

**INACTIVATED FORM OF PURIFIED  
HEMOCYANIN SUBUNIT FROM MALAYSIAN  
HORSESHOE CRAB *TACHYPLEUS GIGAS* AS A  
POTENTIAL ANTIMICROBIAL AGENT**

**JAMES JAM JOLLY**

**MASTER OF SCIENCE  
UNIVERSITI MALAYSIA TERENGGANU**

**2015**

0/9937

1100094798

Pusat Pembelajaran Digital Sultanah Nur Zahirah  
Universiti Malaysia Terengganu.



tesis

QL 447.7 .J3 2015



1100094798

Inactivated form of hemocyanin subunit from Malaysian horseshoe crab *tachylepus gigas* as potential antimicrobial agent / James Jam Jolly.

PUSAT PEMBELAJARAN DIGITAL SULTANAH NUR ZAHIRAH

UNIVERSITI MALAYSIA TERENGGANU (UMT)

21030 KUALA TERENGGANU

1100094798

1100094793

Lihat Sebelah

HAK MILIK  
PUSAT PEMBELAJARAN DIGITAL SULTANAH NUR ZAHIDAH

**INACTIVATED FORM OF HEMOCYANIN  
SUBUNIT FROM MALAYSIAN HORSESHOE  
CRAB *TACHYPLEUS GIGAS* AS A POTENTIAL  
ANTIMICROBIAL AGENT**

**JAMES JAM JOLLY**

**Thesis Submitted in Fulfillment of the Requirement  
for the  
Degree of Master of Science in the School of  
Fundamental Sciences  
Universiti Malaysia Terengganu**

**FEBRUARY 2015**

Approved thesis presented to the Senate of Universiti Malaysia Terengganu  
in partial fulfillment of the requirements for the degree of Master of Science.

**UNSUBSTANTIATED FORM OF HEMOCYANIN SUBUNIT FRAGMEN  
T FROM MALAYSIAN INVASIVE CRAB TACHYRUNUS OEGAS  
AS A POTENTIAL ANTIMICROBIAL AGENT**

BY JAMES JAM JOLLY

March 2014

Chairperson : Associate Professor Dr. Narendranil Jaisel, Ph.D

Panel Members:

Hemocyanin subunit fragmen is the hemocyanin of the horseshoe crab *Tachytrema oegas* and it is believed to serve as an oxygen transporter and defensive agent.

It is believed that truncated form of hemocyanin has

**I WOULD LIKE TO DEDICATE THIS DISSERTATION TO  
MY BELOVED FAMILY**

Several studies showed hemocyanin capping activity that can inhibit adhesion of the virus, that is, polyvalent hemocyanin of horseshoe crab hemocyanin to fibroblast using Auger electron and X-ray determination of antimicrobial properties using the diffusion test (1997) and several inhibitory concentrations (IC<sub>50</sub>) that may vary per participation using both Colloidal Antibody of IgG and IgM based, using FPLC column of Millipore Biogel P-100, 2000 Å, and lastly the binding properties in which protein concentration for each was measured at 1 mg/ml, 2 mg/ml, 5 mg/ml, SDS-PAGE and was validated by NELDE-THIEN water soluble dye method. The hemocyanin was activated to phagocytose and other phagocytosis activities such as hemolytic lytic capacity of C3a, C5a and C5b, 5a, 5b, 5c, diopside sulphate and calcium chloride. Phagocytosis activity was proven through the formation of golden-brown color of emulsion that is a product of

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu  
in fulfillment of the requirement for the degree of Master of Science

**INACTIVATED FORM OF HEMOCYANIN SUBUNIT FROM  
MALAYSIAN HORSESHOE CRAB *TACHYPLEUS GIGAS*  
AS A POTENTIAL ANTIMICROBIAL AGENT**

**JAMES JAM JOLLY**

**March 2014**

**Main Supervisor : Associate Professor Dr. Noraznawati Ismail, Ph.D**

**School: Fundamental Science**

Hemocyanin is abundantly found in the hemolymph of the horseshoe crab *Tachypleus gigas*. Its vital roles are to serve as an oxygen transporter and defensive protein. However, it is believed that inactivated form of hemocyanin has antimicrobial properties as does its analogue, phenoloxidase. Therefore, this research was carried out to compare the antimicrobial response towards the hemocyanin in inactivated form and activated hemocyanin (phenoloxidase). There are three objectives in this study; firstly, purifying hemocyanin in three steps. Secondly, activation of hemocyanin to phenoloxidase using known activators and lastly determination of antimicrobial properties using disc diffusion test (DDT) and minimal inhibitory concentration (MIC). First step was partial purification using Spin Column Affinity of 30K and 100K, second, using FPLC column of HiPrep Sephadex Exclusion 26/60 2-200 HR and last step was desalting procedures to obtain purified hemocyanin. Its purity was validated by electrophoresing on 12% SDS-PAGE and was validated by MALDI-TOF-MS as HC subunit IIIa (72.9 kDa). The hemocyanin was activated to phenoloxidase using known phenoloxidase activators such as hemolymph lysate supernatant, SDS, citric acid, thiourea, EDTA, magnesium sulphate and calcium chloride. Phenoloxidase activities were proven through the formation of reddish-brown color of ortho-quinone as a product of

