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Polypyrrole-Based Xanthine Oxidase Potentiometric Biosensor for Hypoxanthine

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Abstract: A biosensor is developed for the determination of hypoxanthine (Hx) by entrapment of XOD and a mediator, ferrocene or potassium ferrocyanide, ($K_4Fe(CN)_6$, into a PPy film during galvanostatic film formation. The optimum conditions for the formation of PPy-XOD-$Fe(CN)_6^{4-}$ film include 0.4 M pyrrole, 6.2 U mL^{-1} XOD, 40 mM $K_4Fe(CN)_6$, polymerisation period of 200 sec and an applied current density of 0.5 mA cm^{-2}. Potentiometric modes of detection, was investigated. The potential for the potentiometric biosensing of Hx was 0.0 mV vs. Ag/AgCl (3 M KCl) in 0.05 M phosphate buffer. It is revealed that potentiometric detection was sensitive and gave a wider linear concentration range. The PPy-XOD potentiometric biosensor was successfully used for the determination of fish freshness.

Key words: Biosensor, hypoxanthine, fish freshness, polypyrrole, potentiometric and xanthine oxidase

INTRODUCTION

Hypoxanthine (Hx) is the main metabolite of adenine nucleotide degradation in biological materials and can therefore accumulate in biological tissue (International Conference on Microbiological Safety in Food, 1988). Consequently, the amount of Hx present is often used as an index of freshness of meat in the food industries and the pathology of some processes in the human body (Van Raemdonck *et al*., 1996). For example, when a fish is caught and killed it losses freshness during autolysis. In the early stages, the breakdown of adenosine-5-triphosphate (ATP) in fish muscle results in the release of adenosine-5-diphosphate (ADP) and further disintegration product, such as adenosine-5-monophosphate (AMP), ionosine-5-monophosphate (IMP), inosine (HxR), Hx, xanthine (X) and uric acid (Zen *et al*., 2002), IMP is known to contribute to the pleasant flavour of fresh fish, however its degradation to HxR and Hx is responsible for the progressive loss of good flavour and the development of a most unpleasant fishy smell (Okuma *et al*., 1991; Carsol *et al*., 1997; Luong *et al*., 1989; Mulchandani *et al*., 1989). The conversion of Hx to xanthine through the catalytic effect of xanthine oxidase (XOD) together with the production of H_2O_2 and reaction of O_2 was found to be the rate determining step in the overall reaction sequence in fish muscle (Mulchandani *et al*., 1989; Veciana-Nogues *et al*., 1973; Zen *et al*., 2002; Volpe and Mascini, 1997; Hu *et al*., 2000).

Hypoxanthine has been found to accumulate in fish and beef, as well as in human organs, such as heart, kidney and skeletal muscle (van Raemdonck *et al*., 1996; Mulchandani *et al*., 1989; Veciana-Nogues *et al*., 1973; Yano *et al*., 1995a, b; Manthei *et al*., 1995). Therefore, the determination of hypoxanthine (Hx) has considerable importance for quality control of fish and other fish products in the food industry. Thus, from monitoring the concentration of Hx in dead fish or animals, it is possible to predict the time of death and the accumulation of HxR and Hx over time can be used to assess freshness of fish.

Various methods (Nguyen *et al*., 1990; Jones *et al*., 1964; Tarr, 1966) such as spectrophotometry (Jones *et al*., 1964), chromatography and electrophoresis (Nguyen *et al*., 1990) have been used for the determination of hypoxanthine; however some of these methods require pre-treatment and complicated and time-consuming procedures. In response to the limitation of these methods, electrochemical biosensors have been developed to enable a simpler and more rapid determination of Hx (Luong *et al*., 1989; Hu and Liu, 1997; Volpe and Mascini, 1997; Chemnitius *et al*., 1982; Ibrahim *et al*., 1996; Hu *et al*., 2000).

Various enzyme immobilisation methods that have been considered for this purpose include immobilisation of XOD in graphite, carbon paste, nylon mesh with glutaraldehyde (GLA) and covalent attachment to cellulose acetate membrane (Watanabe *et al*., 1983, 1984 and nylon mesh (Moody *et al*., 1987; Mao *et al*., 1999) on platinum and carbon paste electrodes (Yao, 1993). Cross-linking with *bovine serum albumin* (BSA) and GLA is the most commonly used method for the development of an

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amperometric Hx biosensor because of its ability to improve the stability of immobilization (Okuma *et al*., 1991; Moody *et al*., 1987; Mao *et al*., 1999; Yao, 1993; Watanabe *et al*., 1987; Kilinc *et al*., 1998) of any mediator. Mediators such as methylviologen (Sampath and Lev, 1996), acyanoferrate, hydroxylmethylferrocene (Kilinc *et al*., 1998) and colbaltphalcyamine (Hu and Liu, 1997) were co-immobilised or added to the test solution to enhance sensitivity and selectivity of the detection of Hx. Recently, amperometric-mediated biosensors have often been successfully used for the detection of hypoxanthine and xanthine (Mulchandani *et al*., 1989; Nguyen *et al*., 1991; Amine *et al*., 1993; Gonzalez *et al*., 1991; Rehak *et al*., 1994; Foulds and Lowe, 1986; Watanabe *et al*., 2005; Agui *et al*., 2006).

