UNIVERSITI PUTRA MALAYSIA

PURIFICATION AND CHARACTERIZATION OF MEMBRANE-BOUND POLYPHENOL OXIDASES AND PEROXIDASES FROM *METROXYLON SAGU* ROTTB

GALILA HASSAN ONSA

FSMB 2003 16

PURIFICATION AND CHARACTERIZATION OF MEMBRANE-BOUND POLYPHENOL OXIDASES AND PEROXIDASES FROM *METROXYLON SAGU* ROTTB.

By

GALILA HASSAN ONSA

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

May 2003

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctorate of Philosophy

PURIFICATION AND CHARACTERIZATION OF MEMBRANE-BOUND POLYPHENOL OXIDASES AND PEROXIDASES FROM *METROXYLON SAGU ROTTB.*

By

GALILA HASSAN ONSA

May 2003

Chairman: Assoc. Prof. Nazamid bin Saari, Ph.D.

Faculty: Food Science and Biotechnology

The histochemical studies indicated that *Metroxylon sagu* polyphenol oxidases (mPPO: E.C.1.10.3.2) and peroxidase (mPOD: E.C.1.11.1.7) were cellular membrane-bound enzyme. The enzymes were isolated using temperature-induced phase partitioning technique with Triton X-114. The temperature-induced phase partitioning extract was subsequently chromatographed on DEAE-Toyopearl 650M, Butyl-Toyopearl 650 M and Sephadex G-100. Two mPPO isoenzymes designated as mPPO-I and mPPO-II were purified 43.9 and 76.1-fold respectively. On Native-PAGE, both isoenzymes were resolved into two charge isomers, very close in charge density. The molecular masses of mPPO-I and mPPO-II were 38 and 39 kDa respectively. The latency that was observed for the temperature-induced phase partitioning mPPO extract was not detected in purified enzyme and a fully active mPPO was obtained. The optimum pHs of mPPO-I and mPPO-II were 4.5 and 5.0 respectively. mPPO isoenzymes did not react with monophenols but were highly

reactive toward diphenols and triphenols at varying affinities. Ascorbic acid with K_i value of 0.015 mM was the most potent inhibitor for mPPO followed by sodium metabisulfite, L-cysteine, kojic acid, and *p*-coumaric acid. Metal ions tested affected both isoenzymes similarly. The enzyme activity was enhanced in the presence of 1.0 mM Cu^{2+} and hardly affected by 10 mM Ca^{2+}, Al^{3+}, Ni^{2+} and Hg^{2+}. mPPO-II showed high thermal stability with activation energy of heat inactivation (E_a) of 40.34 compared to 32.94 $kcal.mol^{-1}$ for mPPO-I.

mPODs were weakly adsorbed onto DEAE-Toyopearl 650M. The eluent was subsequently chromatographed onto CM-Toyopearl 650M followed by Sephadex G-100. Two isoenzymes; mPOD-I and mPOD-II were purified 76.5- and 37.0-fold respectively. Their molecular masses were of 51.2 and 43.8 kDa respectively. mPOD-1 and mPOD-II had an optimum pH at 6.0 and 5.5 respectively. Both mPOD isoenzymes showed high efficiency of interaction with TMBZ, guaiacol, diphenols and triphenols only in the presence of H_2O_2. Ascorbic acid was the most potent inhibitor of mPOD with K_i value of 0.01 mM, followed by sodium metabisulfite, L-cysteine and *p*-coumaric acid. mPOD-I activity was enhanced in the presence of 1.0 mM Al^{3+}, Ca^{2+}, Fe^{3+}, Ni^{2+} better than mPOD-II and both isoenzymes were not affected by Hg^{2+} and $Cu^{2+}$and moderately inhibited by the presence of 10 mM Zn^{2+} and Co^{2+}. mPOD-I was more thermal stable with an inactivation energy (E_a) of 45.77 $kcal.mol^{-1}$ compared to 40.62 $kcal.mol^{-1}$for mPOD-II. Other thermodynamic parameters such as enthalpy and entropy were also determined and compared.

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

PENULENAN DAN PENCIRIAN POLIFENOL OKSIDASE DAN PEROKSIDASE TERIKAT MEMBRAN DARIPADA *METROXYLON SAGU* ROTTB.

Oleh

GALILA HASSAN ONSA

Mei 2003

Pengerusi: Prof. Madya Nazamid bin Saari, Ph.D.

