COPPER-PEPTIDES MIMICKING CATALYTIC ACTIVITY OF LACCASE IN OXIDATION REACTIONS

SHARIFA ZAITHUN BEGUM

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By

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Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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July 2020

Chairman : Mohd Basyaruddin Abdul Rahman, PhD
Faculty : Science

Laccase, an oxidative enzyme naturally found in fungi and bacteria, has been widely used in the fields of chemical and bio-catalysis. Its active site contains several copper ions making it very interesting for studies related to the structure and catalytic mechanisms. However, extracting laccase uses large amounts of organic solvents to attain low yield; making it a less preferred choice for all green chemistry applications. The significance of this study is to mimic and enhance the catalytic activity of the highest oxidation activity reported laccase from *Trametes versicolor*. The four peptide sequences of the active sites found in most laccases was designed into nona (Np), hepta (Hp), tetrapeptides (Tp1 and Tp2) using computational techniques. The peptides were synthesized using Fmoc SPPS, analyzed and purified using HPLC and LC-MS. Copper(II)-peptides identified as Np-CuC, Np-CuS, Np-CuN, Hp-CuC, Hp-CuS, Hp-CuN, Tp1-CuC, Tp1-CuS, Tp2-CuC and Tp2-CuS were synthesized, crystallized and analyzed using FTIR, Raman, NMR, AAS, XPS and CD. Different molar ratio of peptides to copper(II) ions were analyzed for binding studies. These peptides and copper-peptides were tested for their catalytic activity in oxidation reaction of benzyl alcohol where both oxidation to benzaldehyde and disproportionation to toluene and phenol were achieved simulataneously. Np-CuC was observed to have higher catalytic activity (62.3%, 100% selectivity) than laccase (40.8%, 19.1% selectivity) towards benzaldehyde. They were also used as catalysts in the degradation of twelve pharmaceutical active compounds (PhACs). Tp2-CuS (1:2) proved to be a better catalyst in the degradation of PhACs. Oxidation of 5-ASA and DCA catalyzed by these copper peptides where Hp-CuS provided the highest yield of the oxidized product (46.5%). These activities were compared with laccase (positive control) and no catalyst (negative control). Overall, Np-CuC, Tp2-CuS and Hp-CuS were found to be better catalysts for oxidation reactions compared to their parent peptides and laccase.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KUPRUM-PEPTIDA MEMIMIK AKTIVITI PEMANGKINAN LACCASE DALAM TINDAK BALAS PENGOKSIDAAN