The constructions of these biosensors are commonly based on electrochemical oxidation of H_2O_2 produced by the enzymatic reaction as follows:

$$Hx + O_2 \xrightarrow{XOD} Xanthine + H_2O_2 \quad (1)$$

$$Xanthine + O_2 \xrightarrow{XOD} Uric\ acid + H_2O_2 \quad (2)$$

Another approach, which has not been fully explored for the immobilisation of XOD, is the direct electrochemical entrapment in polypyrrole. This approach has been considered for the immobilisation of XOD but was not used to determine fish freshness (Lui *et al*., 2004; Liang and Shoubin, 2004). However, electrochemical entrapment has been successfully used for the immobilisation of various enzymes, such as GOx (Adeloju and Moline, 2001), penicillinase (Nishizawa, 1992), urease (Adeloju *et al*., 1996), cholesterol oxidase (Govender, 2001) formate dehydrogenase (Yuan, 1989) and sulphite oxidase (Adeloju *et al*., 1994). XOD and purine nucleosides phosphorylase (Adeloju and Lawal, 2005), ATP (Kueng and Kranz, 2004) and alcohol oxidase (Barsan and Bratt, 2008).

In this research, the electrochemical entrapment of XOD in polypyrrole film will be explored for the fabrication of a stable, sensitive and selective Hx biosensor for rapid and reproducible measurement of fish freshness. Important considerations in the development of the Hx biosensor have been focused on two significant aspects: (a) film formation and electrochemical entrapment conditions, such as current density, polymerisation time, effect of XOD and pyrrole concentration and (b) analytical utilisation conditions, such as applied potential, influence of electron mediator, pH and buffer concentration.

MATERIALS AND METHODS

Reagents and standard solutions: Xanthine oxidase (XOD) (EC1.1.3.22 Grade 1) from buttermilk, ferrocene carboxylic acid (Fc), potassium ferrocyanide, pyrrole and hypoxanthine were obtained from Sigma Aldrich Chemical Pty Ltd (NSW). Other chemicals used were reagent grade and all reagents used in this work were prepared without further purification. XOD was stored in the refrigerator at 5°C until required. The pyrrole was distilled under vacuum at 130°C prior to use and this was stored in a closed bottle wrapped with aluminium foil in the freezer to prevent UV degradation until required for use. A stock solution of 0.25 M $K_4Fe(CN)_6$ salt was prepared by dissolving 1.0060 g of the salt in Milli-Q water. The volume was then adjusted to 10 mL. The volumetric flask was then placed in an ultrasonic bath, until the salt dissolved and a clear solution obtained. The solution was stored until required. Stock phosphate buffer solution (0.5 M, pH 7.0) was prepared by neutralising othophosphoric acid solution with sodium hydroxide. This was stored in the refrigerator and diluted when needed. A stock solution of 0.1 M solution of ferrocene carboxylic acid was prepared by dissolving 0.36 g of the salt in 100 mL 0.01 M KOH.

Instrumentation: Electrochemical measurements were performed with a potentiostat/galvanostat designed and constructed in our laboratories. This instrument was used in the galvanostatic mode for the electropolymerisation. A three-electrode system, which consists of a platinum working electrode, a platinum wire counter electrode and a saturated calomel reference electrode (SCE), was employed for electropolymerisation, while a two-electrode system, consisting of platinum working and a reference electrode, was used for potentiometric detection of Hx. The potentiostat was connected to a computer controller (AMD-K6-400 mHz Celeron processors, 32 MB RAM, 8 Gigabyte HD, Hansonl Monitor and Windows 98 keyboard and mouse) and a Brother HL-12707 network laser printer. The solution was stirred when necessary with a Sybron Thermolyne (model S-17410) stirrer.

Glassware: Glassware and polyethylene cells that were used for solution preparation and measurements were soaked in an acid bath (1% HCl: 1% HNO_3) for one week. These were washed with detergent and then soaked in an acid bath (1% HCl: 1% HNO_3) overnight after use. Before being used, each item was rinsed several times with fresh Milli-Q water drained and dried in an air-circulating oven at 80°C.

