UNIVERSITI PUTRA MALAYSIA

LIPOPHILIC ANTIOXIDANTS IN VARIOUS TISSUES OF SELECTED MALAYSIAN FRESHWATER FISH

EZARUL FARADIANNA BT LOKMAN.

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By

EZARUL FARADIANNA BT LOKMAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the Requirement for the Degree of Master of Science

January 2006

Specially dedicated to;

My beloved late father, mother
my family and friends

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman: Associate Professor Juzu Hayati Arshad, PhD

Faculty: Faculty of Biotechnology and Biomolecular Sciences

The total antioxidant activity in the muscle, liver and intestine of eleven species of Malaysian freshwater fish known as *Pangasius polyuranodon*, *Anabas testudineus*, *Channa striatus*, *Clarias batrachus*, *Labeo rohita*, *Tilapia mossambica*, *Leptobarbus hoevenii*, *Trichogaster pectoralis*, *Hemibagrus nemurus*, *Cyprinus carpio carpio* and *Puntius gonionotus* were evaluated using the optimized thiobarbituric acid (TBA) method. The synthetic antioxidant, butylated hydroxytoluene (BHT) was used as positive control. The peroxidation of linoleic acid in the thiobarbituric acid system was markedly inhibited by all the sample extracts compared with the control assay and also showed very low chelating activity with the iron chelator test. This indicated that the tissue samples contained insignificant quantities of iron chelators which would otherwise interfere with the TBA method. All the fish extracts exhibited total antioxidant activity in the order of muscle (61-81%) > liver (51-83%) > intestine (40-70%).

Three fish species identified to have high antioxidant activities in the muscle tissue namely *Anabas testudineus*, *Clarias batrachus* and *Labeo rohita* and a

species with the lowest antioxidant activity, *Hemibagrus nemurus* were selected for determination of the lipophilic antioxidants using the High Performance Liquid Chromatography (HPLC) analysis. The high antioxidant activities found in the muscle of *Anabas testudineus* and *Labeo rohita* were influenced by the presence of relatively high amounts of lipophilic antioxidants namely α-, β-, γ-, δ- tocopherols, retinol and coenzyme Q_{10}. Liver, which was found to have retinol at 0.711 ± 0.09 to 6.05 ± 0.16 μg/g wet weight probably influenced the antioxidant activities obtained. However, the intestine showed the lowest antioxidant activity compared to the other tissues examined. It was found that β-tocopherol (1.316 -3.861μg/g wet weight) was the only lipophilic antioxidant present.

The distributions of the various tocopherol homologues, retinol and coenzyme Q homologues in different tissues of Malaysian freshwater fish indicated that these compounds might be independently regulated in each tissue. The difference in the antioxidant activities in the muscle, liver and intestine of different samples in this study may be influenced by the presence of different types of lipophilic antioxidants in each sample. The three potential Malaysian freshwater fish species with high antioxidant activities identified were *Anabas testudineus*, *Clarias batrachus* and *Labeo rohita.*

In conclusion, the Malaysian freshwater fish species examined which were found to have high antioxidant activities are recommended as part of the diet as they may be able to protect the human body from free radicals and retard the progress of many chronic diseases. The fish extracts found to have lipophilic antioxidants in this study also can be used as accessible source of

natural antioxidants to replace synthetic antioxidants, as possible food supplement as well as in pharmaceutical applications.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANTIOKSIDA LIPOFILIK DALAM BEBERAPA JENIS TISU IKAN AIR TAWAR TERPILIH DI MALAYSIA

