Stability Characteristics and Applications of Native and Chemically-Modified Horseradish Peroxidases

By

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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ABBREVIATIONS

A	electrode area
AA	Amino acid
Å	Angstrom
AA-NHS	Acetic acid N-hydroxysuccinimide ester
Abs	Absorbance
Ag/AgCl	Silver/Silver Chloride
AH_2	Hydrogen acceptors
Arg	Arginine
Asp	Asparagine
C	bulk concentration of substrate
CD	Circular dichroism
C_6H_5OH	Phenol
CO_2	Carbon dioxide
CP	Chlorophenol
CPE	Carbon paste electrode
CSTR	Continuous stirred tank reator
Cys	Cysteine
CV	Cyclic voltammetry
Da	Dalton
DEAE	Diethylaminoethyl
DH	Hydrogen donor
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
E	Applied potential
$E_{½}$	Half-wave potential in voltammetry
EDTA	Ethylenediaminetetra-acetic acid

EG-NHS	Ethylene glycol bis-succinic acid ester of N-hydroxysuccinimide
EIA	Enzymeimmunosassay
ELISA	Enzyme-linked immunosorbent assay
ESR	Electron spin resonance
FIA	Flow injection analysis
GA	Glutaraldehyde
GnCl	Guanidine hydrochloride
H_2O_2	Hydrogen peroxide
His	Histidine
HPLC	High performance liquid chromatography
HQ	Hydroquinone
HRP	Horseradish peroxidase
$HS\text{-}(CH_2)_n\text{-}SH$	Dithiol
H_2SO_4	Sulphuric acid
i, I	Current
I	Inactive state of protein
IAA	Indole acetic acid
ISE	Ion selective electrode
K	Equilibrium constant
K_m	Michaelis-Menten constant
k	Rate constant for protein inactivation
kDa	kilodaltons
Lys	Lysine
M	Molar
mM	millimolar
Mr	Molecular weight
N	Native state of protein
nA	Nanoampere
$Na_2B_4O_7$	Sodium tetraborate
NaH_2PO_4	Sodium dihydrogen orthophosphate
NaOH	Sodium hydroxide

$-NH_2$	Amino group
NH_3	Ammonia
NH_4^+	Ammonium
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
O_2	Oxygen
o-AP	*o*-aminophenol
OPD	*o*-phenylenediamine
Ox	Oxidised form of enzyme
PAP	Poly(*o*-aminophenol)
PEG	Polyethylene glycol
Phe	Phenylalanine
POPHA	*p*-hydroxy-phenylacetic acid
Red	Reduced form of enzyme
rpm	Revolutions per minute
SA-NHS	Suberic acid N-hydroxysuccinimide ester
SCE	Saturated calomel electrode
TMB	3, 3', 5, 5'-tetramethylbenzidine
TNBS	Trinitrobenzenesulphonate
Tris Hcl	Tris(hydroxymethyl)aminomethane hydrochloride
Trp	Tryptophan
Tyr	Tyrosine
U	Unfolded form of enzyme
U.V./Vis.	Ultra violet/Visible spectroscopy
(v/v)	Volume per volume
V_{max}	Maximum rate of enzyme reaction
(w/v)	Weight per volume

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ABSTRACT

Due to its inherent stability, ease of handling and availability, horseradish peroxidase (HRP) will continue to be used more extensively in analytical and industrial situations. It is often used as a model for other peroxidases.

Developments in the fields of protein stabilisation and biosensor construction are discussed in Chapter one; also, various enzymatic methods for treating phenolic effluents are reviewed. The effects of chemical modifiers on native HRP were investigated (Chapter two). Homobifunctional crosslinkers specific for lysine residues were employed. No loss of enzyme activity occured on reaction with such N-hydroxysuccinimide (NHS) compounds. Derivative forms of HRP displayed greater thermostability and a greater tolerance of water-miscible organic solvents. Enhanced resistance towards denaturants was noted. Structural changes in the vicinity of the heme of HRP derivatives were studied by UV/Visible spectrophotometry and fluorimetry. The extent of modification on HRP's six lysines has been determined.

The NHS derivatives of HRP have also been employed in the removal of phenols from aqueous solution (Chapter three). HRP catalyses the oxidation of toxic aromatic compounds in the presence of hydrogen peroxide. Reaction products polymerise to form high molecular weight materials which can be easily separated from aqueous solution. Modified peroxidases displayed greater removal efficiencies of phenols compared to the native enzyme over a wide range of reaction conditions, including high temperatures. For some pollutants, the efficiency of removal is high.

Native HRP has also been used in the development of a biosensor for the selective determination of uric acid (Chapter four). The sensor was found to function efficiently without the necessity for an electron transfer mediator. The mechanism of the sensor's response was thought to be due to direct electron transfer from the electrode to HRP. A monomer, *o*-aminophenol, which was electrodeposited at the working surface of the electrode, was found to protect the biocomponents from interferences and fouling. The sensor was incorporated into a flow injection system for the quantification of uric acid in human serum. Recoveries compared favourably with a standard spectrophotometric method.

CHAPTER 1

INTRODUCTION

1.1 FOREWORD

This first section of this chapter deals with the characteristics and properties of peroxidase enzymes, and especially those of horseradish peroxidase (HRP). The role of peroxidase in physiologically important reactions is discussed, along with its moderate stability in a range of adverse conditions. Although HRP exhibits characteristics similar to that of an ideal enzyme, its catalytic ability could be considerably stabilised by immobilisation techniques and/or specific chemical modification. Use of additives has also been reported to stabilise HRP. The aforementioned strategies have also enhanced HRP's catalytic ability in adverse environmental conditions.

The second part details the various methods for treating phenolic wastes (enzymatic, microbial, incineration, activated carbon, chemical oxidation, etc.) Aspects of soluble and immobilised peroxidases systems are discussed. Strategies towards enhancing the (soluble) peroxidase process are listed. Reactor design has been shown to be crucial in achieving greater efficiency in the system; also, a good understanding of HRP's catalytic mechanism is essential if the removal of phenolic waste from water is to be improved.

The final section of this chapter deals with the development of biosensors and particularly the role of HRP in the entire field. The progress in uric acid sensor research is also reviewed. The applications of various working electrode designs are discussed, as well as procedures for immobilising the biocomponent in the electrode configuration. Most attention is placed on the process of electropolymerisation. The future prospects of such devices are discussed.

1.2 BIOCHEMISTRY AND STABILISATION OF HRP

1.2.1 Introduction

Peroxidases constitute a class of enzymes which are extensively distributed throughout the plant and animal kingdom. Their widespread presence would suggest that they are an essential component of practically all living systems. Peroxidases play an active role in metabolism, i.e. participation in coupled oxidations and in the protection of the cell against peroxide poisoning. They are also known to play an integral role in cell wall biosynthesis. In plant cells they are located mainly in the cell wall, vacuoles, transport organelles and on membrane-bound ribosomes [1]. Peroxidases occur naturally in human saliva, the adrenal medulla and in the liver, kidney and leucocytes. Products from human salivary peroxidase participate in the regulation of oral bacterial growth and metabolism [2]. Peroxidase from a previously unreported source was reported by Shindler *et al.* [3]. The enzyme was found in all specimen human cervical mucus samples collected from different patients. Electrophoretic techniques have shown a type of peroxidase to be a major component of the soluble protein of cervical mucus. The kinetic mechanism proposed for the cervical mucus peroxidase, utilising 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) as a substrate, is identical to a previous mechanism proposed for horseradish peroxidase.

Of all the peroxidases, most research has been devoted to horseradish peroxidase (HRP), which was discovered in 1903 by Bach. Horseradish roots are one of the richest sources of peroxidase enzyme. HRP was the fourth haemoprotein to be crystallised.