Oleh

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Laccase adalah enzim pengoksidaan semulajadi yang terdapat dalam kulat dan bakteria. Ia telah digunakan secara meluas dalam bidang kimia dan bio-pemangkinan. Tapak aktif *laccase* yang mengandungi ion-ion kuprum menjadi komponen penting bagi kajian berkaitan dengan struktur dan mekanisma pemangkin. Walaupun, jumlah pelarut organik yang banyak diperlukan untuk mengekstrak *laccase*, namun hasil ekstrak yang diperolehi adalah sedikit, sekaligus menjadikannya kurang sesuai untuk semua aplikasi kimia hijau. Kajian ini penting untuk cuba menyamai dan menambahbaik aktiviti pemangkinan oleh aktiviti pengoksidaan laccase laccase yang diperoleh *Trametes versicolor*. Empat rangkaian peptida bagi tapak aktif yang biasa didapati dalam kebanyakan *laccase* telah direka dan diubahsuai kepada nona (Np), hepta (Hp), tetrapeptida (Tp1 dan Tp2) menggunakan perisian dan teknik pengkomputeraan. Peptida telah disintesis menggunakan kaedah Fmoc SPPS, dianalisis dan ditulenkan menggunakan HPLC dan LC-MS. Kuprum (II) -peptida iaitu Np-CuC, Np-CuS, Np-CuN, Hp-CuC, Hp-CuS, Hp-CuN, Tp1-CuC, Tp1-CuS, Tp2-CuC dan Tp2-CuS telah disintesis, dihablur dan dianalisis menggunakan FTIR, raman, NMR, AAS, XPS dan CD. Beberapa nisbah molar peptida terhadap ion-ion kuprum(II) yang berbeza telah dianalisis untuk kajian mengikat. Peptida dan kuprum-peptida ini telah diuji untuk aktiviti pemangkin dalam tindak balas ringkas pengoksidaan benzil alkohol dimana kedua-dua pengoksidaan ke benzaldehid dan ketidakseimbangan ke toluene dan fenol telah dicapai serentak. Menurut pemerhatian, Np-CuC mempunyai aktiviti pemangkin yang lebih tinggi (62.3%, kepilihan 100%) daripada *laccase* (40.8%, kepilihan 19.1%) terhadap benzaldehid. Peptida dan kuprum(II)-peptida juga digunakan sebagai pemangkin dalam degradasi 12 sebatian atif farmaseutikal (PhACs). Tp2-CuS (1:2) terbukti menjadi pemangkin yang lebih baik dalam degradasi PhACs. Pengoksidaan asid 5-ASA dan DCA dipangkin oleh kuprum-peptida, dimana Hp-CuS menghasilkan produk oksida (46.5%) yang tertinggi. Aktiviti pemangkinan peptida dan kuprum (II) -peptida ini dibandingkan dengan *laccase* (kawalan positif) dan tiada pemangkin (kawalan negatif). Secara keseluruhan, Np-CuC, Tp2-CuS dan Hp-CuS dilaporkan sebagai pemangkin yang terbaik dalam tindak balas pengoksidaan berbanding dengan peptida dan *laccase* induknya.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

5-ASA	5-aminosalicylic acid
a.a	Amino acid
AAS	Atomic Absorption Spectroscopy
ABTS	2,2`-azino-bis(3-ethylbenzothiazoline-6-sulfonate)
Ala (A)	L-alanine
Arg (R)	L-arginine
Asp (D)	L-aspartic acid
Boc	*Tert*-butyl-Oxycarbonyl
CD	Circular Dichroism
CO_2	Carbon dioxide
Cu-Hp	Copper(II) bound to heptapeptide (HHCGCHH)
Cu-Np	Copper(II) bound to nonapeptide (HHHCGCHHH)
Cu-C	Copper(II) acetate monohydrate
Cu-N	Copper(II) nitrate trihydrate
Cu-S	Copper(II) sulphate pentahydrate
Cu-Tp1	Copper(II) bound to tetrapeptide 1 (HDGC)
Cu-Tp2	Copper(II) bound to tetrapeptide 2 (HMGC)
Cys (C)	L-cysteine
D-a.a	Dextrorotatory amino acid
DCA	Dihydrocaffeic acid/Dihydrocinnamic acid
d-d	*d-d* orbital transition
DI	Deionised water
DIEA	N, N-Diisopropylethylamine
EC	Electron count
EDTA	Ethylenediaminetetraacetic acid
Fe	Iron
Fmoc	Fluorenylmethyloxycarbonyl protecting group
FPLC	Fast Protein Liquid Chromatography
FTIR	Fourier Transform Infrared
GC-FID	Gas Chromatography-Flame Ionization Detector
GC-MS	Gas Chromatography-Mass Spectrometry
Glu (E)	L-glutamic acid
Gly (G)	L-glycine
HCTU	O-(6-Chloro-1-hydroxybenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
His (H)	L-Histidine
H_2O	Water
H_2O_2	Hydrogen Peroxide
HOMO	Highest occupied molecular orbital
Hp	Heptapeptide (HHCGCHH)
HPLC-UV	High Performance Liquid Chromatography-Ultraviolet
HPLC-PDA	High Performance Liquid Chromatography-Photodiode Array
IBD	Inflammatory Bowel Disease
ICP	Inductively Coupled Plasma
L-a.a	Levorotatory-amino acid
LC_{50}	Lethal concentration at 50% of cell death
LC-MS	Liquid Chromatography-Mass Spectrometry
Leu (L)	L-leucine

