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

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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

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iii

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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 The effect of hypoxanthine, hydrogen peroxide and developed. 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.



CONTENT

		PAGE
FRO	NT PAGE	i
DEC	LARATION	ii
CON	FIRMATION	iii
ACK	NOWLEDGEMENT	iv
ABS	TRACT	v
ABS	TRAK	vi
CON	TENT	vii
LIST	OF FIGURES	xi
LIST	OF PHOTOS	xiv
LIST	OF TABLES	xv
LIST	OF ABBREVIATION	xvi
CHA	PTER 1 INTRODUCTION	1
1.1	Introduction	1
1.2	Research Scope	3
1.3	Research Objectives	3
CHA	PTER 2 LITERATURE REVIEW	4
2.1	Biosensor	4
2.2	Dry Chemistry	7
2.3	Assessment Of Fish Quality	8
	2.3.1 Sensory Methods	8
	2.3.2 Biochemical Methods And Chemical Methods	9
	2.3.3 Physical Method	10



	2.3.4 Microbiological Methods	10
2.4	Paper Strip Basic Components	11
	2.4.1 Support	12
	2.4.2 Reaction Zone	12
2.5	Hypoxanthine (Hx)	13
2.6	Xanthine Oxidase (E.C.1.1.3.22)	14
	2.6.1 Reaction Catalyzed By Xanthine Oxidase	16
2.7	3,5,3',5'- Tetramethylbenzidine (TMB)	19
2.8	The Proposed Model And Layer Structure	23
	2.8.1 Spreading Layer	25
	2.8.2 Enzyme Layer	25
	2.8.3 Membrane Layer	25
	2.8.4 Indicator Layer	26
2.9	Reaction In The Paper Strip	26
CHA	APTER 3 METHODOLOGY	29
3.1	Reagents And Apparatus	29
3.2	Test Strip Preparation	30
3.3	Standards And Samples Preparation	32
	3.3.1 Preparation Of Hypoxanthine Solution	32
	3.3.2 Preparation Of Phosphate Buffer Ph 7.6	32
	3.3.3 Preparation Of Indicator Reagent Solution	33
	3.3.4 Preparation Of Hydrogen Peroxide Standard Solution	33



	3.3.5	Preparation Of Xanthine Oxidase	33
	3.3.6	Preparation Peroxidase Enzyme Solution	33
	3.3.7	Immobilization Of Xanthine Oxidase	34
	3.3.8	Immobilization Of H ₂ O ₂ Detecting Reagent	34
3.4	Нуроз	kanthine, Hydrogen Peroxide And Tmb Concentration Effect Test	35
	3.4.1	3,5,3',5'- Tetramethylbenzidine (TMB) Concentration Effect	35
	3.4.2	Hydrogen Peroxide Concentration Effect	36
	3.4.3	Hypoxanthine Concentration Effect	37
СНА	PTER 4	RESULT	38
4.1	Enzyn	ne System	38
4.2	3,5,3'	5'- Tetramethylbenzidine (TMB) Concentration Effect	39
4.3	Hydro	gen Peroxide Concentration Effect	43
4.4	Нуро	canthine Concentration Effect	48
4.5	Paper	Strip Development And Dry Chemistry	53
СНА	PTER 5	DISCUSSION	58
5.1	3,5,3'	5'- Tetramethylbenzidine (TMB) Concentration Effect	58
5.2	Hydro	gen Peroxide Concentration Effect	60
5.3	Нурох	anthine Concentration Effect	64
5.4	Dry C	hemistry System	67