1.2.2 Structure of HRP molecule

Horseradish peroxidase is an oxidoreductase (donor: hydrogen peroxide oxidoreductase; E.C. 1.11.1.7; HRP). Similar to all peroxidases, it functions in the transfer of hydrogen to hydrogen peroxide from hydrogen donors (DH) [4]. As many as 40 isoenzymes have been detected. Evidence suggests that some apparent

isoenzymes may be HRP molecules with variations in carbohydrate composition. Three important isoenzymes exist: isoenzyme A (acidic), isoenzyme C (neutral or slightly basic) and a strongly basic HRP. The latter has been called cyanoperoxidase due to its isolation from plants as a ferric-cyanogenic complex [5]. Native HRP consists of a polypeptide chain containing 308 amino acid residues [6]. The amino terminus is blocked by a pyrrolidene carboxyl residue. C-terminal peptides have been isolated with and without a terminal serine. HRP contains a single iron(III) protoporphyrin IX prosthetic group in which the iron centre is coordinated to a histidine residue in the fifth position. This protoporphyrin IX group is held in place by electrostatic interactions between the propionic acid side chain of the heme and a lysine molecule in the apoprotein. HRP isoenzyme C consists of two compact domains, between which the heme group is positioned [6]. The iron group has six coordination positions; four of which are occupied by porphyrin nitrogen atoms and the fifth by a protein group (histidine) [7]. The sixth position can be occupied by various compounds: peroxidases appear to operate by exchange of substrate in this position [4]. The components of the peroxidase molecule have been described [6]. (Figure 1.1).

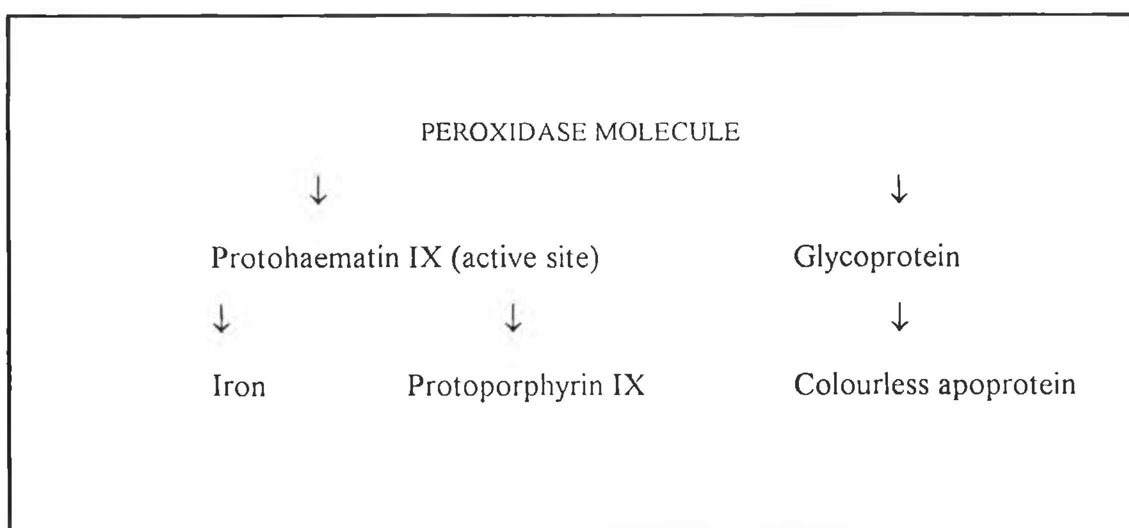


Figure 1.1: Various components of peroxidase molecule

The covalent structure of HRP consists of two domains; one of which incorporates the heme group. Eight neutral carbohydrate side chains are attached through asparagine residues at positions 13, 57, 158, 186, 198, 214, 250 and 268 [6]. The carbohydrate residues are mainly located in the C-terminal half of the polypeptide. There are four disulphide bridges located between the cysteine residues 11-91, 44-49, 97-301 and 177-209. No free amino groups exist and only two titratable histidines occur. The carbohydrate portion (which accounts for approximately 18% of HRP [8]) appears to shield the six lysines on the protein backbone. The enzyme contains a single tryptophan residue that emits fluorescence, but it is not located in the active site [9].

HRP is a metalloprotein, where calcium appears to play a major role in maintaining the structural stability of the enzyme [7]. The protein contains 2 moles calcium per mole of enzyme. Treatment of HRP with 6 M guanidine hydrochloride-10 mM EDTA for approximately 4 hours can remove the bound calcium, which results in a significant decrease in thermal stability. Addition of calcium appears to restore stability. Due to the glycoprotein nature of HRP, it is released from plant cells in a calcium-controlled process [10] which appears to have a role in the elongation of plants through the rigidification of cell walls. The total molecular weight of the enzyme is approximately 44kDa, taking into account the carbohydrate, but not counter ions or bound water. HRP has a working pH range of 4.0 - 8.0 [9].

1.2.3 General peroxidase isoenzymes

Peroxidase isoenzymes are widely distributed throughout the plant and animal kingdom. In callus, vegetative and floral buds of *Nicotiana tabacum* (tobacco plant), 47 isoenzymes have been isolated, with over half of these performing a defined functional role [11]. In plant cells, peroxidases are mainly located in areas such as the cell wall and transport organelles [1]. The various locations of such isoenzymes is usually based on their electrophoretic mobility. The large prevalence of peroxidase in the cell wall has been ascribed to anionic isoenzymes. These are regarded as important in the normal functioning of the cell wall due to their high affinity for lignin precursors.

As a result of this, anionic isoenzymes have been detected predominantly in the stems of highly lignified tobacco plants, but appear to be absent from the callus [12]. Moderately anionic isoenzymes are also found at high levels in similar areas as anionic isoenzymes, but are found in high levels in the callus. Such isoenzymes appear to increase in concentration upon tissue damage in plants. In tobacco cells, cationic isoenzymes are mainly located in vacuoles. Unlike their anionic counterparts, they possess a low affinity towards lignin precursors. They are predominantly located in the root and callus tissue and function in the formation of hydrogen peroxide which may be utilised by other isoenzymes. This classification of isoperoxidases does not hold in other plant systems. For example, in other plants, the majority of peroxidase activity is attributed to cationic isoenzymes.

Shannon *et al.* succeeded in isolating seven peroxidase isoenzymes from horseradish root by purification techniques such as ammoniun sulphate precipitation and column chromatography on carboxymethyl (CM-)cellulose and DEAE-cellulose [13]. Activity in the seven isolated isoenzymes accounted for approximately 86% of the original activity. Due to the glycoprotein nature of HRP, carbohydrate is present in each of the isoenzymes, although it was shown that peroxidase activity occurs in the absence of carbohydrate. The isoenzymes isolated by Shannon *et al.* appear to fall into two individual groups. One group possesses a high content of arginine, a basic amino acid. The second group contains neutral and acidic residues. Spectrophotometric analysis of the isolated isoenzymes displayed two distinctive absorption spectrum patterns.

1.2.4 Functions and Catalysis of peroxidases

Peroxidases have long been associated with a wide range of physiological events, such as active roles in metabolism and in lignin synthesis. The enzymes have also been suggested as having a role in chlorophyll degradation. Peroxidase activity also appears to be involved in indole-3-acetic acid (IAA) catabolism and ethylene biosynthesis [9], both of which are plant hormones. Its presence in IAA catabolism suggests a possible

role for peroxidases in the regulation of plant growth, whilst ethylene is involved in the regulation of aspects in plant growth and development, such as fruit ripening [9].