LMCT	Ligand-Metal Charge Transfer
LMS	Laccase Mediator System
LOMETS	Local- meta-threading server
LUMO	Lowest unoccupied molecular orbital
Lys (K)	L-lysine
m/z	mass/charge ratio
MeOH	Methanol
Met (M)	L-methionine
MLCT	Metal-Ligand Charge Transfer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMP	N-Methyl-2-pyrrolidone
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
Np	Nonapeptide (HHHCGCHHH)
pdb	Protein Data Bank
PhAC's	Pharmaceutically active compounds
Phe (F)	L-phenylalanine
Pro (P)	L-proline
PTFE	Polytetrafluoroethylene
rpm	revolutions per minute
R_T/min	Retention time in minutes
T1 Cu	Type 1 copper (Cu^{+})
T2 Cu	Type 2 copper (Cu^{2+})
T3 Cu	Type 3 copper (Cu-O-Cu)
TFA	Trifluoroacetic acid
Thr (T)	L-threonine
TIS	Triisopropyl silane
TLC	Thin Layer Chromatography
Tp1	Tetrapeptide 1 (HDGC)
Tp2	Tetrapeptide 2 (HMGC)
Trp (W)	L-tryptophan
Tyr (Y)	L-tyrosine
UV	Ultraviolet
UV-Vis	Ultraviolet Visible Spectroscopy
v/v	volume per volume
Val (V)	L-valine
XPS	X-ray Photoelectron Spectroscopy

CHAPTER 1

INTRODUCTION

This research is dedicated to oxidation reactions catalyzed by peptides and copper(II)-peptides for the synthesis of new metabolite drugs and in the degradation of harmful phenolic-based xenobiotics. Laccases, (EC 1.10.3.2, benzenediol: oxygen oxidoreductases), commonly known as the blue oxidase metalloenzymes were used as the basis for the design of various organocatalysts in the form of peptides and copper(II)-peptides. They are excellent catalysts for oxidizing phenols and cyclic substrates in the presence of mediators such as 2,2`-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT) that aids in the oxidation reactions. Without the laccase mediators; also known as electron transfer agents, they have low catalytic activity. They are often found in plants, bacteria, insects and white rot fungi (Call and Mücke, 1997). Laccases are well known for their environmentally benign nature as they only require O_2 as their co-substrate to produce H_2O as the sole by-product. Researchers like to term such oxidation reactions "green catalytic reactions" since they can contribute to a clean environment (Cannatelli, 2017).

The active site of laccase highlighted in Figure 1.1 has four copper ions in mononuclear (Cu^+) and trinuclear sites (Cu^{2+} and Cu-O-Cu) that contribute significantly in oxidation reactions to form new C-C and C-N bonds (Bertrand *et al.*, 2002). The mononuclear Cu^+ is located in a wide, hydrophobic binding pocket containing imidazole rings of histidine which contributes to the high π-electron density. It is also known to be the primary electron acceptor where the organic substrates bind and undergo rapid four-electron oxidation. These electrons are transferred through the tripeptide, His-Cys-His, to the trinuclear site where the reduction of oxygen (O_2) to water (H_2O) takes place (Solomon *et al*., 2008).

