Research Square Preprint: Please note that this article has not completed peer review.

In vivo antimicrobial assay of the PPO system using horseshoe crab as the model animal

CURRENT STATUS: POSTED

Naxin Jiang
National University of Singapore

Nguan Soon Tan
National University of Singapore

Bow Ho
National University of Singapore

Jeak Ling Ding
National University of Singapore

DOI:
10.1038/nprot.2007.482

SUBJECT AREAS
Biological techniques *Microbiology*

KEYWORDS
horseshoe crab, prophenoloxidase (PPO), antimicrobial

Introduction

To demonstrate the ability of microbial factor-activated PPO activity in clearing the invading pathogen *in vivo*, we infected horseshoe crabs in the presence or absence of PO-specific inhibitor, PTU[1] or kojic acid[2]. A comparison of the remnant bacterial load under these conditions should help to clarify the specific contribution of PO, if any, to the antimicrobial activity. Previously, it was reported that HMC/PPO is activated by host intracellular factors released through LPS-dependent degranulation of hemocytes. To avoid provocation of PPO by such cellular components and to unequivocally demonstrate that the microbial factor-activated PPO contributes to the antimicrobial defense, Gram-positive bacteria lacking LPS were used to avoid LPS-induced hemocyte lysis. To this end, the *S. aureus* laboratory strains, PC1839 (V8 protease-producing) and AK3 (V8 protease inactive mutant), were injected into the animals.

Procedure

1. Culture the Gram-positive bacterial strains under pyrogen-free condition as described in protocols #4 and #5.
2. Adjust the bacterial population to 10^6-10^7 cfu/ml with pyrogen free 3% NaCl (isotonic to the horseshoe crab hemolymph).
3. Inject the horseshoe crab intracardially with 10^5-10^6 cfu bacteria /100 gram body, using #23 needle.
4. At 30 min post injection, collect hemolymph from the horseshoe crab by cardiac puncture using #18 needle.
5. Immediately after collection, remove the hemocytes by centrifuging the hemolymph at 150 x g for 10 min at room temperature.
6. Quantify the remnant bacterial load in the extracellular milieu by applying 100 µl of the cell-free hemolymph (from step 5) to the nutrient agar plate and incubate at 37 °C overnight.
7. In order to confirm the contribution of PO activity in the bacterial clearance, include

5 mM PTU or 5 mM kojic acid in the bacterial injection to block *in vivo* PO activity, if any.

Anticipated Results

As shown in Figure 7, at 30 min post-injection, the remnant bacterial load in the cell free hemolymph (injected without PTU or kojic acid) is less than 10^4 cfu/ml. Co-injection of PTU or kojic acid with the bacteria results in significantly higher bacterial load of the extracellular-protease positive strains such as *S. aureus* PC1839. In contrast, the clearance of the extracellular-protease-negative strains such as AK3, is unaffected by PTU or kojic acid.

References

1. Nellaiappan, K. & Sugumaran, M. On the presence of prophenoloxidase in the hemolymph of the horseshoe crab, *Limulus. Comp Biochem Physiol B Biochem Mol Biol*. **113**, 163-168 (1996).
2. Dowd, P. F. Relative inhibition of insect phenoloxidase by cyclic fungal metabolites from insect and plant pathogens. *Natural Toxins*, **7** (6), 337-341 (1999)

Figures

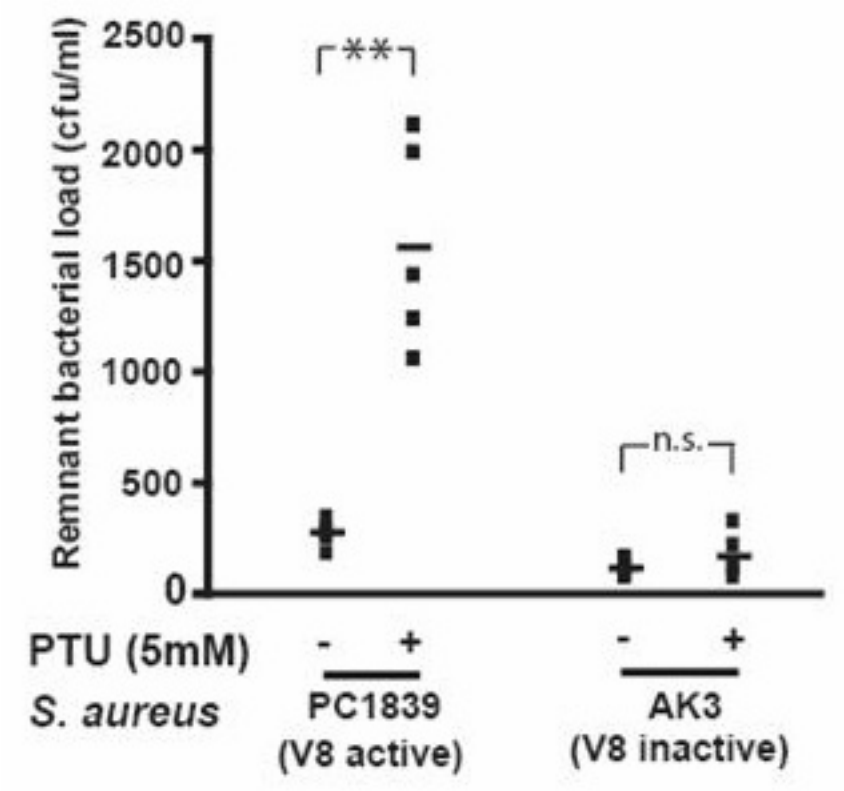

Figure 1

The PO triggered by the microbial protease contributes to in vivo antimicrobial activity. Injection of the _S. aureus_ laboratory strains, PC1839 and AK3, which are active V8 protease-positive and -negative, respectively into the horseshoe crab at 105 cfu/100 gram body weight, in the presence or absence of 5 mM PTU. At 30 min post injection, the remaining bacterial load in the hemolymph was measured. The protease-positive strain which specifically evoked the ROS-production by HMC/PPO, is in turn killed effectively. However, co-injection with PTU inhibited the HMC/PPO activity, and allowed the bacteria to remain viable in the host. On the other hand, the clearance of the V8-inactive strain was unaffected by PTU. This is probably due to the antimicrobial effects of parallel PO-independent mechanisms (see main text for further explanations).

Respiratory protein–generated reactive oxygen species as an antimicrobial strategy

by Naxin Jiang, Nguan Soon Tan, Bow Ho & Jeak Ling Ding

Nature Immunology (13 June, 2007)