DEVELOPMENT OF A NOVEL ENZYMATIC ASSAY FOR THE DETECTION OF HYPOXANTHINE FOR FRESHNESS ESTIMATION

JUNAIPIN BIN AMIN

THIS DISERTATION IS BEEN PRESENTED TO FULLFILL PART OF THE REQUIREMENT TO OBTAIN BACHELOR OF SCIENCE WITH HONOURS

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Ijazah: SARJANA MUDA SAINS (BIOTEKNOLOGI)

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DECLARATION

I affirm that this paper is my own work effort, except for the references and summaries, which have been, cited clearly their sources.

19 November 2006

JUNAIPIN BIN AMIN
HS2003-2856

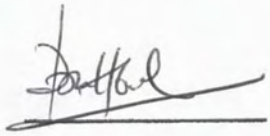
CONFIRMATION BY

Signature

Professor Datuk Dr. Kamaruzaman Ampon _____

(SUPERVISOR)

Dr Roziah Hj. Kambol



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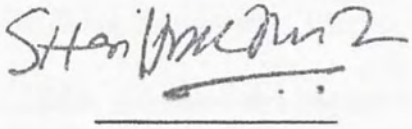

Assc. Professor Dr. Shariff A.K Omang _____

(DEAN)

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ABSTRACT

Hypoxanthine is a naturally occurring purine derivative and one of the substrate for the enzyme xanthine oxides. Xanthine oxidase oxidizes hypoxanthine forming simpler molecule, xanthine. Further oxidation of xanthine by xanthine oxidase produce uric acid and trace amount of hydrogen peroxide as a byproduct. Hypoxanthine derived from common molecule found in muscles (ATP). This paper studies the development of a system to detect hydrogen peroxide in order to establish correlation between the quantity of hydrogen peroxide and the freshness of any ATP containing protein. A system consists of xanthine oxidase, peroxidase and 3,5,3′5′-Tetramethylbenzidine (TMB) is being developed. The effect of hypoxanthine, hydrogen peroxide and 3,5,3′5′-Tetramethylbenzidine (TMB) concentration were studied via real time scanning using spectrophotometer at 660 nm wavelength. Two of these parameters (TMB and hypoxanthine) show direct proportional relation between their respective concentration and maximum absorbance value, while hydrogen peroxide reacted differently. The data obtained in this experiment originated from liquid phase since dry form of this system was not fully optimized to generate detectable result.

ABSTRAK

Hypoxanthine ialah bahan terbitan semulajadi *purine*, dan juga ia merupakan salah satu bahan tindak balas bagi enzim *xanthine oxidase*. Enzim *xanthine oxidase* mengoksidakan *hypoxanthine* membentuk molekul yang lebih ringkas iaitu *xanthine*. Pengoksidaan lanjut *xanthine* oleh enzim *xanthine oxidase* menghasilkan asid urik dan sedikit hidrogen peroksida sebagai hasil sampingan. *Hypoxanthine* adalah bahan yang berasal dari molekul yang ditemui pada otot (ATP). Kertas laporan ini mengkaji pembangunan sistem untuk mengesan kehadiran hidrogen peroksida yang bertujuan untuk mewujudkan satu hubugkait antara kuantiti hidrogen peroksida dan kesegaran sebarang bahan yang mengandungi ATP. Sistem yang terdiri daripada enzim *xanthine oxidase, peroxidase* dan *3,5,3'5'-Tetramethylbenzidine* (TMB) cuba dibangunkan. Kesan kepekatan *hypoxanthine*, hidrogen peroksida dan TMB dikaji menerusi imbasan masa nyata dengan menggunakan spectrofotometer pada jarak gelombang 660 nm. Dua daripada parameter ini (TMB dan Hypoxanthine) menunjukkan keputusan perkadaran secara terus di antara kepekatan dan juga bacaan cerapan maksimum, manakala ujian kepekatan hydrogen peroksida menunjukkan keputusan yang berlainan dengan dua parameter yang terdahulu. Data-data yang diperoleh dalam eksperimen ini merupakan data yang berasal dari ujikaji sistem yang dilakukan dalam bentuk cecair kerana sistem dalam bentuk kering tidak dapat dihasilkan dengan baik untuk menghasilkan keputusan yang boleh dikesan.