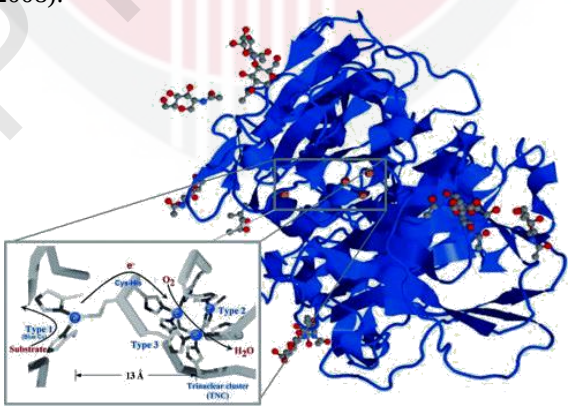

Figure 1.1: Laccase from *Trametes versicolor* and its' active site (PDB: 1gyc)
(Jackson *et al*., 2016)
*Blue spheres refer to copper ions.
*Brown spheres refer to the catalytic active site
* Red spheres refer to oxidation substrates

Laccase-catalyzed oxidations are biochemically versatile and being an enzyme that accepts vast range of substrates is an excellent catalyst for organic synthesis. Laccases are well known to oxidize low-molecular weight, natural phenols to degradable wastes, hence sustaining to the concept of green chemistry. However, due to its lock-and-key mechanism, only specific phenolic substrates can be oxidized to polymers through cross-coupling or via radicals that combine with non-laccase substrates to form dead-end products or degraded aliphatic compounds. The oxidized products play an important role in the food, medical and cosmetic industries (Jeon *et al*., 2012). Due to the limitations mentioned in the problem statement, copper(II) peptides mimicking the active site of laccase were designed, synthesized and applied as organocatalysts in oxidation reactions.

1.1 Problem Statement

Laccases cannot be chemically synthesized due to their extremely long sequences and the complex structure of their active sites. Like all other oxidative enzymes, they are isolated from various bacteria, fungi and plant sources (Muthukumarasamy and Murugan, 2014). Although these raw materials are cheaper than amino acids, large amounts of fungi/bacteria are required to extract a small amount of laccase. Various organic solvents such as 3,4-dimethoxybenzylalcohol are needed to extract the laccase and once extractions are complete, remnants of organic solvents are detected using spectroscopic methods (Thurston, 1994). Although laccase can be recovered and reused, its catalytic activity decreases after each cycle. Being enzymes, laccases are stable at a specific range of temperature. Since denaturation occurs at temperatures higher than 30 °C, it is not suitable for the temperature optimization of catalytic activity (Mikolasch *et al.*, 2002) .

A laccase-lipase co-catalytic system was used to catalyse asymmetric reactions such as Michael addition reactions between catechol and aromatic amines (Witayakran & Ragauskas, 2009). New C-C or C-N bonds can be formed through various asymmetric reactions such as aldol, Michael, *etc* and organic reactions such as Fielder-Crafts acylation, Suzuki coupling reactions, *etc.* All these reactions use vast amounts of organic solvents making oxidation a better option for catalysis. It was said that laccase from fungal *Trametes versicolor* gave the highest oxidation activity (Tominaga *et al.*, 2004) when compared with other extracted oxidative enzymes and organocatalysts. Laccase and peroxidase catalyzed the reaction of 3,3`,5,5`-tetramethylbenzidine with 5-aminosalicylic acid (5-ASA) whereby peroxidase produced a higher yield compared to laccase but hydrogen peroxide was produced as the side product (Touahar *et al.*, 2014).

1.2 Scope of research

This research focuses on the laccases' active site as template for Cu(II)-peptides as catalysts in the oxidation of 5-aminosalicylic acid (5-ASA) with dihydrocaffeic acid (DCA) and in xenobiotic degradation of pharmaceutical waste containing phenolic/aromatic side chains. The scope of the research is shown in Figure 3.1.

