a very low content of organic carbon (1.07%), and a high content of sulfur (0.55%). The area from which the soil was collected had been subjected to liming, but that had not changed the pH of the soil. In that area, geo-mechanical transformation was noted, leading to special hydrological conditions (a high ground water level).

2.2. Modification of lysozyme with carboxyfluorescein using a water-soluble triazine-based coupling reagent (CMpSFAT/NMM/BET)

A solution of 5(6)-carboxyfluorescein (0.188 g; 0.5 mmol) in DMF (2 mL) was treated with pentahydrate of N-(2-(4-aminophenylsulfonate)-4-metoxy-1,3,5-triazyn-6-yl)-4-methyl-morpholinum (CMpSFAT/NMM/BET × $5H_2O$) (0.471 g; 1 mmol) and 4-methylmorpholine (NMM) (110 μl; 1 mmol), and gently stirred at 5 °C for 3 h. The resulting mixture was added dropwise to a solution of lysozyme (0.1 g, 6.94×10^{-6} mol) dissolved in 0.1 M sodium bicarbonate solution (5 mL). A precipitate was formed with a characteristic reddish brown color. After the entire mixture was added, the precipitate was separated from the solution using a centrifuge. The precipitate was suspended in water three times (3 mL × 5 mL), and then separated from the solution using a centrifuge again. After lyophilization, the crude product (110 mg) was obtained as a red-brown powder.

The crude product (5.5 mg) was dissolved in 0.1 M Na_2CO_3 solution, and then purified by gel filtration on a Sephadex G-25 column (14 mm × 900 mm) using 0.1 N Na_2CO_3 solution as the mobile phase. The yellow-orange-colored fraction with 300–420 min retention time was collected, lyophilized, and then desalted using an ion-permeable membrane yielding 1 mg of modified lysozyme. The activity of lysozyme-FITC was measured by *Micrococcus* test (Sigma–Aldrich, USA) in accordance with the test instruction.

2.3. Analysis of E. coli and S. aureus bacteria interactions with lysozyme-FITC

The use of lysozyme-FITC for the staining of *E. coli* and *S. aureus* cells were measured by fluorescence (1) and laser interferometry methods (2).

(1) Cell suspensions containing 10^5 bacterial cells (*E. coli* or *S. aureus*) were incubated with lysozyme-FITC at a concentration of 300 μg/mL at 4 °C for 24 h. After incubation, the suspensions were washed several times (centrifugation for 5 min at 1000 rpm) with phosphate buffered saline (PBS) and fluorescence was analyzed (λ_{ex} = 496 nm; λ_{em} = 521 nm) in supernatants after each washing and in sediments with a TECAN Infinite 200 PRO microplate reader (Tecan Group Ltd., Switzerland). Moreover, washed bacterial sediments (labeled by lysozyme-FITC) were stabilized in agarose gel for fluorescence microscopy analysis. The staphylococci can aggregate, especially in the presence of lysozyme-FITC. To make sure that we examined lysozyme-FITC labeled single cells, we used a protocol for bacterial cell stabilization in a gel structure. After vigorous shaking, bacterial cell sediments were mixed with 100 μl of 2% agarose of low melting temperature at 37 °C, and then placed on a slide pre-coated with a thin layer of 0.5% normal melting agarose. The bacterial cell sediments were immediately covered with a cover glass and the slides were kept at 4 °C for 5 min. to allow the agarose to solidify. The *E. coli* and *S. aureus* cells sediments were analyzed by fluorescence microscopy.

(2) A laser interferometry method for investigations of substance transport was presented previously (Arabski et al. 2007, 2009; Wąsik et al. 2013). Laser interferometry equipment consisted of a Mach-Zehnder interferometer, a membrane system, a TV-CCD camera, and a computer with a system for the acquisition and processing of interference images (Fig. 2S; supplementary data). The membrane system used in the present study was composed of two glass cuvettes separated by a horizontally placed nuclepore membrane with a pore diameter of 0.2 μm. The cuvettes were made of optical glass of high uniformity. In the presented experiments, the upper cuvette held pure water while the lower was filled with lysozyme-FITC at a concentration of 1 mg/mL in PBS following 24 h of incubation with 10^5 bacterial cells (*E. coli* or *S. aureus*). Laser light (Fig. 2) was spatially filtered and transformed by a beam expander into a parallel beam, approx. 80 mm wide, and then split into two beams. The first beam went through the investigated membrane system parallel to the membrane surface, while the second went directly through a compensation plate to the light detection system. As a consequence of the superimposition of these beams, interference images were generated. The images were influenced by the refraction coefficient of the solute, which in turn depended on the substance concentration. If the solute was uniform, the interference fringes were straight, but they bent if a concentration gradient appeared. In this system, water and lysozyme solution diffusing through the membrane led to the formation of concentration boundary layers (CBLs). The computer program used for analyzing these images calculated concentration profiles and CBL thickness. All experiments were performed at 37 °C.

2.4. Cultivation of soil bacteria

First, 1 g of a soil sample (from an arable or non-arable region) was suspended in a sterile Winogradsky salt solution (0.4 g K_2HPO_4, 0.13 g $MgSO_4 \times 7H_2O$, 0.13 g NaCl, 1.52 mg $MnSO_4 \times H_2O$, and 0.5 g NH_4NO_3), and intensively shaken in a magnetic stirrer for 15 min. After sedimentation of soil particles, the soil solution was diluted up to 10^{-6} and spread on the surface of the following solid microbiological media: Luria-Bertani agar (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, pH 4.5), tryptic soy agar (TSA; 1.45% enzymatic digest of casein, 0.5% enzymatic digest of soybean meal, 0.5% NaCl, 1.4% agar), minimal M9 agar (6.4% $Na_2HPO_4 \times 7H_2O$, 1.5% KH_2PO_4, 0.25% NaCl, 0.5% NH_4Cl, 0.024% $MgSO_4$, 0.4% glucose, 0.011% $CaCl_2$, 1.5% agar), King B agar (2% peptone, 0.15% dipotassium phosphate, 0.15% magnesium sulfate, 1% glycerol, 1% agar), soil extract medium (0.5% glucose, 0.5% peptone, 1% soil extract, in case of agar medium-1.5% agar) (each soil extract and its dilutions were plated on respective soil extract agar-SEA). Plates were incubated for up to 1 week at 25 °C, and then the number of aerobic bacteria was determined. Bacterial growth was observed only on LB agar and SEA. Moreover, few morphological types of colonies were noted on SEA. In the case of the soil from the sulfur mine, 3 different morphological types of colonies were found on SEA medium and 1 on LB agar. For arable soil, the corresponding figures were 13 types on SEA and 8 on LB agar.

Therefore, SEA was used as a medium to determine the kinetics of bacterial growth at 25 °C over 24–72 h by the pour plate method (CFU/mL). Additionally, the growth of Gram-positive bacteria isolated from both tested soil samples was examined