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In vitro antimicrobial assay using chemically reconstituted system with hemocyanin or hemoglobin

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Method Article

Keywords: in vitro antimicrobial assay, PPO, ROS

Posted Date: October 31st, 2007

DOI: https://doi.org/10.1038/nprot.2007.466

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Abstract

Introduction

Here we describe the chemically-reconstituted system for evaluating the antimicrobial activity of the microbial protease-mediated ROS production from the hemocyanin-prophenoloxidase \(HMC/PPO)or hemoglobin. The antimicrobial activity is examined by endpoint measurement of the remnant microbial population or by real-time observation of the bactericidal process. To demonstrate the specificity of PO-induced ROS-production which causes the antimicrobial activity, PTU is used here as a specific inhibitor of PO activity.

Procedure

1. Prepare the bacterial culture as described in protocol #4, and then adjust the bacterial population to 10^6 - 10^7 cfu/ml. 2. For the end-point measurement of HMC/PPO: \(a) incubate the bacteria under test at 10^6 cfu/ml with 60 µg purified HMC plus 100 nmol 4-methylcatechol \(4ME) in 100 µl PBS at 37 °C for 1 h. \(b) then enumerate the remnant bacterial population in the reaction mixture by plating 100 µl of serially diluted samples on nutrient agar plates and incubating at 37 °C overnight. Perform the colony count and calculate the bacterial cfu/ml. \(c) add 10 nmol of phenylthiourea \(PTU) to the reactions in order to evaluate the impact of PO activity. \(d) also set up controls by incubating bacteria with HMC/PPO or 4ME or PTU separately, or in combinations. 3. For real-time imaging of the bacterial clearance elicited by HMC/PPO: \(a) mix each bacterial strain at 10^7 cfu/ml with 60 µg HMC/PPO and 100 nmol 4ME in 100 µl PBS. \(b) apply one µl of the mixture to fluorescence microscopy and examine it at magnification of 63 x 1.6, with time-elapse method. \(c) capture images at intervals of 30 s for 1 h, and make a movie. 4. For the end-point measurement of metHb: \(a) incubate the bacteria under test at 10^6 cfu/ml with 36 µg metHb and 1.6 µmol H_2O_2 in 200 µl PBS at 37 °C for 1 h. \(b) then enumerate the remnant bacterial population in the reaction mixture by plating 100 µl of serially diluted samples on nutrient agar plates and incubating at 37 °C overnight. Perform the colony count and calculate the bacterial cfu/ml. \(c) to further prove that the antibacterial activity was indeed attributable to ROS, apply 2 µmol GSH to the incubation mixture to quench the superoxide ions. \(d) set up controls by incubating bacteria with metHb, H_2O_2 or GSH separately or in combinations.

Anticipated Results

In the presence of the HMC/PPO and its substrate, more of the protease-producing bacteria are cleared compared to those of the protease-inactive strains. This difference can be reduced when ROS quenchers \(eg: GSH for both quinone and superoxide anion or SOD for superoxide anion) are applied. When supplemented with exogenous proteases, the antimicrobial effect of the protease-inactive strains is increased dose-dependently. \(Refer to the example shown in figure 5 as a typical result obtained using HMC).

Figures

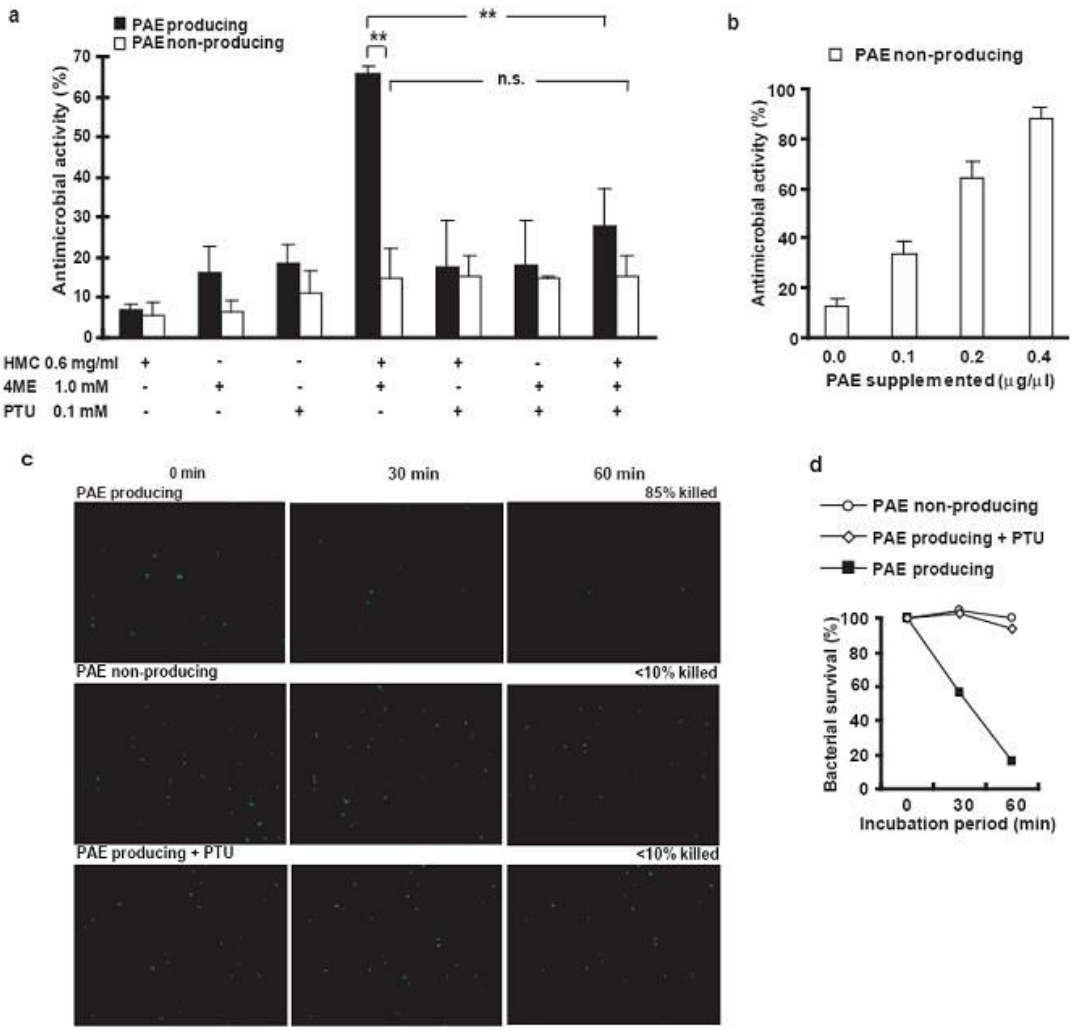

Figure 1

The _in vitro_ antimicrobial action of the bacterial component-activated PO: the end-point measurement and the real-time observation. (a) The end-point antimicrobial activity assay using PAE-producing and PAE non-producing strains of _Pseudomonas aeruginosa_. (b) Supplementation of exogenous PAE

increased the antimicrobial activity against the PAE non-producing strain. (c) The interception of the microscopy images at 0, 30, 30 min during the real-time observation; (d) the bacterial counts at each time-point of observation.