CHAPTER 6	CONCLUSSION	69
REFFERENCES	3	71
APPENDIX 1		74
APPENDIX 2		75
APPENDIX 3		76



LIST OF FIGURES

Figure	e No.	Page
2.1	The main components of colorimetric biosensors	6
2.2	A Commercial instrument for freshness evaluation in use	10
2.3	Schematic of a general paper strip	12
2.4	Chemical structure of hypoxanthine	14
2.5	Structural model of xanthine oxidase	14
2.6	The reaction mechanism of xanthine oxidase in the oxidation of	
	xanthine	17
2.7	A schematic diagram illustrating the pathway by which adenine	
	nucleotides are metabolized with the formation of uric acid as the	
	final product	18
2.8	The chemical structure of TMB	19
2.9	TMB first level of oxidation	20
2.10	The complete reaction of TMB oxidation	22
2.11	The design of the test strip	23
2.12	Side and top view of the test strip	23
2.13	Layers constituent of the test strip	24
3.1	Dimensions of the support	30
3.2	Dimensions of the plastic tube	31
4.1	The effect of different (concentration range from 2 μl to 20 $\mu l/ml$)	
	TMB concentration over maximum absorbance reading in the system	39
4.2	Absorbance reading changes over time for 2 µl/ml of TMB in	
	1 ml of assay solution	40
4.3	Absorbance reading changes over time for 5 µl/ml of TMB in	
	1 ml of assay solution	40



4.4	Absorbance reading changes over time for 8 µl/ml of TMB in		
	1 ml of assay solution	41	
4.5	Absorbance reading changes over time for 11 µl/ml of TMB in		
	1 ml of assay solution	41	
4.6	Absorbance reading changes over time for 15 µl/ml of TMB in		
	1 ml of assay solution	42	
4.7	Absorbance reading changes over time for 20 µl/ml of TMB in		
	1 ml of assay solution	42	
4.8	The effect of different hydrogen peroxide concentration		
	(range: 0.1 M to 1.0x10 ⁻⁷ M) over maximum absorbance reading in		
	the system.	43	
4.9	Absorbance reading changes over time for 0.1 M hydrogen peroxide in		
	1 ml assay solution.	44	
4.10	Absorbance reading changes over time for 0.01 M hydrogen peroxide in		
	1 ml assay solution	44	
4.11	Absorbance reading changes over time for 1x10 ⁻³ M hydrogen peroxide in		
	1 ml assay solution.	45	
4.12	Absorbance reading changes over time for 1x10 ⁻⁴ M hydrogen peroxide i	in 1	
	ml assay solution	45	
4.13	Absorbance reading changes over time for 1x10 ⁻⁵ M hydrogen peroxide in		
	1 ml assay solution	46	
4.14	Absorbance reading changes over time for 1x10 ⁻⁶ M hydrogen peroxide	in	
	1 ml assay solution	46	
4.15	Absorbance reading changes over time for 1x10 ⁻⁷ M hydrogen peroxide in		
	1 ml assay solution	47	
4.16	The effect of different hypoxanthine concentration (range: 0.5 mg/ml to		
	0.001 mg/ml) over maximum absorbance reading in the system.	48	
4.17	Absorbance changes over time for 0.5 mg/ml hypoxanthine in		
	1 ml of assay solution	49	
4.18	Absorbance changes over time for 0.3 mg/ml hypoxanthine in		
	1 ml of assay solution	49	



xii

4.19	Absorbance changes over time for 0.25 mg/ml hypoxanthine in	
	1 ml of assay solution	50
4.20	Absorbance changes over time for 0.1 mg/ml hypoxanthine in	
	1 ml of assay solution	50
4.21	Absorbance changes over time for 0.01 mg/ml hypoxanthine in	
	1 ml of assay solution	51
4.22	Absorbance changes over time for 0.005 mg/ml hypoxanthine in	
	1 ml of assay solution	
4.23	Absorbance changes over time for 0.001 mg/ml hypoxanthine in	
	1 ml of assay solution	52
5.1	Absorbance reading changes over different TMB concentrations	59
5.2	Absorbance value (660 nm) changes over concentration of	
	hydrogen peroxide	61
5.3	Pathway equation of TMB oxidation process	62
5.4	Absorbance changes over addition of hydrogen peroxide into	
	TMB solution in a titration process	63
5.5	Absorbance changes over series of hypoxanthine concentration	65