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LIST OF ABBREVIATION

rpm	Rotation Per Minute
ml	millilitre
µl	microliter
mg/ml	milligram per millilitre
g/ml	gram per millilitre
g/L	gram per litre
N	Normality
L	litre
mg	milligram
µM	micromolar
mM	milimolar
cm	centimeter
^0C	degree Celsius
cells/ ml	cells per milliliter
v/v	volume per volume
min	minute
mm	millimeter
M	Molar
%	percent
et al.	*et alia*
$O_2^{\bullet-}$	Oxygen free radical

Fe^{3+}	ferric ion
dH_2O	distilled water
H_2O_2	hydrogen peroxide
$NaH_2PO_4.H_2O$	sodium dihydrogen phosphate dehydrate
K_2HPO_4	dipotassium hydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
NaOH	sodium hydroxide
$CuSO_4.5H_2O$	copper (II) sulfate pentahydrate
H_2SO_4	sulfuric acid
XO	xanthine oxidase
BOD	Biological oxygen demand
Etc.	Et cetera
A.D	Anno Domini
TMA	Trymethylainine
DMA	Dimethylainine
Hx	Hypoxanthine
Da	Dalton
FAD	flavin adenine dinucleotide
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
XDH	xanthine dehydrogenase
EC	enzyme commission

CHAPTER 1

INTRODUCTION

1.1 Introduction

Fisheries product is not only important in terms of nutrition and health but it is also important in the field which can bring profit to certain people such as fishermen, exporters etc. who depend on fisheries industries for survival.

Fisheries products available in the global market are estimated to be more than 50 billion dollars (RM 190 billion) in recent years (Venugopal, 2001). Statistical research shows increment in demand on this commodity since a few years back. There are 120 million metric tones of fish production and shows that there are insufficient supplies of this commodity even though the increment and the total global fisheries items are very huge. It seems that the supplies failed to cater for all global demand according to a review on seafood safety and quality by Vengopal (2001).

Relatively, fisheries items are harder to be transported between countries as to other product that are not related to fish like chicken based product, beef related or lamb related product. This is because fish are very diverse in many aspects that can contribute

to the rate of fish deterioration. These factors include species, sex, maturity, habitat, enzymatic activity and the availability of microbes in the dead fish (Venugopal, 2001).

The level of fish freshness cannot be estimated accurately or precisely by merely by a physical appearance. Fish that is physically good cannot be guaranteed in term of the freshness of that particular fish. Nowadays, sellers or traders especially those who sell directly to consumer use chemicals to prolong the physical condition of the fish. This is not fair to the consumer because the real condition of the fish cannot be based on what they saw from the physical appearance. Common tricks that the sellers often use include moisturizing the gill with blood so that it looks fresh, use specially made powder to make the eyes look clear and use chemical to prevent the formation of ammonia which makes the fish market smelly. Thus, the fisheries industry needs an instrument or a procedure to identify or determine the real condition of a fish and estimates the fish's freshness level without interruption caused by the preservatives which is only effective on its physical appearance but not to its overall quality. This instrument aims to help the fisheries industry to develop a standard or a reference for the level of fish freshness and quality and also to help in freshness estimation of fish rapidly in the field in order to overcome the deteriorated fish supply in the market.

Generally fresh fish is a fish that is structurally excellent, looks good and taste good (Venugopal, 2001). However, freshness characteristics are subjective and depend on individual opinions and preference. The evaluation of fish freshness in every individual is

different and depends on the level of response or reaction towards the tested characteristics (Dalgaard, 2000).