Peroxidases catalyse the oxidation of a range of substrates. The process is characterised by the formation of Compounds I and II (Figure 1.2), which are active intermediates. The initial step of the reaction involves the two electron oxidation of the heme group of the enzyme by hydrogen peroxide [14]. This interaction, between the resting enzyme ferriperoxidase (Fe_p^{3+}) and H_2O_2, results in the formation of an unstable intermediate, known as Compound I. This intermediate interacts with an electron donor with the loss of one oxidising equivalent to form Compound II. In the absence of an electron donor, or at low concentrations of hydrogen peroxide, Compound I (the product of the oxidation reaction) decomposes slowly [2]. Suitable electron donors employed in the formation of Compound II would include most chromophores used in peroxidase activity assays. A reductive step returns Compound II to the resting state. This entire cycle is common to most peroxidase reactions. An excess of hydrogen peroxide results in total enzyme inactivation. However, in the absence of H_2O_2, oxidase reactions have been found to be catalysed by peroxidases. O_2 is reduced to a superoxide radical ($O_2^{\bullet -}$) in a reaction where Compounds I and II do not participate. Two forms of peroxidase (ferroperoxidase and Compound III) are involved. A reaction between hydrogen peroxide and Compound II results in the formation of Compound III [15], thus substrate inhibition is usually the reason for the appearance of Compound III (red in colour). It is generally accepted that Compound III is relatively inert, due partially to the fact that it is not an essential part of the peroxidase cycle [16].

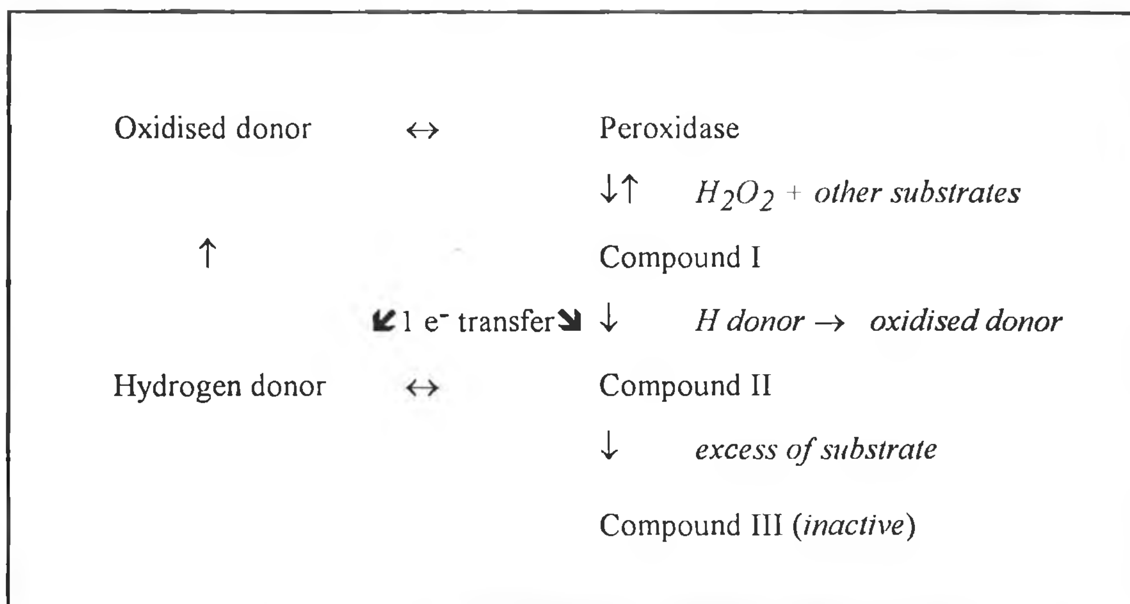


Figure 1.2: Scheme of peroxidase catalysis

1.2.5 Stability of HRP

Native HRP exhibits characteristics similar to that of an ideal enzyme. Its catalytic activity is maintained for long periods of time at room temperature. Activity may also be maintained in buffers of varying ionic strength and in a pH range of 4 - 11 [9] for short intervals of time, even though its optimum pH is between 6 - 8.

It has been demonstrated that peroxidase can function when suspended in water miscible organic solvents, even with minimal water bound to the protein surface [17]. Whilst enzymes can function in anhydrous organic solvents such as hexane or toluene [18], water miscible solvents often lead to protein inactivation. An enzyme's catalytic activity is affected only by its bound water and not by the free water in the particular solvent. The presence of this bound water ensures enzymatic activity in organic media [19]. Water is involved in all non-covalent interactions that help to maintain protein conformation. It has been suggested that enzymes such as HRP require approximately 10^3 molecules of water per enzyme molecule. This layer of water is thought to act as lubricant or plasticiser, with the ability to form hydrogen bonds with various functional groups.

Different considerations apply to water-miscible organic solvents; however, Gorman and Dordick described the desorption of tritiated water bound to HRP in a range of organic solvents such as methanol or hexane [19]. Desorption of tritiated water was almost immediate, with most of the desorbable T_2O released in the first five minutes. This phenomenon, known as "water stripping" [20], appears adequate to account for the catalytic sensitivity of nearly all enzymes in water-miscible organic media. It has also been reported that suspending enzymes in organic solvents can alter a number of their fundamental properties [21]. Substrate specificities can be significantly modified by placing enzymes in such environments. It has also been suggested that the pH dependence of an enzyme can be changed, i.e. the activity of an enzyme in an organic medium depends on the pH of the solution from which the enzyme was lyophilised. A significant increase in thermostability of an enzyme is possible in the presence of water-miscible organic solvents. Low volumes of water miscible organic solvents such as dioxane or acetonitrile, appear to cause partial denaturation in HRP [19]. Fluorimetric analysis illustrates a conformational difference in HRP's sole tryptophan residue when exposed to organic solvents. Absorbance and EPR spectroscopy suggest exposure of the active site to the organic solvent, which results in reduced local polarity and enhanced H-bonding of phenolic residues to the enzyme.

The thermal inactivation kinetics of horseradish peroxidase have been studied extensively. Chang *et al.* found that HRP's thermal inactivation profile in the presence of sugars deviates from first order kinetics over a temperature range of 60-94^{0}C [23]. The process was estimated to be 1.5-order. The authors also demonstrated, using electrophoretic techniques, the presence of four HRP isoenzymes with a reaction order of 1.5. This deviation from a pure first order process has been attributed to such factors as the formation of enzyme aggregates with varying heat stabilities, the presence of heat-stable and heat-labile isoenzymes and series type enzyme inactivation kinetics. It was shown, by the use of differential scanning calorimetry (DSC), that sucrose, which is a non-reducing sugar, stabilised the enzyme against thermoinactivation, where as reducing sugars such as fructose, glucose and lactose

brought about an increase in thermal inactivation. Hendrickx *et al.* observed biphasic thermoinactivation for solid state HRP in the temperature range 140-160^{0}C. This was ascribed to the presence of two fractions of HRP displaying first order kinetics [24]. Enhanced thermostability has been reported by Ugarova *et al.* [25]. Horseradish peroxidase was chemically modified with carboxylic acid anhydrides and picrylsulphonic acid at the epsilon-amino groups of the lysines. First order thermal decay kinetics were observed for HRP derivatives at a temperature of 56^{0}C.

Calcium is a vital constituent of the peroxidase molecule and has a role in maintaining the structural stability. In spite of the fact that calcium does not participate in the peroxidase catalytic reaction, the thermal stability of the enzyme has been reported to decrease significantly when exposed to guanidine hydrochloride made up in EDTA [7]. Calcium removal results in a disturbance in the vicinity of the heme group as determined by NMR spectroscopy [26]. Peroxidase, which functions in plant growth through the rigidification of the cell wall, is released from cells in a Ca^{2+}-controlled process [10]. Thus, peroxidase with a deficient calcium content could lead to plant injury and death.

Manganese, phenols and hydrogen peroxide are all known to affect the stability of peroxidase. They have also been shown to act as promotion agents in IAA oxidation. As stated previously, peroxidase is considered the main enzyme responsible for IAA catabolism. Manganese was found to have a stimulatory role on oxidation of IAA by HRP [27]; however the general function of Mn^{2+} is unclear. Activation of the IAA/peroxidase reaction by phenols such as *p*-coumaric acid was due to its ability to reduce Compound III, producing ferriperoxidase and H_2O_2 for the peroxidative cycle. It is thought that the optimum concentration range of hydrogen peroxide to which HRP can catalyse efficiently is narrow. Low concentrations of hydrogen peroxide result in decreased HRP activity, whilst too high a concentration is inhibitory. Cyanide and sulphide reversibly inhibit HRP but carbon monoxide does not. The enzyme is quite sensitive to bacteria, bacteriostatic agents and other chemicals found in tap water.