Fakulti: Sains Makanan dan Bioteknologi

kajian histokimia menunjukkan polifenol oksidase (PPO: E.C.1.10.3.2) dan peroksidase (POD, E.C. 1.11.1.7) *Metroxylon sagu* adalah enzim membran-terikat. Enzim ini telah dipencilkan dengan teknik rangsangan suhu fasa sekatan menggunakan Triton X-114. Ekstrak rangsangan suhu fasa sekatan tersebut telah berturutan ditulen menggunakan kromatografi DEAE-Toyopearl 650M, Butyl-Toyopearl 650M dan Sephadex G-100. Dua isoenzim mPPO dikenalpasti sebagai mPPO-I dan mPPO-II telah dipencil dan ditulenkan masing-masing 43.9 kali dan 76.1 kali. mPPO-I dan mPPO-II telah dipisahkan kepada dua galur yang aktif di atas PAGE-native; terlalu hampir dalam ketumpatan casnya dengan berat molekul masing-masing 38 dan 39 kDa. Setelah penulenan dijalankan, mPPO yang aktif sepenuhnya telah diperolehi. pH optima bagi mPPO-I dan mPPO-II masing – masing 4.5 dan 5.0. Isoenzim mPPO tidak bertindakbalas dengan monofenol tetapi sangat aktif terhadap difenol dan trifenol pada afiniti yang berbagai. Asid askorbik

dengan nilai K_i 0.015 mM adalah perencat yang paling berpotensi diikuti oleh sodium metabisulfite, L-sistein, asid kojik dan *p*-komarik. Ion logam juga mempengaruhi kedua-dua isoenzim ini. Aktiviti enzim ditingkatkan dengan kehadiran 1.0 mM Cu^{2+} tetapi tidak dipengaruhi oleh 10 mM Ca^{2+}, Al^{3+}, Ni^{2+} dan Hg^{2+}. mPPO-II adalah lebih tahan kepada penyahaktifan terma dengan tenaga pengaktifan (E_a) 40.34 berbanding 32.94 kcal.mol-1 untuk mPPO-I.

mPOD ini mempunyai daya lekatan yang lemah kepada DEAE-Toyopearl 650M. Eluen ditulenkan dengan kromatografi menggunakan CM-Toyopearl 650 M diikuti dengan Sephadex G-100. Dua peroksidase membran-terikat mPOD-I dan mPOD-II ini telah ditulenkan masing-masing sebanyak 76.5 dan 37 kali dengan berat molekul masing-masing 51.2 dan 43.8 kDa. mPOD-I dan mPOD-II menpunyai pH optima masing-masing 6.0 dan 5.5. Kedua-dua isoenzim mPOD ini menunjukkan kecekapan interaksi yang tinggi dengan TMBZ , guaikol, difenol dan trifenol hanya dengan kehadiran H_2O_2. Asid askorbik merupakan perencat paling berpotensi untuk mPOD dengan nilai K_i 0.01 mM, diikuti sodium metabisulfite, L-sistien dan asid *p*-komarik. Aktiviti isoenzim mPOD-I ditingkatkan dengan kehadiran 1.0 mM Fe^{3+}, Ca^{2+}, Al^{3+}, Ni^{2+} lebih baik dari mPOD-II dan kedua isoenzim ini tidak dipengaruhi oleh Hg^{2+} dan Cu^{2+}serta direncat dengan sederhana oleh kehadiran 10 mM Zn^{2+} dan Co^{2+}. mPOD-I lebih stabil dengan tenaga pengaktifan (E_a) 45.77 kcal.mol^{-1} berbanding 40.62 kcal.mol^{-1} untuk mPOD-II. Parameter termodinamik yang lain juga seperti entalpi dan entropi telah ditentu dan dibandingkan.

ACKNOWLEDGMENT

All praise due to Allah, the most gracious and merciful for giving me the strength, health and determination to complete this research. I would like to express my deepest appreciation and gratitude to the chairman of my supervisory committee, Assoc. Prof. Dr. Nazamid Saari, Department of Food Science, for his patience, invaluable guidance and suggestions throughout the planning and extension of this research. I am eternally grateful to my co-supervisors, Prof. Dr. Jinap Selamat and Assoc. Prof. Dr. Jamilah Bakar for their attention, support and constant encouragements. My gratitude and appreciations were also extended to Assoc. Prof. Dr. Kharidah Muhammad and Dr. Zaiton Hassan for their guidance and advice during planning of this research proposal. I would like to acknowledge Dr. Lee Kong Hung, Department of Food Biotechnology, UPM, for his assistance in editing the final draft of this thesis.

I am indebted to Dr. Shamsul Elbahri and Mrs Chan Jee Leene, Biotechnology and Strategic Research Unit, Rubber Research Institute of Malaysia and the staff of Electron Microscopy Unit, Universiti Putra Malaysia for their technical assistance to accomplish the enzyme histochemistry as part of this research. My deepest gratitude to Haj Mahmoud and his wife the owner of sago plantation in Batu Pahat, Johore State for their hospitality and kind assistance in collecting the sample used in this research. My thanks and great appreciations are also due to all graduate students in the Department of Food Science for giving moral encouragement and support. Special

thanks are extended to Halifa Mat Yasin and Ermina Sari for Bahasa Malaysia translation of the abstract.

I am grateful to the Government of Sudan, Ministry of Agriculture and Forestry and Ministry of Labor Force for giving me the chance to pursue my study. I would like to acknowledge the Ministry of Science, Technology and Environment, Government of Malaysia for IRPA grant that financed this study.

