



UNIVERSITI PUTRA MALAYSIA

***BIOGENIC AMINES AND UROCANIC ACID IN KEROPOK LEKOR, AND
THEIR EFFECTS ON CYTOTOXICITY AND PROINFLAMMATORY
MEDIATOR SECRETION OF MACROPHAGE CELL CULTURE***

HUSNIZA BINTI HUSSAIN

FSTM 2018 24



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By

HUSNIZA BINTI HUSSAIN

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

August 2017

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

BIOGENIC AMINES AND UROCANIC ACID IN *KEROPOK LEKOR*, AND THEIR EFFECTS ON CYTOTOXICITY AND PROINFLAMMATORY MEDIATOR SECRETION OF MACROPHAGE CELL CULTURE

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August 2017

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Scombroid fish poisoning (SFP) or histamine fish poisoning (HFP) is caused by consumption of mishandled fish or fishery products containing high contents of histamine and leads to allergy-like reaction. *Keropok lekor* or Malaysian fish sausage is a widely consume fish product. In fish products, some spoilage indicators are used including the levels of biogenic amines (BAs), urocanic acid (UCA), and presence of microorganisms. This thesis work aimed to develop rapid and robust methods for quantification/screening of nine BAs, UCA isomers and amino acid decarboxylase-producing bacteria in *keropok lekor* processed at different stages. Furthermore, the cytotoxicity and induction of proinflammatory mediator secretion by individual or mixture of the investigated compounds were carried out on RAW 264.7 macrophage cell culture. The modified Ultra High Performance Liquid Chromatography enabled rapid verification measurement of BAs and UCA regardless of inclusion of internal standard. The validated Liquid Chromatography-Mass Spectrometry using high strength silica polyfluorophenyl column allowed simultaneous measurement of all investigated compounds. The Møller Decarboxylase Micro-Method for screening of BAs-producing bacteria showed cost-effectiveness with small volume of growth media used, less laborious with reduce waste disposal. The developed method for determination of simultaneous cells viability using multilabel microplate reader may serve as an alternative application of acridine orange and propidium iodide in addition to their common usage in confocal microscopy imaging. This study revealed that BAs levels did not increase following the heat treatment i.e. boiling and frying of the *keropok lekor*. Nonetheless, frying process was showed to be best eliminating the amino-acid decarboxylase-producing bacteria. Several microorganisms were identified in both the fresh and processed *keropok lekor*. These include *Proteus vulgaris*, *Proteus mirabilis*, *Kocuria rhizophila*, *Kocuria kristinae*, *Lactococcus garvieae*, *Granulicatella elegans* and *Candida parapsilosis*. The study on the effects of BAs on RAW 264.7 macrophage cell culture showed that individual tyramine, tryptamine, spermine and spermidine induced inflammation in the macrophages led to apoptosis and necrosis, but no progression of inflammation nor secretion of proinflammatory mediators were observed with the *keropok lekor* extracts and its BAs mixtures. Spermine and

spermidine were the most cytotoxic and the inhibition concentrations at 50% cell viability (IC_{50}) were 28.35 $\mu\text{g/ml}$ and 56.01 $\mu\text{g/ml}$, respectively. Taken together, this thesis work develops several modified methods that are rapid and robust for quantifying and screening of BAs, UCA and microorganisms in fishery products i.e. *keropok lekor*. The study also suggests that effective heat treatment i.e. frying could improve the microbiological quality of *keropok lekor* but not the contents of BAs. In addition, different food-related BAs exhibit different cytotoxicity effects on the RAW 264.7 macrophage cell culture. Future research on *in vitro* cytotoxicity effects of BAs in fishery products or other foods using different cell lines to resemble *in vivo* study is warranted.



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

**AMINA BIOGENIK DAN ASID UROCANIC DI DALAM KEROPOK LEKOR,
DAN KESAN TERHADAP SITOTOKSISITI DAN PEREMBESAN MEDIATOR
PRO-INFLAMASI DARI KULTUR SEL MAKROFAJ**