1.2 Research Scope

The scope of this research is to develop a novel enzymatic assay for freshness estimation by detecting hypoxanthine quantitatively or semi quantitatively

1.3 Research Objectives

The objectives of this research are to prepare a working enzyme sensor consisting of xanthine oxidase (XO) and hydrogen peroxide detecting reagent and also to determine the concentration of hydrogen peroxide based on the reaction.

CHAPTER 2

LITERATURE REVIEW

2.1 Biosensor

Basically, biosensor is a device that measures changes upon certain parameters that are specific for the integrated sensing element, and the integrated sensing element is made of biological component (Venugopal, 2001).

According to the IUPAC definition,
"Biosensor is a self-contained integrated device that is capable of providing specific quantitative analytical information using biological recognition element which is in direct spatial contact with a transduction element" – IUPAC 1996 (Inventory Of Definitions In Analytical Chemistry, Division Of Analytical Chemistry, ed: 2002).

Biosensor is a device that uses biological materials to detect certain substances qualitatively, semi-quantitatively or quantitatively. Generally, biosensor possesses several components as mentioned in the table below.

Component	Function
The detected parameters	The specific substrate that will be detected by the specific biological component
Biological component	Produce products from substrates. Example: enzyme
Transducer	To change chemical reaction signal into an understandable form such as electrical signal, color formation etc.
Detector	To detect and to record changes that occur in the transducer
Amplifier	To amplify the electrical signal received from transducer

Table 2.1 important components in a general biosensor (Gopel *et al.*, 1991).

The principle of biosensor is simple and is based on the concept that an enzyme is specific for certain substrate. With this basic concept, an accurate and precise biosensor can be develop. Below is the working principle of a biosensor based on the change of electric current (S. Zhang *et al*, 1999).