xiii

LIST OF PHOTOS

Photo	No.	Page
4.1	Color formation when 1 ml of hydrogen peroxide (with different	
	concentration) reacted with 5 μ l of TMB coloring solution.	53
4.2	Paper disc immobilized with peroxidase and TMB reacted with	
	hydrogen peroxide with different concentration	53
4.3	Paper disc immobilized with peroxidase and TMB treated with	
	0.1 M of hydrogen peroxide (in circle)	54
4.4	Paper disc immobilized with peroxidase and TMB treated with	
	0.01 M of hydrogen peroxide (in circle)	54
4.5	Paper disc immobilized with peroxidase and TMB treated with	
	0.001 M of hydrogen peroxide (in circle)	54
4.6	Paper disc immobilized with peroxidase and TMB treated with	
	1x10 ⁻⁴ M of hydrogen peroxide (in circle)	54
4.7	Paper disc immobilized with peroxidase and TMB treated with	
	1x10 ⁻⁵ M of hydrogen peroxide (in circle).	55
4.8	Paper disc immobilized with peroxidase and TMB treated with	
	1x10 ⁻⁶ M of hydrogen peroxide (in circle)	55
4.9	Paper disc immobilized with peroxidase and TMB treated with	
	1x10 ⁻⁷ M of hydrogen peroxide (in circle)	55
4.10	The actual model of the paper strip immobilized with peroxidase,	
	TMB and xanthine oxidase. The strip were treated with distilled	
	water as the negative control	56
4.11	Three strips immobilized with enzymes and TMB ready to be use	56
4.12	Three strips treated with controls and sample.	57
4.13	Paper strip immobilized with xanthine oxidase, peroxidase and	
	TMB treated with 0.1 M hypoxanthine	57



LIST OF TABLES

Table	No.	Page
2.1	Important component in a general biosensor	5
3.1	The content of 7 different test tubes for TMB test	35
3.2	The content of 8 different test tubes for hydrogen peroxide test	36
3.3	The content of 8 different test tubes for hypoxanthine test	37
4.1	Alphabet symbol with their represented value	53
5.1	TMB concentration with respective maximum absorbance reading	59
5.2	Hydrogen peroxide concentration with recorded maximum	
	absorbance value	61
5.3	Hypoxanthine concentration series with maximum recorded	
	absorbance value	65



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
μМ	micromolar
mM	milimolar
cm	centimeter
⁰ C	degree Celsius
cells/ ml	cells per milliliter
v/v	volume per volume
min	minute
mm	millimeter
М	Molar
%	percent
et al.	et alia
O2°-	Oxygen free radical



Fe ³⁺	ferric ion
dH ₂ O	distilled water
H_2O_2	hydrogen peroxide
NaH ₂ PO ₄ .H ₂ O	sodium dihydrogen phosphate dehydrate
K ₂ HPO ₄	dipotassium hydrogen phosphate
KH ₂ PO ₄	potassium dihydrogen phosphate
NaOH	sodium hydroxide
CuSO ₄ .5H ₂ O	copper (II) sulfate pentahydrate
H ₂ SO ₄	sulfuric acid
хо	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).



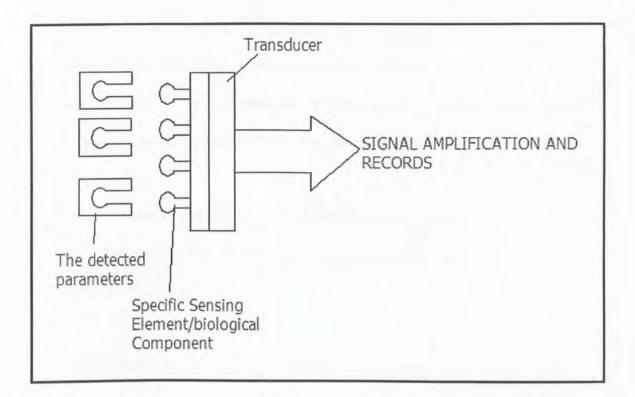


Figure 2.1 The main components of colorimetric biosensors (Elizabeth, 1991)

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



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