1.2.6 Peroxidase activity assays

Peroxidases are capable of catalysing the oxidation of a range of substrates. Essentially, HRP functions in decomposing two molecules of hydrogen peroxide, the natural substrate of the enzyme, into oxygen and water by a two electron oxidation step, i.e. hydrogen peroxide is reduced in the presence of a hydrogen donor. HRP has a low affinity for the second molecule of hydrogen peroxide and other electron donors may be employed [28]. The native HRP is regenerated by the hydrogen donor, which is oxidised. This is known as the oxidation/reduction type reaction [29]. The concentration of hydrogen peroxide in the reaction can be estimated by monitoring the concentration of the oxidised hydrogen donor.

A wide range of indicator molecules or substrates exist, which are capable of assessing peroxidase activity. Substrates may be incorporated into colorimetric, luminescent, fluorimetric, electrochemical and hydroxylation reactions, employed in the detection of HRP and hydrogen peroxide. The best assays provide a direct measure of enzyme activity, usually by coloured product formation (colorimetric assays). When chromogenic substrates are used as hydrogen donors, oxidation results in the formation of a coloured product. Chromogenic substrates for HRP include 3,3',5,5'-tetramethylbenzidine (TMB), a non-carcinogenic chromogen which yields a blue reaction product at sites of HRP activity when oxidised by the HRP/H_2O_2 complex [30]. It has also been used as a chromogen for HRP in enzyme-immunoassay [31]. Sustrates such as o-phenylenediamine (OPD), *o*-dianisidine and mesidine have also been used; however, their oxidation products are carcinogenic. Chlorpromazine and 4-methoxy-alpha-naphthol have been quoted as efficient substrates, as has 2,2'-azino-di-(3-ethyl-benzothiazoline-(6)-sulphonic acid), known as ABTS. Other chromogenic substrates include 4-chloro-1-naphthol (4-CN), 3,3'-diaminobenzidine (DAB) and 3-methyl-2-benzothiazoline hydrazone hydrochloride (MBTH) [28]. An ideal chromogenic substrate should be non-carcinogenic and easy to use. It also should be readily soluble in aqueous solutions, stable and exhibit high molar absorptivity [32],

unlike guaiacol (a H-donor still in widespread use) which produces an unknown mixture of oxidation products.

HRP also possesses an intrinsic ability to catalyse chemiluminescent type reactions. Chemiluminescence involves the emission of light during a reaction. Luminol, which is one of the most commonly used chemiluminescent reagents, can be oxidised by HRP in an alkaline solution to yield 3-aminophthalate and light [33] in the presence of hydrogen peroxide. It is possible to detect extremely low levels of H_2O_2 by employing excess HRP and luminol in the reaction mixture. Intensity may be measured at 425nm. Chemiluminescence has also been used in the determination of superoxide dismutase [34]. Superoxide dismutase can be determined as it acts by inhibiting the chemiluminescence produced by a HRP/luminol reaction. The detection limit for the system reached a level as low as nanogram quantities. Reagents such as luminol can be used in conjunction with such enhancers, to determine hydrogen peroxide in flowing streams or bioreactor situations. A HRP/luminol reaction has been used in the determination of alkaline phosphatase [35]. This system involves the use of 5-bromo-4-chloro-3-indoyl phosphate as a substrate. The assay has found applications in enzyme immunoassay and DNA probe assays.

HRP and hydrogen peroxide may also be detected by fluorimetric methods. Fluorescent substrates such as 1,2-diarylethylenediamines and catechols, when incubated with HRP and H_2O_2 at 37^0C for a 10 minute period, can determine peroxidase activity [36]. Fluorimetric intensities were measured at 500 nm, with an excitation wavelength of 360 nm. Hydrogen peroxide was reported to have been detected in a system incorporating fluorescein dye, which contained peroxidase and 3-(N-morpholine)propanesulphonic acid (MOPS buffer). Light emission was detected by a photomultiplier tube. The detection limit was comparable to systems involving luminol [37]. Another fluorimetric technique for hydrogen peroxide determination involves p-hydroxy-phenylacetic acid (POPHA), which reacts with HRP to form a fluorescent dimer. The amount of this dimer present is proportional to the concentration of H_2O_2. Excitation and emission wavelengths were 320 and 400 nm

respectively [38]. Modification of this assay may allow peroxidase activity determination. It is feasible to determine hydrogen peroxide, by fluorimetric means, in the absence of HRP. The feasibility of using hematin, as a substitute for HRP was explored by Genfa *et al.* [39] with *p*-cresol being used as a substrate. In a flow-injection system, hematin achieved a limit of detection of 5nM H_2O_2.

The concentration of H_2O_2 in a reaction can also be determined electrochemically, in the presence of HRP. Such reactions involve HRP utilising a hydrogen donor that can be monitored voltammetrically upon electrochemical reduction [40]. Hydrogen donors that act as electron mediators and can be easily monitored voltammetrically are essential. Electron mediators employed in such situations include hydroquinone, *o*-toluidine, resorcinol and catechol.

1.2.7 Other functions of HRP

Horseradish peroxidase possesses the ability to carry out a large range of reactions, such as the catalysis of one-electron oxidations of phenols. Phenols, such as *p*-cresol and *p*-hydroxyphenylacetate are converted to phenoxy radicals, which spontaneously result in polymer formation [41]. HRP reactions in organic solvents include hydroxylations, N-demethylations, sulphoxidations and other oxidations of various organic substances. HRP can catalyse the hydroxylation of certain aromatic compounds by molecular oxygen where dihydroxyfumaric acid acted as a hydrogen donor [42]. This topic is dealt with in the second section of this chapter. The ability of the enzyme to function in a number of organic solvents is a well reported phenomenon [17]. Enzyme activity is maintained even when the bulk water is replaced by organic solvents. The determination of physiologically important analytes is feasible in organic media (e.g. cholesterol determination using cholesterol oxidase and HRP; a bienzyme system [43].

1.2.8 Protein stabilisation - general overview

Proteins, such as HRP, find many applications in medical and industrial fields, such as diagnostics, therapeutics, bioreactors, fine chemicals, enzyme-based electrodes and biosensors. In applied enzymology, the most important goal is to obtain proteins which exhibit a high degreee of reliability and reproducibility. Enzymes can be stabilised by a range of methods, including immobilisation, chemical modification, use of additives and protein engineering. Enzymes exhibit properties similar to that of an ideal catalyst. The relative advantages of an enzyme over conventional catalysts are summarised in Table 1.1. The problems of industrial related enzymes may be overcome by pursuing a specific stabilisation method which would be compatible with the particular enzyme.

Table 1.1

Relative advantages and disadvantages of an enzyme over conventional catalysts

Advantages	Disadvantages
High catalytic activity	Thermal inactivation
High degree of substrate specificity	Inactivation by chemical reagents
Operation under mild conditions	Radiation modification
Minimal bi-product formation	Environmental modification
Low cost bulk production	-

1.2.9 Naturally existing stable enzymes

Enzymes exist that possess relatively high stability. Such proteins may occur in readily available organisms which are capable of living at elevated temperatures (55-100^{0}C). In such microorganisms (thermophiles), or indeed halophiles (exist in high salt conditions), the cell's supramolecular structures, e.g. ribosomes, are very stable in extreme conditions [44]. The presence or absence of calcium and substrate is believed

to affect the thermostability of amylases. The latter acts in the rigidification of the protein's conformation while calcium ions have an activation and stabilisation role on amylase. Bound ions function in holding the enzyme structure together [45]. Thermostable enzymes have been isolated from the fungus *Aspergillus niger*, which displays an optimum temperature of 65^{0}C. Reports also exist on the glycolytic enzyme lactate dehydrogenase from *Thermotoga maritima*, a thermophilic eubacterium. The enzyme is stable up to 90^{0}C and displays an extremely high tolerance of guanidine hydrochloride (GnCl) [46]. Such thermostable enzymes often display relatively high activity in the presence of protein denaturants, such as GnCl, urea, detergents, organic solvents and proteolytic enzymes.