Oleh

EZARUL FARADIANNA BT LOKMAN

Januari 2006

Pengerusi: Professor Madya Juzu Hayati Arshad, PhD

Fakulti: Fakulti Bioteknologi dan Sains Biomolekul

Jumlah aktiviti antioksida dalam otot, hati dan usus sebelas spesis ikan air tawar di Malaysia dikenalpasti sebagai *Pangasius polyuranodon*, *Anabas testudineus*, *Channa striatus*, *Clarias batrachus*, *Labeo rohita*, *Tilapia mossambica*, *Leptobarbus hoevenii*, *Trichogaster pectoralis*, *Hemibagrus nemurus*, *Cyprinus carpio carpio* dan *Puntius gonionotus* dinilai menggunakan kaedah asid thiobarbiturik (TBA) yang optimum. Antioksida sintetik, butylated hydroxytoluene (BHT) digunakan sebagai kawalan positif. Pengoksidaan asid linoleik di dalam sistem asid tiobarbiturik nyatanya direncat oleh ekstrak sampel-sampel berbanding dengan asai kawalan dan juga menunjukkan aktiviti pengelat besi yang rendah. Ini menunjukkan bahawa tisu sampel mengandungi kuantiti pengelat besi yang tidak signifikan. Semua ekstrak tisu menunjukkan jumlah aktiviti antioksida mengikut turutan otot (61-81%) > hati (51-83%) > usus (40-70%).

Tiga spesis ikan dikenalpasti mengandungi aktiviti-aktiviti antioksida yang tinggi di dalam otot iaitu *Anabas testudineus*, *Clarias batrachus* and *Labeo rohita* dan sejenis spesis aktiviti antioksida yang terendah, *Hemibagrus*

nemurus dipilih untuk menentukan antioksida lipofilik menggunakan Kromatografi Cecair Prestasi Tinggi (HPLC). Aktiviti antioksida yang ditemui tinggi dalam otot *Anabas testudineus* dan *Labeo rohita* dipengaruhi oleh kehadiran secara relatif jumlah antioksida lipofilik yang tinggi seperti α-, β-, γ-, δ- tokoferol, retinol and coenzyme Q_{10}. Hati, yang dikenalpasti mengandungi retinol pada 0.711 ± 0.09 to 6.05 ± 0.16 μg/g berat basah berkemungkinan mempengaruhi aktiviti antioksida yang diperolehi. Walaubagaimanapun, usus menunjukkan aktiviti antioksida yang terendah berbanding dengan tisu-tisu lain yang dikaji. Dikenalpasti bahawa hanya β- tokoferol (1.316 – 3.861 μg/g berat basah) sahaja lipofilik antioksida yang hadir.

Taburan homolog tokoferol, retinol dan homolog coenzyme Q di dalam beberapa jenis tisu ikan air tawar di Malaysia menunjukkan bahawa sebatian-sebatian ini berkemungkinan dikawal atur secara tersendiri di dalam setiap tisu. Perbezaan aktiviti antioksida dalam otot, hati dan usus bagi sampel berbeza di dalam kajian ini mungkin dipengaruhi oleh jenis lipofilik antioksida yang berbeza di dalam setiap sampel. Tiga spesis ikan air tawar yang dikenalpasti berpotensi dengan aktiviti antioksida yang tinggi ialah *Anabas testudineus*, *Clarias batrachus* and *Labeo rohita.*

Pada kesimpulannya, ikan air tawar Malaysia yang dikaji mengandungi aktiviti antioksida tinggi digalakkan untuk dijadikan sebagai sebahagian daripada diet disebabkan kebolehan antioksida untuk melindungi badan manusia daripada radikal bebas dan merencat pembentukan peyakit-penyakit kronik. Ekstrak ikan yang mengandungi lipofilik antioksida di dalam kajian ini juga boleh digunakan sebagai sumber antioksida semulajadi untuk

menggantikan antioksida sintetik, sebagai makanan tambahan serta di dalam aplikasi farmaseutikal.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to the Almighty Allah for His infinite mercy and abundant graces.

I would like to give my sincere appreciation to my supervisor, Associate Professor Dr Juzu Hayati Arshad for her guidance, critical suggestions, help and encouragement from time to time. I am also very thankful for the help, support and guidance given by my co-supervisors, Associate Professor Dr Johari Ramli and Associate Professor Dr Radzali Muse. I wish to thank Dr Mohd Yunus for his kind help and suggestions while doing the HPLC work.

I wish to thank all my labmates, Mohd Zahari Tajul Hassan, Jawahir Jamid, Suhaida Ahmad, Norazlina Masdor, Lailatul Jumaiyyah, Vanitha Mariappan and Sumirah Mohd Dom for their constant support and encouragement they have given to me. I also want to thank Encik Jasni from the hatchery of Universiti Putra Malaysia for providing me some Malaysian freshwater fish needed in this project and all the staffs in the Department of Biochemistry for providing me support and lab facilities.

