Purification of the horseshoe crab hemocyanin

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Method Article

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Abstract

Introduction

This protocol describes the isolation and purification of hemocyanin from the horseshoe crab. In the open-circulating system of the horseshoe crab, hemocyanin is the most abundant extracellular protein, constituting >90% of the total proteins. Since hemocyanin exists as a 48-mer holo-protein \(MW= 3,500 kDa)[1], its sheer size makes it readily separated by gel-filtration chromatography from other major plasma proteins, such as the C-reactive protein which exists as a pentamer of 150 kDa, or α-2-macroglobulin which exists as a monomer of 180 kDa[2,3].

Procedure

1. By cardiac puncture of the horseshoe crab, collect the hemolymph into pyrogen-free tubes. The animals are handled under national and institutional guidelines of \(National Advisory Committee for Laboratory Animal Research, Singapore). Only partial bleeding of the horseshoe crab is performed and the animal is returned alive, to its natural habitat \(the estuary). 2. Carefully remove the hemocytes by centrifugation at 150 x g for 10 min at 4 ˚C. 3. To select plasma from uninfected individuals for further hemocyanin purification, at this juncture, spread 100 µl of the supernatant on nutrient agar plate and culture overnight at 37 ˚C. Only plasma samples from individuals that showed sterile hemolymph may be used for further HMC/PPO purification. 4. Further clarify the supernatant at 9000 x g for 10 min at 4 ˚C. 5. Quick-freeze the second supernatant \(referred to as plasma) in liquid nitrogen and store at -80 ˚C until further use. 6. Centrifuge the plasma twice at 40,000 x g for 3 h each at 4 ˚C. 7. Resuspend the plasma containing enriched HMC/PPO in 0.5 M pyrogen-free NaCl, and further dialyze against 0.1 M Tris-HCl, pH 8.0, 5 mM $CaCl_2$, 5 mM $MgCl_2$, at 4 ˚C overnight. 8. Further purify the hemocyanin by gel filtration chromatography through Superdex S200 column \(2 x 150 cm) using elution buffer containing 0.1 M Tris-HCl, pH 8.0, 5 mM $CaCl_2$, 5 mM $MgCl_2$. Collect the eluate in 1 ml fractions. 9. Analyse the protein profile for each peak by electrophoresis on 12% SDS-PAGE. 10. Pool the first half of the large leading peak which contains purified hemocyanin. 11. Determine the concentration of the purified hemocyanin by the method of Bradford using bovine serum albumin \(Sigma, Fraction V) as standard.

Anticipated Results

The purified hemocyanin shows a doublet, with apparent molecular weight of 72-74 kDa on 12% SDS-PAGE. The purity of the hemocyanin should be no less than 98%.

References

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Figures

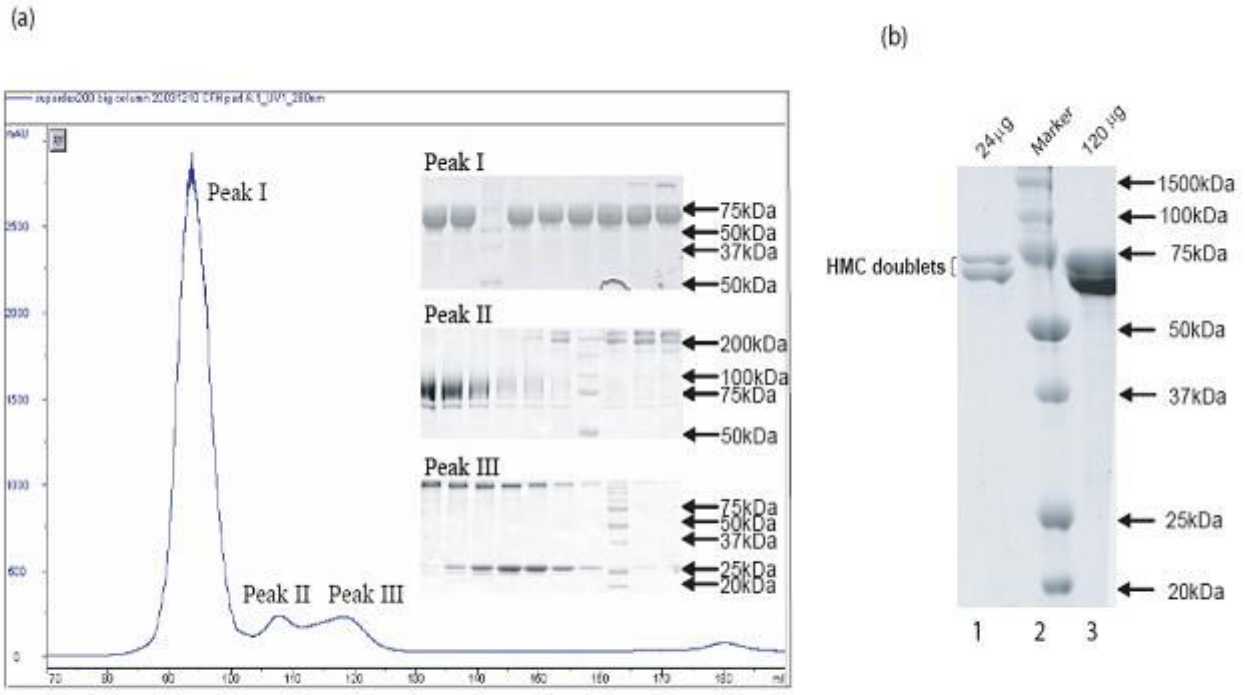

Figure 1

Purification of hemocyanin from horseshoe crab hemolymph. (a) Elution profile of the horseshoe crab plasma proteins from gel-exclusion chromatography. Two ml of plasma containing 64 mg total proteins were applied to a 2×150 cm Superdex^TM^ 200 column. The OD~280nm~ was monitored. The proteins were eluted with 0.1 M Tris-HCl, pH 8.0, 5 mM CaCl~2~, 5 mM MgCl~2~ at a flow rate of 9 ml/h. The inset shows reducing SDS-PAGE of the protein profiles collected from each peak. The first half of peak I, containing virtually pure hemocyanin was pooled as purified HMC/PPO. (b) SDS-PAGE profile of

the purified horseshoe crab HMC/PPO. Lanes 1 and 3 contain 24 μg and 120 μg respectively of purified HMC/PPO. The molecular weight markers are shown on lane 2.