1.2.10 Denaturation

The activity of any enzyme requires that the structural and functional integrity of the active site remains intact. However, the tertiary structure can be irreversibly disrupted by a range of physical, chemical and biological forces. In addition to denaturation at high temperatures, proteins may also denature at low temperatures. Denaturation refers to the conformational change that results in the loss of molecular function, which may be reversible or not. Inactivation results from changes in the degree of association or aggregation of the molecule, as well as modification of amino acid side chains. Mozhaev *et al.* put forward a number of causes of protein inactivation, such as thiol-disulphide exchange, cleavage of S-S bonds, dissociation of oligomeric proteins into subunits and conformational changes in the macromolecule [47]. Thus, stability in a protein is a balance between stabilising (hydrophobic) interactions and the loss of conformational entropy as the protein is unfolded [48].

Reactivation of proteins may take several hours and require quick or slow cooling or prolonged incubation at intermediate temperatures. Denatured enzymes may become only partially or fully active after hours of exposure to normal temperatures. If high temperatures exist for long periods of time, enzyme activty will not return as irreversible inactivation processess will have occurred [49].

1.2.11 Enzyme immobilisation

The immobilisation of enzymes or proteins onto insoluble matrices forms the fundamental step of many biotechnology processes and analytical devices. By definition, an immobilised enzyme is one that is physically localised in a certain position or converted from a water soluble mobile state to a water insoluble immobile one [44]. Immobilised enzymes can show different thermal kinetic values relative to their soluble counterparts. As immobilised enzymes simulate the state of enzymatic proteins within the intracellular microenvironments of living cells, they can provide us with a good model system to study. Information concerning the primary, secondary, tertiary and quaternary structure of proteins has been obtained through the use of immobilised enzymes. Furthermore, information on enzyme reactions and intracellular microenvironmental properties, among other aspects, has been elucidated through such investigations. A number of immobilisation techniques exist:

1. binding to carriers by covalent bonds or by adsorptive interactions;
2. entrapment in gels, beads or fibres;
3. crosslinking or co-crosslinking with bifunctional reagents;
4. encapsulation in microcapsules or membranes [44].

Immobilisation can be used as a method to stabilise an enzyme (Section 1.4.4). Optimisation of such a procedure with respect to a number of factors such as pH, reaction time and temperature is necessary to achieve maximum attachment and activity. Studies on the multipoint covalent attachment of trypsin to agarose gels were performed by Blanco *et al.* [50]. Immobilisation techniques have been assessed on native HRP. Stabilisation towards extremes in temperature was achieved by encapsulation of the enzyme with a low molecular weight polymeric glutaraldehyde followed by crosslinking with a second layer consisting of polyacrylamide derivatives. This bilayered synthetic cage, surrounding the enzyme, appears to enhance its intrinsic stability. It is then possible to immobilise the enzyme onto a polyacrylamide-hydrazine

gel by crosslinking reactions [51]. Martinek *et al.* observed an increase in thermostability in immobilised derivative forms of chymotrypsin and trypsin by seven hundred fold in the temperature range 60-100^{0}C [52]. Enzymes were firstly acylated with acryloyl chloride and then copolymerised with acrylamide. It was deduced that the greater the number of attachment points between the enzyme (due to the preliminary modification step) and the polymer support, the greater the stabilisation achieved. Rigidification of enzymes such as trypsin prevents unfolding (and therefore activity loss) in extreme conditions.

Chemical compounds, such as urea, are potent protein denaturants. They compete with water in binding to the polypeptide and so disrupt inter- and intra-chain hydrogen bonds which help maintain the native protein structure. Weng *et al.* reported a decrease in thermostability of immobilised HRP at elevated temperatures in the presence of organic solvents such as dodecane, decanol and tetradecane. The Z value (temperature increment needed for a 10-fold reduction of the D value of the agent, ^{0}C, where D is the decimal reduction time[in minutes]) of peroxidase was changed from 26.3^0 C to 14.1^{0}C by the method of immobilisation. This was lowered to 11.1^{0}C in the presence of an organic solvent [53].

1.2.12 Chemical modification of proteins

Soluble enzymes can be chemically modified by a number of methods so as to alter their structural and kinetic properties. Bifunctional or crosslinking reagents, which find uses in the preparation of conjugates for immunoassays, diagnostic imaging and other applications, can be used to chemically modify and thereby stabilise protein molecular structure [54]. Bifunctionally reactive compounds include bis-imidates, bis-succinimides and bis-maleimides. The latter compounds react specifically with thiols whilst the first two crosslink amino groups. These are regarded as homobifunctional reagents, in that they possess a similar type of reactive group at either end of the molecule. Heterobifunctional reagents have different reactive groups at each end of the molecule. Such reagents introduce both inter- and intra- molecular bridges in proteins

[55]. Such intramolecular crosslinks act in the stabilisation of the tertiary structure of a protein. In crosslinking reactions, thiol and amino groups are predominantly targetted. Such functional groups are reactive and occur frequently in protein primary structures. Other groups that can be targetted include carboxyl groups on glutamyl and aspartyl resudues, guanidino groups on arginine residues and phenol groups on tyrosines. As opposed to site-directed mutagenesis and protein engineering, relatively little structural information is required regarding the target protein. Such modification experiments are often simple to carry out and protocols may be implemented and data obtained rapidly [45]. In crosslinking reactions, the protection of the active site residues by substrate or reversible inhibitors is important. It is also imperative that functional groups targetted for reagents are not involved in catalysis. Potential target sites must be distant from the active site of the enzyme and not buried in the folded protein structure

It is known that unfolding is an essential step in protein denaturation [56]. Thus, denaturation can be retarded if molecular "braces" are placed across the protein backbone so as to prevent unfolding [57]. Molecular rigidification, employing bifunctional reagents, is achieved by crosslinking functional groups on the protein backbone. In theory, a crosslinked enzyme should be more stable than the native form. The extent of crosslinking that occurs depends largely on the length of the reactive molecule, and hence on the distance between the molecular centres to be linked [56]. For example, bis-succinimides are capable of forming successful crosslinks [56, 58]. Data from crosslinking experiments carried out on HRP, shows that the longer ethylene glycol derivative (EG-NHS), which is 14.0Å long, stabilises the enzyme to a greater extent than the suberic acid derivative (SA-NHS) which only spans 11Å. Imidoesters shorter than 11Å have failed to stabilise native HRP [59]. These results would indicate that there is a threshold or minimum distance to obtaining a stable crosslink in HRP. In the same way, the modification of chymotrypsin by Torchillin *et al.* was successful for some diamines used, but not for others of differing molecular lengths [56]. In order to prove the theory, chymotrypsin was reacted with equal quantities of dithiols of $HS\text{-}(CH_2)_n\text{-}SH$, (n ranges from 4 to 10), i.e. same type

molecules but with differing molecular lengths. This study showed that intramolecular crosslinking renders the enzyme more tolerant of extreme conditions such as temperature (as observed by Ryan *et al.* [59]) salt action and denaturing agents. Ugarova *et al.* also reported that chemical modification of the epsilon-amino groups on lysine residues could alter the thermal stability of HRP [25]. The enzyme was modified with anhydrides of monocarboxylic acids and by anhydrides of dicarboxylic acids and by picrylsulphonic acid (TNBS). The native HRP possessed greater catalytic activity around 50^0C, whereas the modified enzyme displays a maximum activity in the range 55-65^0C.