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Keracunan ikan Scombroid (SFP) atau keracunan ikan histamine (HFP) disebabkan oleh pengambilan ikan membusuk atau produk perikanan yang mengandungi kadar histamin yang tinggi dan menyebabkan tindak balas seperti alergi. Keropok lekor atau sosej ikan Malaysia adalah produk ikan yang popular. Dalam produk ikan, beberapa petunjuk tahap kerosakan digunakan termasuk tahap amina biogenik (BA), asid urocanic (UCA), dan kehadiran mikroorganisma. Tesis ini bertujuan untuk membangunkan kaedah yang cepat dan mantap untuk kuantifikasi/pemeriksaan kehadiran sembilan BA, isomer UCA dan bakteria penghasil enzim decarboxylase asid amino di dalam keropok lekor yang pada peringkat pemprosesan yang berbeza. Selain itu, kesan sitotoksiti dan induksi rembesan mediator pro-inflamasi oleh kompaun individu atau sebatian telah dikaji pada kultur sel makrofaj RAW 264.7. *Ultra High Performance Liquid Chromatography* (UHPLC) yang diubah suai membolehkan kuantifikasi pantas BA dan UCA bersama/tanpa kemasukan standard dalaman (IS). *Liquid Chromatography-Mass Spectrometry (LC-MS)* yang divalidasikan menggunakan kolum silika polifluorofenil berkekuatan tinggi membenarkan pengukuran seiring semua sebatian yang dikaji. *Møller Decarboxylase Micro-Method* untuk pemeriksaan kehadiran bakteria penghasil BA memperlihatkan keberkesanan kos dengan menggunakan jumlah media pertumbuhan yang kecil, lebih ringkas dan kurang penghasilan sisa. Kaedah yang dibangunkan untuk menentukan viabiliti sel dan *non-viability* sel menggunakan pembaca mikroplat multilabel boleh berfungsi sebagai aplikasi alternatif *acridine orange* (AO) dan propidium iodida (PI) sebagai tambahan kepada aplikasi terdahulu untuk pengimejan melalui mikroskopi *confocal*. Kajian ini menunjukkan bahawa tahap BA tidak meningkat berikutan pendidihan dan penggorengan keropok lekor. Walau bagaimanapun, proses penggorengan menunjukkan bahawa ia adalah terbaik untuk menghapuskan bakteria penghasil enzim decarboxylase asid amino. Beberapa mikroorganisma telah dikenal pasti di dalam kedua-dua keropok lekor yang segar dan diproses. Ini termasuk *Proteus vulgaris*, *Proteus mirabilis*, *Kocuria rhizophila*, *Kocuria kristinae*, *Lactococcus garvieae*, *Granulicatella elegans* dan *Candida parapsilosis*. Kajian mengenai kesan BA pada kultur sel makrofaj RAW 264.7 menunjukkan bahawa tyramine, tryptamine, spermine

dan spermidine individu yang menyebabkan inflamasi pada makrofaj yang menjurus kepada apoptosis dan nekrosis, tetapi tiada perkembangan inflamasi atau rembesan mediator pro-inflamasi diperhatikan dengan ekstrak keropok lekor dan sebatian BA. Spermine dan spermidine adalah yang paling sitotoksik dan kepekatan yang menyebabkan kerencatan viabiliti sel pada kadar 50% (IC₅₀) masing-masing adalah 28.35 µg/ml dan 56.01 µg/ml. Secara keseluruhan, tesis ini menghasilkan beberapa kaedah ujikaji yang diubahsuai dan divalidasi agar lebih cepat dan mantap untuk kuantifikasi BA dan UCA dan penyingkapan mikroorganisma dalam produk perikanan keropok lekor. Kajian ini juga menunjukkan bahawa aplikasi haba yang berkesan iaitu penggorengan boleh menghapuskan mikroorganisma dalam keropok lekor tetapi tidak ke atas kandungan BA. Di samping itu, BA yang berbeza memberikan kesan sitotoksiti yang berbeza pada kultur sel makrofaj RAW 264.7. Kesan sitotoksik *in vitro* BA dalam produk perikanan atau makanan lain dengan menggunakan kultur sel yang berbeza untuk menyamai kajian *in vivo* adalah wajar dijalankan di masa hadapan.

ACKNOWLEDGEMENTS

In the name of Allah, the Beneficent, the Merciful.

Read: In the name of thy Lord who createth,
Createth man from a clot. [Surah 96 (Al-Alaq), Ayat 1-2].

First and foremost, I would like to express my utmost gratitude to Allah for bestowing numerous sustenance through camaraderie with the best acquaintances to whom I have crossed my path with, and for giving me the knowledge which I could convey to others for the benefit of mankind. May all of them be rewarded sufficiently.

With sincere heartfelt, I would like to thank my supervisors, Prof. Dr. Hasanah Mohd. Ghazali, Prof. Dr. Jamilah Bakar, Dr. Wan Nazaimoon Wan Mohamud and Dr. Mohd. Fuat Abd. Razak for their continuous guidance and teachings throughout my ten years of post-graduate study in the Universiti Putra Malaysia.

Special thanks to my companions whom with their presence has added joy to my life and makes my journey very meaningful; to Zarafina Abdul Rahman, Dr. Too ChunLai, Ms. B. Vimala d/o R.M.T. Balasubramaniam and Ms. Lee Sin who constantly being there in my rainy days, and willingly sharing knowledge and expertise in completing this dissertation; to Hafeezah for being strong through ups and downs, and continue believing in me; to Hafeezudin for standing by me and being my protector; and to IA who holds endeavours in bringing hopes to reality.

