HLS was prepared with the hemocytes of juvenile crabs at intermolt stage, as described [1] and further precipitated using saturated ammonium sulfate (SAS) [2–5]: SAS was added to HLS at 30% final solution (v/v) and mixed on ice for 20 min by constant stirring (~1000 rpm), centrifuged at 15,000 g at 4ºC for 20 min and then, the protein pellet was retrieved and resuspended in 50 or 100 µl of ice-cold working buffer. The remaining solution was used as source for a new ammonium sulfate precipitation at 50, and 65%. The protein concentration of each SAS fraction was measured and evaluated in its PO activity as described [1].

HLSs (2 µg) were separated on a 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane (Bio-Rad) and further processed using the procedure as described [6]. In brief, the membrane was blocked with 7% nonfat milk (NFM) in PBST (1X PBS, 0.05% Tween 20) at RT for 2 h. The primary antibody (α-CasPPO-hemo; 1: 2,000 dilutions in NFM+PBST) was incubated overnight at 4ºC. After washing with PBST (three times per 20 min each), the membrane was then incubated with a secondary antibody, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Jackson Immuno-Research) at 1:10,000 dilutions for 1 hr at RT. The signals on the autoradiography film (HyBlot CL, Denville Scientific Inc) were detected by chemiluminescence using luminol as a substrate.

References