Preparation of XOD electrode

Electrode preparation: A 320 μm aluminium oxide powder was used to polish the platinum working electrode with a soft polishing pad, to remove any previous film and then finally polished with 5 μm aluminum oxide. The platinum electrode surface was washed thoroughly with Milli-Q water, rinsed under a stream of acetone and finally rinsed thoroughly with Milli-Q water to remove any of the remaining aluminum oxide. The electrode was dried with fibre-free tissue paper prior to use.

Electropolymerisation of PPy-XOD film: Three-electrode voltammetric cell was used to perform electropolymerisation of the PPy film. Platinum wire and Ag/AgCl (3 M KCl) were used as the auxiliary and reference electrodes respectively while the working electrode was a platinum electrode (0.17 cm^2).

Xanthine oxidase (6.2 unit mL^{-1}) and ferrocene or ferrocyanide were immobilised into the polypyrrole film by electropolymerisation of pyrrole in a solution which contained 0.1-0.5 M of the monomer and 40 mM ferocene carboxylic acid or 20 mM potassium ferrocyanide at various current densities and a polymerisation time of 200 sec. After the galvanostatic film formation, the polymer electrode was washed several times under a stream of Milli-Q water to remove any weakly bound XOD or $K_4Fe(CN)_6$ molecules prior to use.

Potentiometric measurements: Potentiometric measurements were performed with a conventional two-electrode system, respectively. The measuring cells contained 20 mL of phosphate buffer, which was stirred in the two methods of measurement. The potentiometric responses were measured after each addition of the standard hypoxanthine solution to the cell under different conditions.

Determination of Hx: In the past, the quantitative Hx assay was performed by measuring the O_2 consumed (Suzuki *et al*., 1989) or the H_2O_2 formed from the XOD catalysed reaction (Cayela *et al*., 1989; Adeloju and Lawal, 2005), as previously illustrated by Eq. 1 and 2. The hydrogen peroxide is detected by potentiometric measurement during oxidation of H_2O_2 at the enzyme electrode as follows:

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \quad (3)$$

In this study, the potentiometric measurement was performed in 50 mL cell, which contained 20 mL of 0.05 M phosphate buffer (pH 7.0). The enzyme and reference electrodes were immersed into the buffer solution. A zero current was applied and, after potential stabilisation (3 min), a standard analyte solution, or sample, was added to the gently stirred buffer and the potential-time response curves were recorded. The magnitude of the change in potential was correlated to concentration of analyte in the cell.

Hx determination in fish: Several extraction solutions, including perchloric acid (Jones *et al*., 1964), water (Watanabe *et al*., 1987) and trichloroacetic acid (TCA) (Pearson, 1975), have been used to prepare fish samples for Hx analysis. Distilled water was used in this study. Many researchers realised that the tedious procedure of acid digestion was not necessary for measurement of hypoxanthine (Watanabe *et al*., 1987). Five grams of fish meat from Blue Grenadier fish fillet was homogenised in 20 mL water at room temperature and filtered through 0.2 μm filter membrane to obtain the fish extract in the filtrate. This was adapted for potentiometric biosensing of Hx. A mixture containing equal volumes of the fish extract and 0.1 M phosphate buffer was subjected to Hx analysis. The Blue Grenadier was analysed after storage under different conditions with the Hx biosensor. The same procedure was used for Lake Entrance Flathead and Sword shark.

RESULTS AND DISCUSSION

Response to Hx: The XOD-catalysed reactions taking place at the Hx biosensor have been illustrated in Eq. 1-3. In the presence of molecular oxygen XOD catalysed the oxidation of Hx and produced hydrogen peroxide, which can be detected by the PPy-XOD-Fc or PPy-XOD-$Fe(CN)_6^{4-}$ electrode. The potentiometric response decreased with increasing Hx concentration. However, the magnitude of the resulting response was influenced by several factors, such as applied potential, type and concentration of mediator, galvanostatic polymerisation conditions, pH and buffer concentration, XOD concentration and applied potential

Potentiometric detection: Figure 1 shows a typical potentiometric response for the PPy-XOD-$Fe(CN)_6^{4-}$ biosensor. The potentiogram shows that the baseline for the response is distinct. Figure 2 show the potentiometric Hx biosensor has a linear range from 5-25 μM. The minimum detectable concentration of Hx with of PPy-XOD-$Fe(CN)_6^{4-}$ electrode by potentiometric detection was 4.9 μM. Table 1 clearly shows the established optimum conditions for potentiometric detection of Hx with PPy-XOD-$Fe(CN)_6^{4-}$ electrode and enabled the detection of lower concentrations of Hx. From the above results,