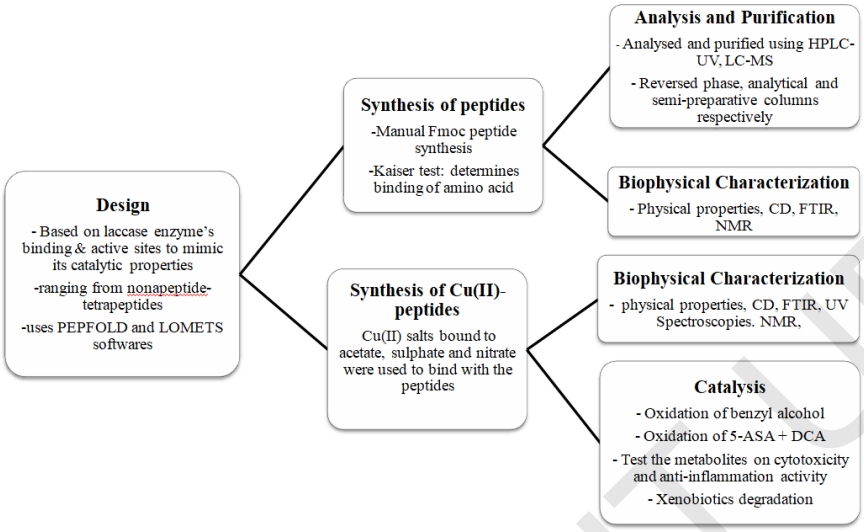

Figure 1.2: Scope of research

1.3 Aim, significant of study and objectives

The primary objective of this project is to enhance the catalytic activity of laccase without using any organic solvents and mediators that are potentially harmful to the environment. Mimicking the design of peptides and chemically synthesizing the active site of laccase with slight modifications is not only time-saving but it is expected to produce compounds that are more structurally-stable than the parent enzyme especially at higher temperatures The aim of this project is to design and synthesize copper-peptides modified from the active site of laccase from *Trametes versicolor* to be used as catalysts in the oxidation of benzyl alcohol, degradation of phenolic pharmaceutical waste into biodegradable ones and in the reaction of 5-aminosalicylic acid (insoluble drug) and dihydrocaffeic acid to obtain soluble metabolite of the drug.. The significance of this study is that these copper-peptides will achieve equal or higher catalytic activities in oxidation reactions of laccase without the use of any organic mediators. The copper-peptides should be able to be recovered and reused several times as they can be purified through chromatographic methods. The potential advantages of persuing a copper-peptide strategy in oxidation reactions were:

1) Since the sequences of these mimicked peptides are much shorter (<10 amino acids) than natural laccases that are made up within the range of 300-700 amino acids (Hakulinen *et al.*, 2002), they are said to be more stable than the tertiary structures of the protein in the enzyme, especially at extreme ends of temperature and pH ranges.
2) These copper-peptides, classified as organocatalysts, have activities higher than or similar to catalytic laccases that utilize lock and key mechanism.

3) The copper peptides would not only mimic part of the active sites of laccases, but also mimic the spectroscopic, structural and redox features of the parent enzyme.
4) The coordination chemistry of copper binding to certain amino acids that act as ligands, such as imidazole ring in histidine (H), carboxylic group in aspartic (D) and glutamic acid (E), the C-S ligands in cysteine (C) and methionine (M) in oligopeptides (Plegaria *et al.*, 2015) is similar to the coordination chemistry of copper ions bound to the amino acids found in active site of laccases.

The objectives of this project are:

1) To design and synthesize nona-, hepta-, tetrapeptides (Np, Hp, Tp1 and Tp2) and copper(II)-peptides designed from the active site of laccase.
2) To characterize and elucidate the structures of structures copper(II)-peptides.
3) To determine the catalytic activity of the synthesized peptides and copper(II)-peptides in oxidation reaction of benzyl alcohol
4) To determine the activities of the synthesized peptides and copper(II)-peptides in xenobiotics degradation of pharmaceutical waste containing phenolic compounds.
5) To employ the peptides and copper-peptides as catalysts in the reaction of 5-aminosalicylic acid (5-ASA) with dihydrocaffeic acid (DCA) and the conjugated products tested for the cytotoxicity (MTT) and the anti-inflammatory activity.

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