Finally, I am thankful for having supportive parents, Hjh. Siti Zaayah Osman and Hj. Hussain Zainal Abidin, whom have shown me the meaning of love and being my first two persons whom I could rely on.

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

AGM	Agmatine
ANOVA	Analysis of Variance
ARG	Arginine
BA	Biogenic amines
BGB	Brilliant green bile
CAD	Cadaverine
CI	Combination index
CO ₂	Carbon dioxide
CYS	Cysteine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ESTD	External standard
FBS	Foetal Bovine Serum
H ₂ O	Water
HBSS	Hank's Buffered Salt Solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIM	Histamine
HIS	Histidine
HPLC	High Performance Liquid Chromatography
hr	Hour
IDL	Instrument Detection Limit
IFN- γ	Interferon-gamma
IL-12	Interleukin-12
IQL	Instrument Quantification Limit
ISTD	Internal standard
KLAB	<i>Keropok lekor</i> after boiling
KLAC	<i>Keropok lekor</i> after cooling
KLAF	<i>Keropok lekor</i> after frying
KLD	<i>Keropok lekor</i> dough
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
L-NAME	L-Nitro-Arginine Methyl Ester
LoB	Limit of Blank
LoD	Limit of Detection
LoQ	Limit of Quantification
LSB	Lauryl sulphate broth
LYS	Lysine
MDBB	Moeller decarboxylase base broth
MDL	Method Detection Limit
MFM	Minced fish meat
min	Minute
MQL	Method Quantification Limit
MWCO	Molecular weight cut-off
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NaOH	Sodium hydroxide

NO	Nitric oxide
ORN	Ornithine
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween 20
PC	Principal component
PCA	Plate count agar
PCA*	Principal Component Analysis
PGE ₂	Prostaglandin E ₂
PHE	Phenylalanine
PHM	2-phenylethylamine
PLA ₂	Phospholipase A ₂
PUT	Putrescine
RBC	Rose-Bengal chloramphenicol
RKL	Rod-shaped <i>keropok lekor</i>
RT	Room temperature
s	Second
SKL	Sliced <i>keropok lekor</i>
SPD	Spermidine
SPM	Spermine
TNF- α	Tumor necrosis factor-alpha
TPM	Tryptamine
TRP	Tryptophan
TYM	Tyramine
TYR	Tyrosine
UCA	Urocanic acid
UHPLC	Ultra High Performance Liquid Chromatography
VRBGA	Violet red bile glucose agar

CHAPTER 1

INTRODUCTION

Eating fish regularly can be very beneficial for our health, and it can help reduce risk for some diseases, particularly those related to the heart. However, toxins in contaminated fish can cause health problems such as scombroid fish poisoning (SFP). SFP is caused by eating fish that has not been properly refrigerated or preserved and thus contains high contents of histamine. Without adequate cooling or improper preservation, some symbiotic bacteria such as *Escherichia coli*, *Morganella morganii*, *Proteus* and *Klebsiella* species can convert histidine, present in the fish tissues, to histamine. In raw fish left at room temperature, these bacteria multiply rapidly; increase the histidine-to-histamine conversion rate, raising the histamine levels and reaching toxic concentration within 12 hours. In SFP cases, histamine is thought to produce the clinical manifestations of illness, thus also termed as histamine fish poisoning (HFP).

Although SFP is caused by bacterially-generated toxins i.e. histamine in the fish, its symptoms usually resemble an allergic reaction, such as flushing of the face, headache, heart palpitations, itching, blurred vision, cramps, diarrhoea and a burning sensation or peppery taste in the mouth. Thus, SFP are often misdiagnosed as allergy reaction (Vlieg-Boerstra et al., 2005). SFP cases were rarely reported in Malaysia. Nonetheless, isolated cases were documented in various developed countries (Merson et al., 1974; Stratton et al., 1991).

The main biogenic amines (BAs) encountered in foods include histamine (HIM), 2-phenylethylamine (PHM), tyramine (TYM), tryptamine (TPM), putrescine (PUT), cadaverine (CAD), spermine (SPM), spermidine (SPD) and agmatine (AGM). These compounds were studied extensively in SFP-related food toxicity (Taylor, 1989; Til et al., 1997; Ansorena, et al., 2002). The four former amines have important physiological effects in human, either psychoactive which affect the nervous system or vasoactive which act on the vascular system (Lovenberg, 1973), whilst other secondary amines (i.e. agmatine, spermine and spermidine) in fish, meat and vegetable products are known to be converted to carcinogenic *N*-nitrosamines with the presence of nitrite. Consumption of foods containing high amount of these amines can be toxic (Shalaby, 1996).