Last but not least, I would like to thank my beloved late father, Lokman Ahmad, my mother, Zailah Bukari, and all my siblings, Emirul Adrianne, Eleanor Daniella, Eleanna Zahara and Ezaideen Ahmad for supporting and inspiring me. Not forgetting, Hamezan Bin Muhammad. Thanks for being with me always.

Without all of the people mentioned above, who kindly took time to participate and to help me gather information, this project would not have been accomplished.

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LIST OF ABBREVIATIONS

ATF	Alpha tocopherol
BHT	Butylated hydroxytoluene
CV	Cyclic voltammetry
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic
FRAP	Ferric reducing ability of plasma
$FeCl_2$	Ferrous chloride
HPLC	High Performance Liquid Chromatography
MADA	Muda Agricultural Development Authority
MS	Mass chromatography
ORAC	Oxygen radical absorbance capacity
PUFA	Polyunsaturated fatty acid
SDS	Sodium Dodecyl Sulphate
TAA	Total Antioxidant Activity
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity

CHAPTER 1

INTRODUCTION

All aerobic organisms require protection against deleterious effect of oxygen radical produced from metabolic oxidation reaction in the cell. These reactive oxygen species (ROS) are capable of damaging lipids, DNA, nucleic acid and protein (Di Giulio *et al.*, 1986 and Tyrell, 1991). Unsaturated membrane lipids are the main cellular targets of ROS damage, oxidation of which impairs normal metabolic functions (Slater, 1984).

Living organisms are usually protected from reactive oxygen species by several defence mechanisms, including antioxidant enzymes and low molecular weight antioxidants (Pascual *et al.*, 2003). Biochemical defences against reactive oxygen species include hydrophilic (glutathione, ascorbic acids and uric acids) and lipophilic compounds (vitamin E, ubiquinol and retinol) that scavenge radical species. In contrast to these low molecular weight scavengers, antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase can specifically remove active species leading to the initiation of lipid peroxidation and oxidation of other cellular biomolecules (Ahmad, 1995).

Current sources of natural lipophilic antioxidants include vegetables, plants, and animal tissues (Ruperez *et al.*, 2001). The most commonly used synthetic antioxidants to preserve food are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), *tert*- butylhydroquinone (TBHQ) and α- tocopherol (ATF). However, synthetic antioxidants have been implicated for several diseases such as liver damage and carcinogenesis

(Gulcin *et al.*, 2004). Therefore, the utilization of more effective lipophilic antioxidants of natural origin are needed. Few studies on the lipophilic antioxidants in fish species were reported in the previous studies particularly in the marine fish species (Giardina *et al.* 1997; Erickson, 1992; Marcon and Filho, 1999, Gieseg *et al.*, 2000; Dunlap *et al.*, 2002). However, no study has been carried out on the antioxidant activity and lipophilic antioxidants in the Malaysian freshwater fish.

Therefore, the objectives of this study were;

1) to screen the antioxidant activity in the muscle, liver and intestine of eleven Malaysian freshwater fish species using the optimized thiobarbituric acid (TBA) assay (via inhibition of lipid peroxidation)

2) to identify and quantify the lipophilic antioxidants in the selected fish using High Performance Liquid Chromatography (HPLC) analysis.

The Malaysian freshwater fish selected in this study were patin (*Pangasius polyuranodon*), puyu (*Anabas testudineus*), haruan (*Channa striatus*), keli (*Clarias batrachus*), rohu (*Labeo rohita*), tilapia (*Tilapia mossambica*), jelawat (*Leptobarbus hoevenii*), sepat (*Trichogaster pectoralis*), baung (*Hemibagrus nemurus*), lee koh (*Cyprinus carpio carpio*) and lampam jawa (*Puntius gonionotus*). The fish species used in this study were chosen based on its popularity amongst Malaysian.

CHAPTER 2

LITERATURE REVIEW

2.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive chemicals, containing oxygen that can react easily with other molecules resulting in potentially damaging modifications. Reactive oxygen species are formed by several different mechanisms, which include cellular respiration and the interaction of ionizing radiation with biological molecules.