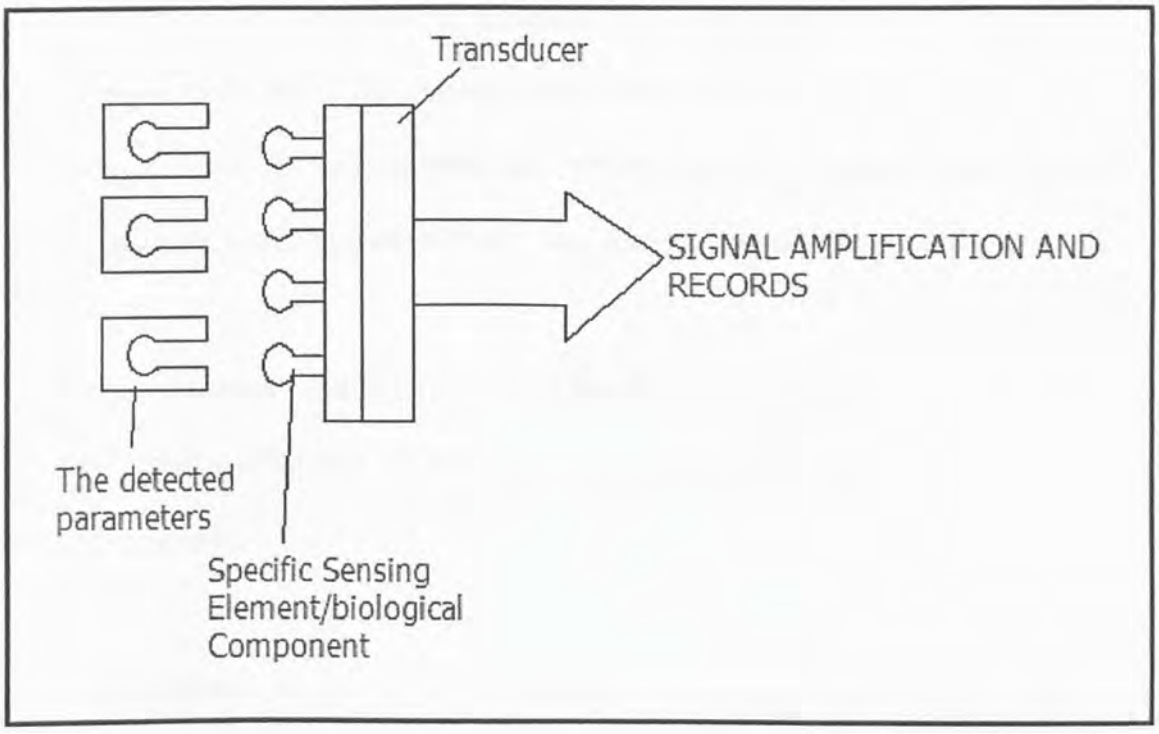

Figure 2.1 The main components of colorimetric biosensors (Elizabeth, 1991)

Biosensor is applicable to many fields. Despite its simplicity and rapidness of procedure, it also has the advantage in terms of accuracy and selectivity. The potential applications of biosensors are as follow:

- Clinical diagnostic and biomedical
- Agricultural, horticultural, veterinary analysis
- Pollution, water, microbial contamination analysis
- Fermentation analysis and control
- Industrial gasses
- Toxic gases explosives and military arena
- Flavors, essences and pheromones

REFERENCES

Ashok, K., Ukeda, H., Kawana, D., Kawamura, M., 1999. Spectrophotomtric flow injection analysis forhypoxanthine based on the detection of superoxide anion. *Analytical Sciences* **Vol.15.**

Borges, F., Fernandes, E., and Roleira, F., 2002. Progress Towards the Discovery of Xanthine Oxidase Inhibitors. *Current Medicinal Chemistry* **9**, 195-217.

Dalgaard, P., 2000. Freshness, quality and safety in seafoods, *Danish Institute for Fisheries Research.*

Dimcheva, N., Horozova, E., and Jordanova, Z., 2002. An Amperometric Xanthine Oxidase Enzyme Electrode Based on Hydrogen Peroxide Electroreduction.

Elizabeth, A.H.H., 1991. Overview Of Biosensors, Biosensors and Chemical Sensors. *ACS Symposium Series* **487.**

Fortier, G., Chen, J.W., Belangor, D., 1991. Biosensors based On Entrapment Of Enzyme in Water-Dispersed Anionic Polymer. *Biosensors and Chemical Sensors, ACS Symposium Series* **487.**

Fraser, O., and Sumar, S., 1992. Compositional changes and spoilage in fish – an introduction. *Food Research Centre, South Bank University, London, UK.*

Guizani, N., Abdallah, M., Al-Busaidy, Al-Belushi, I.M., Mothershaw, A., Rahman, M.S., 2005. The effect of storage temperature on histamine production and the freshness of yellowfin tuna (Thunnus albacares). *Food Research International* **38,** 215–222.

Jason, A.C., and Richards, J.C.S., 1975. The development of an electronic fish freshness meter, *Journal of Physics Electronic: Scientific Instruments* **8.**

Jiang, Z.Y., Alison, C., Woollard, S. and Wolff, S.P., 1990. Hydrogen peroxide production during experimental protein glycation. **268** (1), 69-71.

Josephy, P.D., Eling, T., and Mason, R.P., 1982. The Horseradish Peroxidase-Catalyzed Oxidation Of 3,5,3',5'- Tetramethylbenzidine Free Radical And Charge-Transfer Complex Intermediates. *The Journal Of Biological Chemistry* **257** (7).

Li, H., Samouilov, A., Liu, X., and Zweier, J.L., 2001. Characterization of the Magnitude and Kinetics of Xanthine Oxidase-catalyzed Nitrite Reduction. **276** (27), 24482–24489.

Li, L., 2001. Xanthine Oxidase. *Free Radicals in Biology and Medicine* **77** (222).

Llaudet, E., Botting, N.P., Crayston, J.A., Dale, N., 2002. A three-enzyme microelectrode sensor for detecting purine release from central nervous system, *Biosensors and Bioelectronics* **18**, 43-52.