In addition to the presence of histamine through consumption of mishandled fish or seafood-based products, there is another mechanism which had been suggested contributing to SFP i.e. the presence of urocanic acid (UCA). Urocanic acid is an intermediate in the catabolism of L-histidine. Urocanic acid is found predominantly in the stratum corneum of the skin and is likely that most of it is derived from filaggrin catabolism (a histidine-rich protein) (Moodycliffe et al.,

1992). When exposed to ultraviolet B (UVB) irradiation, *trans*-urocanic acid is converted *in vitro* and *in vivo* to the *cis* isomer. *Cis*-urocanic acid is the triggering agent in the UVB induced immunosuppression. It stimulates the release of neuropeptides which in turn cause the release of endogenous histamine and tumour necrosis factor- α (TNF- α) in mast cells. The release of this histamine also elicits allergic-like reaction.

Histamine can be formed endogenously in the human body and it is released by mast cells in an allergy reaction (Johnson & Erdös, 1973). When histamine is consumed in food due to microbial contamination, it is inactivated by amine oxidases in the gut (Halász *et al.*, 1994; Stadnik & Dolatowski, 2010). The enzyme activity can however be inhibited in individuals with susceptible genetic risk factors, gastrointestinal diseases, or consumed certain medicines and alcohol (Maintz & Novak, 2007).

Cases on HFP had been reported in various developed countries. Thus, research on biogenic amine levels on *keropok lekor*, the popular fish-based food in Malaysia needs to be undertaken. In Malaysia, the study of BAs or microorganisms in fish-based foods including the widely consumed snack named *keropok lekor* had been scarce (Hassan *et al.*, 2009; Mahmud *et al.*, 2009). Thus, it is crucial to determine and identify the main microorganisms that generate the BAs in the *keropok lekor* to prevent the occurrence of SFP. This study is a revisited study over the investigation done by Mahmud *et al.* (2009a & 2009b) on *keropok lekor*. Their findings showed that the biogenic amine levels were < 100 mg/kg. The observed low level may not reflect the whole *keropok lekor* industry in Malaysia. Various reports also had stated that biogenic amines were present in high level in fish-based foods (Tsai *et al.*, 2005; Saaid *et al.*, 2009; Zaman *et al.*, 2011), thus, the inconsistencies would likely be present in different manufacturing sites.

The roles of biogenic amines and urocanic acid compounds in food intolerance and toxicity is poorly understood (De Meulenaer, 2006). However, the involvement of immune response in food intolerance or food toxicity had been discussed in relation to the cytokine levels (Gallardo *et al.*, 1994; Jacobsen *et al.*, 2000; Chang & Adami, 2006). Our body defenses against microbial infection, injury and toxic compounds in food allergy, food intolerance and food toxicity led to inflammation (Genuis, 2010; Langerholm *et al.* 2011). Macrophages are among the first immune cells confronting absorbed food compounds. Thus, the study of the effects for BAs and urocanic acid compounds on macrophages is needed to understand their roles in initiation of inflammation due to consumption of mishandled fish.

In food industry, low-histamine technology in food processing has been introduced and practiced (Bodmer *et al.*, 1999). It is timely and relevant to determine the levels of biogenic amines and urocanic acid in *keropok lekor* during its processing, after frying and those which are commercially available in markets. Taken

together, we can compare the freshness and levels of biogenic amines/urocanic acid of differently processed or commercially available *keropok lekor* in the markets based on the Biogenic Amines Index (Mietz & Karmas, 1977; Veciana-Nogues *et al.*, 1997).

Different methods based on liquid chromatography have been used for the detection of biogenic amines and urocanic acid independently in food. Thus, there is a need to develop a robust liquid chromatography method for quantification of biogenic amines and urocanic acid simultaneously in a single assay as well as developing a better screening method for amino acid decarboxylase-producing bacteria in foods.

This study was done to visit another *keropok lekor* manufacturing site and to determine the microbiological quality and biogenic amine contents in the minced fish meat obtained by the manufacturer and the quality of fresh, processed and fried *keropok lekor*. This study is carried out based on the following objectives:

- (i) To develop quantification/screening methods for biogenic amines, *trans*- and *cis*-urocanic acid and amino acid decarboxylase-producing bacteria in *keropok lekor*,
- (ii) To determine biogenic amines and *trans*- and *cis*-urocanic acid contents in fresh, processed and fried *keropok lekor*,
- (iii) To enumerate and identify the amino acid decarboxylase-producing bacteria present in *keropok lekor*,
- (iv) To determine the cytotoxic and induction of pro-inflammatory mediator secretion effects of BAs, *trans*- and *cis*-UCA, and *keropok lekor* extracts on RAW 264.7 macrophage cell.

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