Reactive oxygen species are free radicals, also known as radicals (molecules having an unpaired electron in the outer orbit and unstable), which include hydroxyl, alkoxyl, hydroperoxyl, peroxyl, nitric oxide and superoxide. The non- radical oxygen species include peroxynitrite, hypochlorite, hydroperoxide, singlet oxygen and hydrogen peroxide (Abuja and Albertini, 2001). Hydroperoxides (a non- radical species) will lead to the formation of alkoxyl and peroxyl radicals in the presence of transition metal ions. The lists of radical and non- radical species are shown in Table 1.

Radicals can give their unpaired electron to other compounds and may cause chain reactions, polymer breakage and lipid peroxidation (Özben, 1997). Free radicals may cause oxidative damage to lipid, protein DNA and nucleic acids. Unsaturated membrane lipids are the main cellular targets of ROS damage, oxidation of which impairs normal metabolic functions (Slater, 1984) which may lead to many biological complications including carcinogenesis, mutagenesis, aging and artherosclerosis (Halliwell and Gutteridge, 1989).

Table 1: Reactive oxidant species (Abuja and Albertini, 2001)

Reactive	Symbol	Non-radical	Symbol
Hydroxyl	$^{\bullet}OH$	Peroxynitrite	$ONOO^-$
Alkoxyl	L (R)$O^{\bullet}$	Hypochlorite	^-OCl
Hydroperoxyl[a]	$HOO^{\bullet}$	Hydroperoxide[b]	L (R)OOH
Peroxyl	L(R)$OO^{\bullet}$	Singlet oxygen	$^{1\Delta}O_2$
Nitric oxide[c]	$NO^{\bullet}$	Hydrogen peroxide[d]	H_2O_2
Superoxide	$O_2^{\bullet-}$		

[a]Hydroperoxyl radical is the conjugated acid of superoxide anion and it is present in aqueous solution at concentrations dependent on pH.

[b]In the presence of transition metal ions, hydroperoxides will lead to the formation of alkoxyl and peroxyl radicals.

[c]$NO^{\bullet}$ itself is rather unreactive and is often regarded as an antioxidant in lipid peroxidation processes. In the presence of superoxide, it forms peroxynitrite, which is strongly oxidizing.

[d]Superoxide is not a good oxidant, rather it is a reductant. It's importance in biological oxidation is a consequence of its ability to both oxidize and reduce transition metal ions, which leads to the formation of H_2O_2 leading to the production of $^{\bullet}OH$ in the presence of reduced transition metal ions.

2.2 Mechanism of Free Radicals on Polyunsaturated Fatty Acids (PUFA)

The polyunsaturated lipid peroxidation process initiates by reactive oxygen species can be divided into initiation, propagation and termination phases as shown in Figure 1. Initiation takes place through a transition metal-induced (or radiation-induced) abstraction of a hydrogen atom from a methylene group of a fatty acid containing two or more separated double-bonds, forming a carbon-centered alkyl radical ($L^{\bullet}$), with a simultaneous rearrangement of the double-bonds to become conjugated ("diene conjugation"). The $L^{\bullet}$ formed reacts with O_2 rate giving rise to a peroxyl radical ($LOO^{\bullet}$).

Propagation involves the abstraction of a hydrogen atom from an adjacent unsaturated fatty acid by $LOO^{\bullet}$, resulting in the formation of a lipid hydroperoxide (LOOH) and a new $L^{\bullet}$ radical. LOOH can react with Fe^{2+}, producing the alkoxyl radical ($LO^{\bullet}$). This radical, which is more reactive than $LOO^{\bullet}$, can again reinitiate lipid peroxidation by hydrogen abstraction from an adjacent polyunsaturated fatty acids, with the formation of $L^{\bullet}$ and alcohol (LOH) at the end product.

LOOH can also undergo degradation into hydrocarbons (ethane, pentane), alcohols, ethers, epoxides and aldehydes. Among the latter, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are of special importance since they can cross-link phospholipids, proteins and DNA. Termination of the lipid peroxidation process is generally believed to take place by interaction between two radicals, to form a non-radical product (Özben, 1997).