A variety of other chemical modification procedures exist apart from the use of bifunctional crosslinking reagents [60]. These are predominantly based on the strengthening of hydrophobic interactions by non-polar reagents and the introduction of new polar or charged groups that give additional ionic or hydrogen bonds to the enzyme [45]. It was reported that the monofunctional methyl acetimidate was used to alter 17 of the available 24 lysine residues of lactate dehydrogenase from pig heart. Acetamidinaton has no effect on net charge; however, a shift in pK values occurs from 10.2 to 12.5. The enzyme derivative displayed enhanced tolerance of heat and alkalinity [61]. Other chemical modifcation methods involve the hydrophilisation of the protein surface to reduce unfavourable surface hydrophobic contacts with water [62]. Dramatic stability enhancement has been reported for surface-hydrophilised derivatives of chymotrypsin (1000 fold at 60^0C). Alkylation of the enzyme with glyoxylic acid followed by cyanoborohydride reduction introduces -$NHCH_2COO^-$ groups which are not hydrophobic to the same extent as existing -NH_2 groups on the protein surface. Treatment with anhydrides or chloroanhydrides of aromatic carboxylic acids followed resulting in the addition of up to five carboxylic groups to each lysine residue previously altered. Acylation results in a more hydrophilic protein with increased thermostability. This effect may possibly be explained by a decrease of non-polar clusters located on the protein surface; contact of such clusters with water molecules has been reported to destabilise the enzyme [44, 63]. A wide range of reagents are

available with selectivity for specific groups on amino acids which are summarised in Table 1.2. [45].

TABLE 1.2

Modification reagents used in protein stabilisation

Residue for modification	Reagent	Reaction
Amine (Lys)	O-methylisourea	Guanidination
	Acid Anhydrides	Acylation
	Imidates	Amidination
	Iodoacetic acid	Alkylation
	Borohydrides and carbonyl compounds	Reduction
		Alkylation
Carboxyl (Asp, Glu)	Carbodiimides	Amidination
Guanidino (Arg)	Dicarbonyls	Not known
Imidazole (His)	Diethylpyrocarbonate	Addition
Indole (Trp)	N-bromosuccinimide	Oxidation
Thiol (Cys)	Maleimido compounds	Addition
	Iodoacetic acid	Redn. & S-carboxymethyln.
	N-ethylmaleimide	Alkylation

1.2.13 Enzyme stabilisation in organic solvents

It was formerly accepted that organic solvents destabilise protein molecules. However, many enzyme processes located *in vivo* occur in chemical compositions very much different to that of aqueous media. Use of soluble enzymes in biphasic aqueous-organic systems and in anhydrous organic solvents may be a novel development, but it is not so

surprising. Klibanov proposed a number of rules to ensure an enzyme's activity in organic media [64]:

1. the particular solvent should be hydrophobic and show little affinity for water;
2. homogenous dispersion of the enzyme in the organic solvent;
3. lyophilisation of the enzyme from solution of its optimum pH prior to its use in an organic solvent.

Ahern and Klibanov also pointed out that water plays an integral role in thermoinactivation reactions of proteins, such as deamidination of asparagine residues or destruction of cysteine residues [49]. Therefore, the less water available, such reactions are unlikely to occur (Section 1.2.5).

Zaks and Russell reported that suspending enzymes in a variety of organic solvents can alter a number of their fundamental properties [21]. For example, the activity of chymotrypsin in an organic medium depends entirely on pH of the solution from which the particular enzyme was lyophilised. Substrate specificities of subtilisin and chymotrypsin were altered significantly when placed in the presence of organic solvents. The thermostability of many enzymes, including HRP, is enhanced in the presence of certain solvents. However, partial denaturation of HRP occured in low volumes of water-miscible organic solvents such as dioxane, methanol and acetonitrile [19].

The conformations of enzymes in organic media can also be influenced by "ligand memory" [21]. Subtilisin, freeze-dried from aqueous solutions containing specific ligands, such as N-acetyl-L-phenylalanine, was found to be much more stable at 111^{0}C in octane than subtilisin prepared without a ligand. It was also observed that, in the case of chymotrypsin, up to 65% butanol could be tolerated without any effect on catalytic activity. Activity lost above a critical solvent concentration could be completely recovered, by dilution in buffer. This leads to the conclusion, that unlike

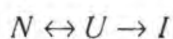
thermoinactivation of proteins at high temperatures, the solvent effect is due to reversible denaturation, rather than to irreversible inactivation [45].

1.2.14 Stabilisation through the use of additives

The stabilisation of enzymes in soluble form is an important technological goal. Such soluble enzymes are used in detergents, the food and textile industries, cosmetic applications and in the diagnostic and medical fields. Compounds such as neutral detergents, sodium azide, maltodextrin and special buffers have been employed as additives to prevent alteration of the native structure of proteins through every step of protein purification. Additives may also be used to overcome the problem of pure-protein storage. Lyophilisation, in conjunction with a range of inorganic salts, is a common preservation technique. Purified enzymes can also be stabilised with sucrose, glycerol, sorbitol or ethanol and stored below 0^0C [45].

The ultimate aim or goal of an additive or preservative, is to increase the stability of the enzyme. Gray defined the term "additive", in this context, as being a soluble species which has an effect on the thermal stability of the protein structure [65]. Sucrose, for example, has long been a recommended additive in the stabilisation of soluble chymotrypsin. Other additives that may be used to stabilise an enzyme include bivalent metal ions, ammonium sulphate, ethylene glycol and various surfactants [66]. Such compounds can strongly bind to proteins because they are substrates or products of the enzyme reaction, allosteric effectors, coenzymes or coenzyme derivatives. The stabilising effect produced by the use of such additives may be explained in two different ways.

1. The binding of the additive (through single/multipoint attachment) to the active portion of the native enzyme as opposed to the inactive unfolded polypeptide, causes a shift in equilibrium to the former, having a beneficial effect on the entire enzyme (see reaction 1.1) [65].



$$N \leftrightarrow U \rightarrow I$$

2. If lower internal energies arise due to the binding of the additive, the resulting "enzyme-substrate" complex is more resistant towards denaturing agents.

The protective effect on enzymes exhibited by certain additives has been explained in terms of the influence that such compounds have on water activity and localisation. Interactions with water molecules results in the formation of water clusters, thus leading to an overall net reduction in the volume of free water in and around the protein structure. A similar theory was put forward by Ahern *et al.*, in that the presence of water contributes to the process of thermal inactivation; a decreasing volume of water acts in retarding protein inactivation [49]. At the same time, such water clusters could possibly reduce potential collisions between the protein and any solvent present in the microenvironment.

1.2.15 Other stabilisation techniques

An increase in stability is observed when enzymes are bound to large molecular weight polyhydroxy compounds, such as polyethylene glycol [67]. Gibson *et al.* reported the stabilisation of HRP and other enzymes involving a polymer-carbohydrate system [68]. Enzymes are dried under vacuum at room temperature in the presence of a soluble polymer, such as DEAE-dextran (carbohydrate sugar). Activity was maintained for two months at 37^0C, in the presence of stabilisers. Control activity (for unstabilised HRP) dropped to approximately 26%.

Modification of HRP with ethanol-methoxypolyethylene glycol resulted in a shift in optimum pH from 5.0 for native HRP to 3.5 for the derivative. The modified derivative alone was active in such organic solvents as toluene, dioxane and methylene chloride. The authors noted that the derivative was also more sensitive to hydrogen

peroxide inhibition in the presence of toluene. Data suggests that 2 to 3 amino groups were altered in the modification process [69].

The carbohydrate moiety on HRP can be used as a target for conjugation of short aliphatic chains (hexadecylamine and octylamine). The optimum temperature for biocatalysis of the former (HRP-C16) was increased by 10 degrees. HRP-C16 displayed increased solubility in toluene, chloroform and dimethylsulphoxide. The octylamine form, HRP-C8, appeared to be less soluble. The addition of the short C8 chain introduced only 3 octylamine chains into the carbohydrate moiety, and as a result, had little effect on the catalytic properties of the enzyme. The activity of the HRP-C16 derivative remained high in toluene and chloroform, even at 70^0C [70].