Lougovoisa, V.P., Kyranasb, E.R., Kyranaa, V.R., 2003. Comparison of selected methods of assessing freshness quality and remaining storage life of iced gilthead sea bream (Sparus aurata). *Food Research International* **36**, 551–560.

Martin, S.P., Lynch, J.M., Reddy, S.M., 2002. Optimisation Of The Enzyme-Based Determination Of Hydrogen Peroxide Using The Quartz Crystal Microbalance. *Biosensors And Bioelectronics* **17**, 735-739.

Mesulam, M., Hegarty, E., Barbas, H., Carson, K.A., Gower, E.C., Knapp, A.G., Moss, M.B., and Mufson, E.J., 1980. Additional Factors Influencing Sensitivity In The Tetramethyl Benzidine Method For Horseradish Peroxidase Neurohistochemistry. *The Journal Of Histochemistry And Cytochemistry* **28**, 1255-1259.

Metelitza, D.I., Karasyova, E.I., Grintsevich, E.E., Thorneley, R.N.F., 2003. Peroxidase-Catalyzed Co-Oxidation Of 3,3,5,5-TetramethylbenzidineIn The Presence Of Substituted Phenols And Their Polydisulfides. *Journal Of Inorganic Biochemistry* **98**, 1–9.

Okuma, H, Watanabe, E., 2002. Flow system for fish freshness determination based on double multi-enzyme reactor electrodes. *Biosensors & Bioelectronics* **17**, 367–372.

Rye, D.B., Saper, C.B., and Wainer, B.H., 1983. Stabilization Of The Tetramethylbenzidine (TMB)Reaction Product: Application For Retrograde And Anterograde Tracing. *The Journal Of Histochemistry And Cytochemistry*, 1145-1153.

Ryu, K., and Dordick, J.S., 1992. How Do Organic Solvents Affect Peroxidase Structure And Function, *Journal of Biochemistry*, 2588-2598.

Sato, N.H., Usui, K., Kobayashi, T., Watanabe, E., 2005. Quality assurance of raw fish based on HACCP concept. *Food Control* **16**, 301–307.

Scrimgeour, K.G., 1977. Chemistry And Control Of Enzyme Reaction. *Academic Press Inc.*, 3-5.

Tatzber, F., Griebenow, S., Wonisch, W., and Winkler, R., 2002. Dual Method For The Determination Of Peroxidase Activity And Total Peroxides-Iodide Leads To A Significant Increase Of Peroxidase Activity In Human Sera. *Analytical Biochemistry* **316**, 147–153.

Veitch, N.C., 2004. Structural Determinants Ofplant Peroxidase Function, Phytochemistry, Reviews. *Kluwer Academic Publishers* **3**, 3–18.

Venugopal, V., 2002. Biosensors in fish production and quality control (review). *Biosensors & Bioelectronics* **17**, 147–157.

Watanabe, E., Tamada, Y., Sato, N.H., 2005. Development of quality evaluation sensor for fish freshness control based on KI value, *Biosensors and Bioelectronics*.

Zen, M., Lai, Y.Y., Yang, H.H., Kumar, A.S, 2002. Multianalyte Sensor For The Simultaneous Determination Of Hypoxanthine, Xanthine And Uric Acid Based On A Preanodized Nontronite-Coated Screen-Printed Electrode. *Sensors And Actuators*, 237–244.

Zhang, S., Wright, G., Yang, Y., 2000. Materials and techniques for electrochemical biosensor design and construction. *Biosensors & Bioelectronics* **15**, 273–282.

Zhao, J., O'Daly, J.P., and Henkens, R.W., 1996. A xanthine oxidase/colloidal gold enzyme electrode for amperometric biosensor applications. *Biosensors & Bioelectronics* **11**, 493—502.

ZOÚUL, Y.,.ZOÚUL, F., 2000. Degradation Products of Adenine Nucleotide in Rainbow Trout (Oncorhynchus mykiss) Stored in Ice and in Modified Atmosphere Packaging, F*ood Research Centre, UK,*