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

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Lihat Sebelah

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I WOULD LIKE TO DEDICATE THIS DISSERTATION TO MY BELOVED FAMILY

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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 Sephacryl 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.

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

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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 Sephacryl Exclusion 26/60 2-200 HR. Langkah terakhir ialah penyingkiran garam untuk mendapatkan hemosianin tulen. Ketulenannnya 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 'orto-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.