1.2.16 Applications of HRP

HRP has long been an invaluable tool in the life sciences. It functions as an indicator in oxidase-based coupled enzyme assays, in enzyme immunoassays, neurohistochemistry, DNA labelling and in biosensor construction. The enzyme is particularly suitable for diagnostic sensors due to the absence, under normal circumstances, of HRP inhibitors in serum and urine [32] Production of easily detectable compounds promotes HRP's use in clinical, analytical and industrial situations.

HRP possesses several properties that make it suitable as an enzyme label in enzyme linked immunosorbent assays (ELISA) [4], such as high turnover number and reasonable stability upon storage The enzyme is capable of working well in varying assay conditions (i.e variations in pH of solution, ionic strength, buffer types and radical temperature changes) Tijssen also reported HRP as having the lowest cost per unit mass, of all commercially available enzymes used in EIA systems. Both HRP and urease produce easily detectable products, in a system where clear-cut visible endpoints are essential The products of enzymes such as β-galactosidase are less strongly coloured. HRP can be easily detected using a wide range of substrates, in colorimetric, flourimetric, luminescent and various other assays.

In ELISA-type systems, the enzyme (HRP) is labelled or attached onto a second layer of antobodies [71]. This enzyme-second antibody conjugate is capable of converting an added colourless substrate into a coloured product or indeed, a non-fluorescent substrate, into an intensely fluorescent product. The amount of product formed is proportional to the concentration of antigen present. Less than a nanogram of protein can be detected by ELISA detection systems.

A number of antibody-enzyme conjugation methods have been reported. Nakane and Kawaoi used sodium periodate to conjugate HRP to antibodies, where HRP's carbohydrate residues are oxidised by periodate [72]. This produces aldehyde groups which bond with unprotonated amino $-NH_2$ on the antibody. Nilsson *et al.*. conjugated HRP and immunoglobulins using N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), which is a heterobifunctional reagent [73]. Other methods of conjugation have been reported [4].

HRP also finds uses in the area of neurohistochemistry. It has been frequently used in the study of the neural network in the central nervous system (CNS). Such a technique involves the tracing of neural connections after injection of HRP into the CNS [74]. Tetramethylbenzidine, an insoluble chromogen, has been used to stain for peroxidase activity in fixed brain-slice specimens. Sites of HRP activity in tissue specimens can give us information about which nerves in the brain transport HRP and, therefore, proteins. HRP also finds uses in the study of fluid pinocytosis, which is defined as the uptake of media by enclosure in small membrane vesicles that bud from the cell surface. This phenomenon occurs in practically all cells. HRP movement in the cell can monitored microscopically, through the visualisation of insoluble reaction products. The enzyme acts as a suitable marker since it is soluble and does not interfere with cellular activities [75].

1.3 USE OF PEROXIDASE IN WASTEWATER TREATMENT

1.3.1 Introduction

Biological treatment of organic waste is a well established technology which has produced many beneficial products and contributed towards conservation through recycling of substrates for different final uses. Water covers over 80% of the earth's surface. However 90% of water is contained in the oceans (making it unusable directly), 2% in the polar ice caps and the remainder beneath the earth's surface. Thus, only a small fraction is available for human consumption. Pollution has been defined as the "introduction by man into the environment of substances or energy liable to cause hazards to human health, harm to living resources and ecological damage, or interferences with legitimate uses of the environment" [76]. Pollutants may be certain metals, a range of organic compounds or gases. Their relative importance in pollution terms is linked to their perceived toxicity to humans. Therefore, the effective treatment of waste is an important requirement, especially in highly industrialised and populated areas, where waste disposal has become a problem due to intensive farming, the growth of the agro-industry and urban centres. In 1976, the United States Resource Conservation and Recovery Act (RCRA) attempted to address the proper management of hazardous wastes by identifying the origin of such wastes, developing appropriate technologies to manage such compounds and implementing regulations to protect public health and the environment in an effort to gain a response from both the public and industry sectors [77]. The EC through its Community Action Programme on the environment, has developed similar strategies. The main objectives involved reducing the quantity of non-recoverable waste, recovering and reusing waste for raw material and energy purposes and to manage non-reusable waste and dispose of it safely [78]. Although constant progress is being made, there is much room for improvement. Systems have been put in place where the movement of hazardous waste can be traced from the point of origin to the place of final disposal. Under 1984 amendments to the 1976 RCR act, the United States Environmental Protection Agency (EPA) is required

to evaluate and, if necessary, prohibit land disposal of certain wastes. The EC places the most dangerously toxic compounds on its "Black List", the less toxic on a "Grey List" [79]. Similarly, the U.S. EPA lists 129 chemicals on its list of priority pollutants. Included on the list are 11 extractable organic compounds including phenol (a monohydroxy derivative of benzene) and related compounds. Ingestion of small amounts of carbolic acid (phenol) can cause nausea, vomiting, paralysis and sometimes death from respiratory failure or cardiac arrest. Such compounds may also bioaccumulate and thus affect the food chain [80]. Phenol and its derivatives are a mark of industrial pollution. In drinking water containing chlorine and phenolics, a chlorophenol taste can arise at phenol concentrations below $0.1 \mu g\ L^{-1}$. Pure phenols cannot be tasted in drinking water at $1 \mu g\ L^{-1}$ levels [81]. Tastes and odours from water can be the result of organic matter, minerals, specific compounds such as phenol or mercaptans, or chlorine and its derivatives. Such compounds become troublesome at very low levels (e.g. <2 ppb for phenol)

1.3.2 General peroxidase catalysed reactions

Peroxidase is capable of catalysing the oxidation of a wide range of compounds, due to the strong redox properties of its oxidised form and also the long distance electron transfer processes that occur in proteins. No apparent limit in the size of the substrate exists, ranging from phenols [82] to biopolymers such as lignin. Dordick *et al.* have reported that HRP is capable of depolymerising lignin in an organic medium such as dioxane but not in aqueous solution [83]. The rate of peroxidase-catalysed breakdown of lignin in dioxane was shown to be much lower under anaerobic conditions, unlike classical peroxidative coupling of phenols in water which is unaffected by molecular oxygen. Harkin *et al.* have shown that peroxidase is the only known enzyme capable of polymerising *p*-coumaryl alcohol, a lignin precursor, into lignin-like polymers in the presence of hydrogen peroxide [84]. Under certain conditions, HRP can catalyse the hydroxylation of some aromatic compounds by molecular O_2 in the presence of dihydroxyfumaric acid acting as a hydrogen donor. L-3,4-dihydroxyphenylalanine (L-

Dopa), a drug used in the treatment of Parkinson's disease, has been produced with yields of up to 70% from the enzymatic hydroxylation of L-tyrosine. The rate of conversion is dependent on the presence of molecular O_2, reaction temperature and the concentration of the mediator [42].

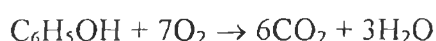
Until recently, very few studies have been carried out on the O-demethylation reactions catalysed by peroxidases. Contrary to prior expectations, a peroxidase system (HRP and H_2O_2) is capable of catalysing such a reaction on the cytotoxic agent, 9-methoxyellipticine. The near complete conversion of the substrate is observed with peroxide in the reaction mixture. The decrease in the quinone-imine concentration during the conversion is related to a copolymerisation of the quinone-imine and the starting material. It was also noted that methanol is formed during such reactions [85]. Gillette *et al.* reported the first N-demethylation-type reaction involving peroxidase. It involved the catalysis of aminopyrine(4-dimethyl-aminoantipyrine) in the presence of peroxide (which was generated from the oxidation of glucose by glucose oxidase) [86]. Its oxidative capacity on *o*-phenylenediamine (OPD) has resulted in the development and optimisation of an activity assay which is sensitive to 16ng L^{-1} of peroxidase and is linear in the 16-200ng L^{-1} range [87].