My gratitude is extended to my beloved mother, sisters and brothers for their patience encouragement and supports. I remember my late father in this occasion with deepest respects, who wished me to get a higher education. Deepest appreciation to Dr. Mahmoud Hassan Onsa (University of Khartoum, Sudan), for his encouragement to accomplish this study. I owe my special thanks to my husband Salah Eldeen for his moral encouragement, understanding, support, love and sacrifices which had been the biggest motivation for undertaking and completing this research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid)
$\Delta G^{\#}$	Change in free energy
$\Delta H^{\#}$	Change in enthalpy
°K	Absolute temperature in Kelvin
$\Delta S^{\#}$	Change in entropy
BSA	Bovine serum albumin
CM	Carboxymethyl
cm	Centimeter
D_{70}	The decimal reduction time at 70°C
DEAE	Diethylaminoethyl
E_a	Thermal inactivation energy
EA	Egg albumin
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast performance liquid chromatography
HLB	Hydrophil-LipophilBalance
hr	hour
k	Thermal inactivation rate constant
kcal	Kilo Calorie
kDa	Kilo Dalton
kg	Kilo Gram
K_i	Inhibition constant
K_m	Michaelis and Menten constant
L^*	Lightness by Hunter system
L-DOPA	L-3, 4-dihydroxyphenylalanine
LMW	Low molecular weight protein marker
mA	Milli Ampere
mM	Milli Molar
mPOD	Membrane-bound peroxidase

mPPO	Membrane-bound polyphenol oxidase
pH	Hydrogen ion concentration
POD	peroxidase
ppm	Part per million
PPO	Polyphenol oxidase
PVP	polyvinyl pyrolydone
R_f	Relative mobility
RM	Malaysian Ringgit
SIRM	Standard and Industrial Research Institute Malaysia
sPOD	Soluble peroxidase
sPPO	Soluble polyphenol oxidase
TEM	Transmission electron microscope
TEMED	N'N'N'N' tetramethylethylenediamine
TMBZ	Tetramethyl benzidine
TX-114	Triton X-114
uv	ultraviolet
V_m	Maximum velocity

CHAPTER 1

INTRODUCTION

Sago palm (*Metroxylon sagu*) is grown mainly for its starch storage trunk to provide the stable food for people in South East Asia. This palm is considered as a high yielding crop in terms of caloric yield per hectare, accumulating a huge amount of starch in the trunk. Sago palm has the ability to grow in water logged acidic soils and deep peat soil where few other plants can survive with minimum of technological input. The starch produced is almost pure; it contains 88% carbohydrate, 0.5% protein, and minute amounts of fat (Encyclopedia Britannica, 1997). Sago starch is ranked as the fourth largest revenue earner among other agricultural commodities, after oil palm, pepper and cocoa (Chew et al., 1999).

Recently, sago starch attracted researchers and policy makers' interest to cope with the predicted food shortage due to increase of world population (Flash, 1997). Due to its unique quality, sago starch can compete with other tropical starches such as tapioca and cassava as it has lower viscosity and higher gelatinization temperature. The starch has been used as major ingredients in various foods, soups, cakes, pudding and sauce thickener. It is also used as an ingredient in the production of monosodium glutamate and sweeteners such as high fructose syrup and caramel. However, the demand for sago starch is much lower compared to other starches. It was reported that the degree of whiteness of sago starch deteriorates markedly upon storage and processing (Yatsugi, 1986). The discoloration of sago starch is attributed to the enzymatic oxidation of the endogenous phenolic compounds present in sago

pith (Okamoto et al., 1991; Takamura, 1991). This oxidation is usually highly undesirable from food processors and the consumers' perspectives. Therefore, browning of the starch has been one of the most critical problems facing the sago starch industry. To create high demand for the starch, some efforts should be done to improve the quality of sago starch.

Enzymatic browning has been a subject of great interest to many food scientists. It is believed that the deterioration of many other food commodities is mediated by polyphenol oxidases (PPO; EC 1.10.3.2) and peroxidases (POD; E.C. 1.11.1.7) (Mayer and Harel, 1978; Vamos-Vigyazo, 1981; Robinson, 1991; Whitaker, 1994). PPO and POD are members of oxidoreductases that oxidize the indigenous phenolic compounds naturally present in plant tissues (Mayer and Harel, 1978; Robinson, 1991). PPOs catalyze the hydroxylation of monophenols and the oxidation of *o*-diphenols to the corresponding *o*-quinones at the expense of molecular oxygen. The resulting quinones formed are further polymerized to produce a brown pigment. POD is another oxidative enzyme, with the ability to catalyze the oxidation of phenolic compounds in the presence of H_2O_2 (Robinson, 1991; Whitaker, 1994; Nicolas et al., 1994). The primary products of the oxidized phenolic compounds are the quinones similar to that obtained with PPO (Robinson, 1991).

Polyphenol oxidases from higher plants are generally considered as a plastid enzyme and located in the tylakoid membrane (Mayer and Harel, 1978). Sanchez-Ferrer et al. (1989) and Marques et al. (1994) describe mPPO as a hydrophilic protein with a short hydrophobic tail that anchors to the membrane. On the other hand, membrane bound peroxidase (mPOD) has a strong electrostatic interaction with the cellular