catechol reduction. The presence of two protein bands from the reaction between hemocyanin and phenoloxidase activators were detected when electrophoresed on 12% SDS-PAGE, as an indication of proteolytic cleavage. Thirdly, the determination of antimicrobial properties of hemocyanin and phenoloxidase were done using DDT on bacteria (*E. coli*, *S. aureus* and *K. pneumoniae*) and fungi (*S. cerevisiae*, *Penicillium* sp. and *A. niger*). All bacteria and fungi strains have shown susceptibility response towards inactivated HC. As for PO, different responses ranging from resistant, intermediate and susceptible were recorded accordingly. The 'break-point' of hemocyanin was determined by MIC as 0.005 g/ml for *E. coli*, *S. aureus* and *K. pneumonia*; 0.01 g/ml for *S. cerevisiae* and 0.02 g/ml for both *Penicillium* sp. and *A. niger*. These results could be a promising indication of producing a new and potential antimicrobial agent against pathogenic microorganisms.

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu sebagai memenuhi keperluan untuk Ijazah Sarjana Sains.

**HEMOSIANIN SUB-UNIT TIDAK AKTIF DARIPADA BELANGKAS  
MALAYSIA *TACHYPLEUS GIGAS* SEBAGAI AGEN  
ANTIMIKROB YANG BERPOTENSI**

**JAMES JAM JOLLY**

**Mac 2014**

**Penyelia Utama : Profesor Madya Dr. Noraznawati Ismail, Ph.D**

**Pusat Pengajian: Sains Asas**

Hemosianin banyak terdapat di dalam hemolimf belangkas *Tachypleus gigas*. Peranannya yang paling utama ialah sebagai pembawa oksigen dan protein pertahanan. Adalah dipercayai, hemosianin yang tidak aktif mempunyai sifat antimikrob seperti analognya fenoloksidase. Kajian ini mempunyai tiga objektif utama; pertama, penulenan hemosianin yang melibatkan tiga peringkat. Kedua, pengaktifan hemosianin kepada fenoloksidase dan yang ketiga menentukan sifat anti-mikrob hemosianin dan fenoloksidase menggunakan ujian ‘Disc Diffusion Test’ dan ‘Minimal Inhibitory Concentration’. Langkah pertama ialah penulenan separa hemosianin menggunakan “Spin Column” bersaiz 30K dan 100K. Kedua, penulenan menggunakan turus FPLC Hiprep Sephadex Exclusion 26/60 2-200 HR. Langkah terakhir ialah penyingkiran garam untuk mendapatkan hemosianin tulen. Ketulenanannya disahkan dengan menggunakan teknik elektroforesis pada 12% SDS-PAGE dan jalur protein yang terhasil disahkan oleh MALDI-TOF-MS sebagai hemosianin subunit IIIa (72.9 kDa). Hemosianin kemudian diaktifkan kepada fenoloksidase menggunakan supernatan lisat hemolimf, SDS, asid sitrik, thiourea, EDTA, magnesium sulfat and kalsium klorida dan aktiviti fenoloksidase dibuktikan dengan kehadiran warna cokelat-kemerahan ‘ortho-quinone’ yang terhasil daripada proses penurunan katekol. Kehadiran dua jalur protein yang terhasil daripada

tindakbalas antara hemosianin dan pengaktif fenoloksidase dalam 12% SDS-PAGE membuktikan pemotongan proteolitik hemosianin telah berlaku. Ketiga ialah penentuan sifat anti-mikrob hemosianin dan fenoloksidase dengan menggunakan ujian DDT ke atas bakteria (*E. coli*, *S. aureus* dan *K. pneumoniae*) dan kulat (*S. cerevisiae*, *Penicillium* sp. dan *A. niger*). Semua bakteria dan kulat menunjukkan respon yg rentan atau "susceptible" terhadap hemosianin. Manakala untuk PO, terdapat pelbagai respon diperolehi termasuklah respon rintang (resistant), perantara (intermediate) dan rentan (susceptible). "Titik-pecah" bagi hemosianin untuk ujian MIC ialah 0.005 g/ml untuk *E. coli*, *S. aureus* dan *K. pneumoniae*; 0.01 g/ml untuk *S. cerevisiae* dan 0.02 g/ml untuk kedua-dua *Penicillium* sp. dan *A. niger*. Keputusan ujian DDT dan MIC ini membuktikan bahawa hemosianin berpotensi dijadikan sebagai agen anti-mikrob untuk melawan organisma patogenik.