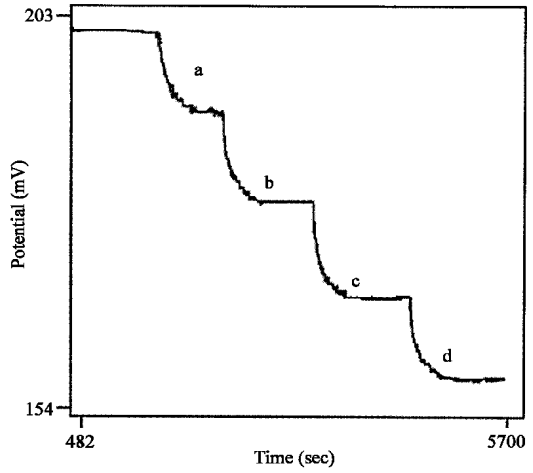

Fig. 1: Typical potentiometric (potential-time) response of PPy-XOD-Fe(CN)$_6^{4-}$ biosensor to Hx. (a) 10, (b) 20, (c) 40 and (d) 60 mM Hx. The monomer solution contained 0.4 M pyrrole, 6.2 U mL^{-1} of XOD, current density: 0.5 mA/cm^2 and polymerisation period: 200 sec

Table 1: Established optimum conditions for potentiometric detection of Hx with PPy-XOD-Fe(CN)$_6^{4-}$ electrode

Optimum condition	Potentiometric mode
[Pyrrole]	0.5
[$K_4Fe(CN)_6$]	50 mM
[XOD]	6.2 U mL^{-1}
Film formation condition polymerisation time	200
Current density	0.75 mA cm^{-2}
Measurement condition applied potential	0
Minimum detectable amount [Hx] µM	4.5
Linear range 1	5-25 µM

sensitive quantification of Hx can therefore be achieved with potentiometric mode of detection.

Analytical application: A linear relationship was also observed in Fig. 2 between the potentiometric response and the Hx concentration. The potentiometric response was linear from 5-25 µM. The minimum detectable concentration of Hx is 5 µM. This is approximately 16-fold lower than that of the oxygen-based biosensor developed by Watanabe *et al.* (1984). These linear ranges are suitable for the determination of Hx in biological and clinical samples.

One of the areas where this biosensor can be useful is in assessing fish and meat freshness. The quality of fish meat can be estimated from an increase in Hx concentration and it has been reported that analysis based on Hx alone is an adequate indicator of freshness (Tarr, 1966). Figure 3 shows the potentiograms obtained for the determination of Hx in three fish samples, namely Flathead, Blue grenadier and Sword shark,

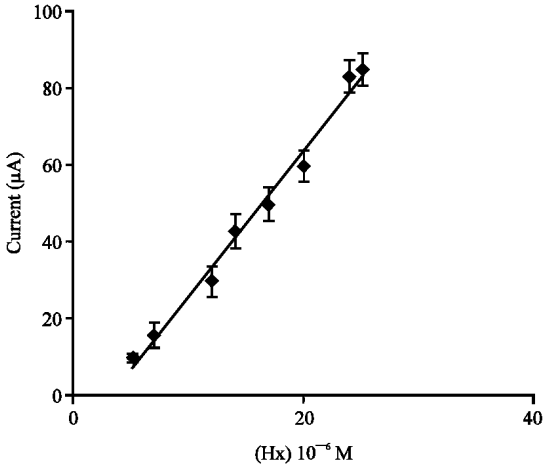

Fig. 2: Calibration plot obtained for the potentiometric biosensing of Hx with the PPy-XOD-Fe(CN)$_6^{4-}$ electrode

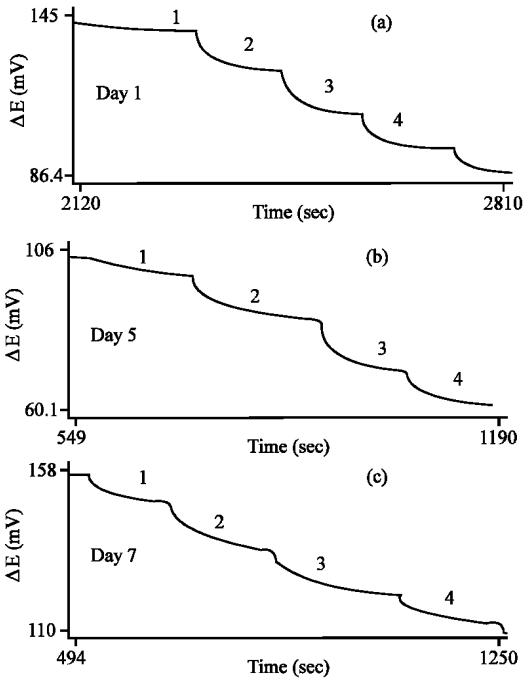

Fig. 3: Potentiometric detection of Hx in (a) Flathead (b) Blue Grenadier and (c) Sword Shark fish samples with PPy-XOD-Fe(CN)64-electrode. (1) sample only, (2) + 0.2, (3) + 0.4, (4) +0.6 mM Hx

with the PPy-XOD-Fe(CN)$_6^{4-}$ electrode based on potentiometric detection. The differences in the [Hx] found in Sword shark (8.7±0.7 µmol g^{-1} at about day 7), Blue grenadier (4.1±0.9 µmol g^{-1} at about day 5) and Flathead (2.1±0.3 µmol g^{-1} at about day 1) could be due

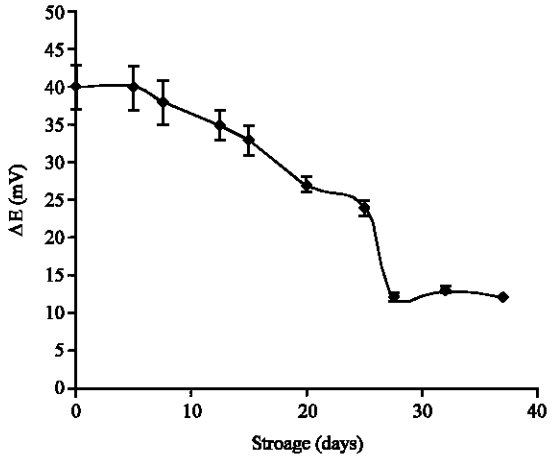

Fig. 4: Stability of the potentiometric response of Hx obtained with the PPy-XOD-$Fe(CN)_6^{4-}$electrode. (Hx) was 10 mM

to post-catch handling and the inability to get an accurate date of catch or death of the fish. But the hypoxanthine found in the three different fish muscles ranged from 2.1 to 8.7 μmol g^{-1}. The results are within the range of values previously reported for common fish fillet by Zen *et al.* (2002) (0.5-8.8 μmol g^{-1}) after day one and Mulchandani *et al.* (1989) (0.4-6 μmol g^{-1}) on day one. Such agreement suggests that the PPy-XOD-$Fe(CN)_6^{4-}$ electrode is useful for reliable, simple and economical detection of Hx as an indicator of fish freshness. Recovery studies were carried out by adding Hx to the buffer after the fish extract addition and the recoveries were satisfactory with an average value of 99.5±4.0%.

The inability to get the correct date of catch made all attempts to look at one fish over the course of 7 days for different fish difficult. The stability of the PPy-XOD-$Fe(CN)_6^{4-}$ electrode, as shown in Fig. 4, in potetiometric mode, shows the sensitivity of the response did not start to decline until after 7 days. The loss in sensitivity may be due to the leaching of XOD and/or $Fe(CN)_6^{4-}$ from the electrode with increasing storage time. However, despite the reduction in sensitivity with time, the biosensor can still be used for routine determination of fish freshness, provided this is done by the standard additions method.

Ascorbic Acid (AA) and Uric Acid (UA) are considered to be major interferents in biological samples (Mao *et al.*, 1999) have coated nafion on the electrode to eliminate both AA and UA interferences.

CONCLUSION

Potentiometric biosensors for the determination of Hx have been fabricated by immobilisation of XOD and Fc or $Fe(CN)_6^{4-}$ into polypyrrole films by galvanostatic polymerisation. The optimum conditions for the formation of the PPy-XOD-$Fe(CN)_6^{4-}$, as given in Table 1 include 0.4 M pyrrole, 6.2 U mL^{-1} of XOD, 50 mM $K_4Fe(CN)_6$, a polymerisation time of 200 sec and an applied current density of 0.75 mA cm^{-2} enabled the detection of a wider linear concentration range of Hx. The biosensor had a minimum detectable amount of 4.5 μM in the potentiometric mode. A linear concentration range for the biosensor in potentiometric mode was 5-25 μM, the biosensor was applied successfully to the determination of hypoxanthine in fish muscle. The concentration of hypoxanthine found in three different fish samples ranged from 2.1-8.7 μmol g^{-1} over a 7-day period. The results suggest that the biosensor can be reliably used for the assessment of fish meat freshness.

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