1.3.3 Methods for treating phenolic wastes

1.3.3.1 Microbial: under properly controlled conditions, microorganisms can be developed that are suitable for treating or removing phenols from waste. Biological means for treating such compounds are feasible over a wide concentration range. Lewandowski and colleagues have reported on three bacterial species, *Klebsiella pneumoniae*, *Serratia liquefaciens* and *Pseudomonas putida*, as being capable of degrading phenol. It was noted that commercial preparations of these species were not capable of similar behaviour [88]. Microbial cells are often immobilised in polysaccharide gels. This results in a higher initial biomass concentration and faster reaction times than with free cells. Loadings of greater than 10^{10} cells per ml of gel

have been reported [89]. Immobilised cells offer the advantage of repeated use if the system is properly designed and operated. Kokubu *et al.* have reported an extended half-life for *Streptomyces fradiae* activity provided optimum environmental conditions are maintained [90]. Microbial methods, however, do suffer from drawbacks such as the slow diffusion of substrate (phenol) through the cell wall, cell lysis and cell reactions yielding products which may affect the primary reaction. However, use of microbial cells avoids the neccesity for expensive enzyme purification processes and also, inside a cell, all the enzymes involved in a reaction are arranged in a logical fashion. It is not possible to mimic such arrangements when immobilising enzymes in a reactor.

1.3.3.2 Incineration: incineration processes burn organic matter in sludge and produce an inert ash as a result. Since the combustible portion of most sludges is below 75%, a substantial proportion of ash remains for disposal. The incineration products of phenol are carbon dioxide and water according to the following reaction:



$$C_6H_5OH + 7O_2 \rightarrow 6CO_2 + 3H_2O$$

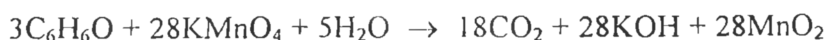
Due to the low solubility of phenol in water (approx. 10%), mechanical mixing is necessary. To sustain the required combustion temperature of 871^0C, a supplemental fuel oil is added [91].

1.3.3.3 Activated carbon: this technique is useful for the treatment of lower phenol concentrations. It is quite versatile and may remove other organic materials present in the phenol-containing stream by adsorption. Powdered activated carbon takes the form of grains between 10 and 50 μM in size [92]. It has a finite capacity for removing phenol from wastewater which can then be regenerated by chemical or thermal means. These processes are sometimes not considered as they are expensive and not applicable for many wastewater types. A relatively low energy-consumption technique has been

developed where supercritical fluid CO_2 passes through the loaded activated carbon and removes the adsorbed material by putting it into solution. This technique is particularly useful for pesticides as they can be resold as dry pesticides [93]. Activated carbon cannot be regenerated when mixed with hydroxide wastes. The factors affecting activated carbon as an option for phenol removal have been discussed [91].

1.3.3.4 Chemical oxidation methods: chemical oxidising agents such as chlorine dioxide, ozone, potassium permanganate and hydrogen peroxide have been applied to convert phenol into less harmful substances. Oxidising agents have also been used in water treatment to remove iron and manganese and to reduce tastes and odours. H_2O_2 in the presence of iron salts is an effective oxidiser of phenols over a wide concentration range. The reaction appears not to be temperature dependent [91]. The advantage of using peroxide is that it has no adverse effect on the environment.

Ozonation, a fairly recent entry into the American wastewater treatment field, offers certain advantages as a method of coal-conversion wastewater clean-up. Ozone has been shown to be about as twice as effective than H_2O_2 in the destruction of phenols. The end products are carbon dioxide and water. The potency of ozone is unaffected by pH; however, a major disadvantage is that it decomposes rapidly to molecular oxygen. It can be employed in either a batch or continuous treatment system and due to its instability, it must be generated on site by passing dry air through an electrical charge with a power consumption of approximately 15Kw hour per kg of ozone produced [94]. Potassium permanganate has been used for treating paint stripping wastes. It oxidises phenol adequately by employing a nine times greater weight of $KMnO_4$. The oxidation of phenol is carried out according to the following reaction scheme. The reaction works best in solutions of moderate alkalinity [91].



$$3C_6H_6O + 28KMnO_4 + 5H_2O \rightarrow 18CO_2 + 28KOH + 28MnO_2$$

1.3.3.5 Aeration: phenol can be removed to some extent from water by aeration. An important factor is the rate at which air enters the water and also the rate at which it is transferred to phenols in solution. Aeration can be of quiescent or forced means. Typical aeration methods would be classed as (a) diffused, (b) submerged turbine and (c) surface. Aerating devices in reactors should be designed so that air can be transferred at a sufficient rate to satisfy the demands of the technique [95]. Liquid depths of reactors are usually no more than 5 metres. The air requirements for a reactor should be based on the mass transfer coefficients for the particular device. Relative efficiencies for various reactor set ups have been discussed [91].

1.3.4 Use of immobilised peroxidases in phenol removal from aqueous solutions

A recent development has been the use of immobilised enzymes in wastewater treatment. The most important features in this area are the low cost of the solid, the production of reusable material and the elimination of waste-disposal problems. A process based on immobilised rather than soluble enzymes is thought to be more effective in treating highly contaminated phenolic waters. Considerable financial savings due to reuse of enzymes are possible. Siddique *et al.* immobilised HRP on a cellulose disk by coupling the enzyme with periodate. Nylon balls and tubing were also compared as insoluble reactor matrices. More than 80% of 4-chlorophenol was removed for all enzyme supports so long as HRP activity was not limiting. Removal efficiencies were not affected by reactor flow rates [96]. HRP has been immobilised on granular chickenbone for the complete removal of phenol from aqueous solutions. The insoluble polyphenols that are produced due to the peroxidative oxidation of phenol are collected on the porous support. The mass transfer properties of this immobilisation technique permit the processing of large volumes of wastewater [97]. A similar strategy involves the immobilisation of peroxidase onto a filter paper via the precursor (adipic acid dihydrazide) of an immobilising agent. The insoluble precipitate is then removed from the filter paper. The reaction is not temperature dependent and is

capable of removing aromatic amines, naphthalene and phenol from aqueous solution [98].

Entrapment of HRP in alginate beads resulted in substantial increases in colour removal from phenolic industrial effluents. Further increases were noted when laccase and HRP were co-entrapped in gel beads which demonstrates both oxidases potential for such applications [99]. HRP has been incorporated on its own into glass beads by precipition of enzyme solution with glass powder. This methodology was used for the determination of phenols and aromatic amines in organic solvents containing H_2O_2. Such a technique may have future applications by direct packing of the peroxidase-modified glass beads in a reactor for the removal of phenols from aqueous effluents [43]. HRP immobilised on a CNBr-Sephadex 4B column with 1mM HCl has been shown to improve the removal of colour from Kraft effluent. Removal, by this technique, is enhanced by a factor of 2.6; thus it would appear that the removal of soluble phenolic compounds is dependent on the immobilisation of the enzyme so that it is biochemically stabilised and reusable [100]. Some examples are given below of surfaces used for the covalent attachment of enzymes [101].

TABLE 1.3

SUPPORT MATERIAL	MODIFICATION REACTION
Poly-*p*-aminopolystyrene	Diazotization
Maleic anhydride	Copolymerised with vinyl ether
Polyacrylamide	Amino ethyl and hydrazide derivatives
Methacrylamide	Allyl glycidyl ether
Polyacrylamide	Diazotization
Nylon	O-alkylation
Nylon	N-alkylation

1.3.5 Use of soluble peroxidases in phenol removal from aqueous solutions

The industrial use of soluble enzymes as biocatalysts dates back to the early 1900's with the development of enzyme processes to manufacture cheese products, bread, beer and wine. Enzymes are able to differentiate between chemicals of closely related structures (hydroxytoluenes in the case of HRP) [96] and can catalyse reactions over a wide temperature range (0-110^{0}C) and in the pH range 2-14 Various enzymes have been shown to perform similar reactions such as removal of amines and phenols from aqueous solution, e.g. HRP and tyrosinase can oxidise catechol in the presence of peroxide to produce *o*-quinones [102].

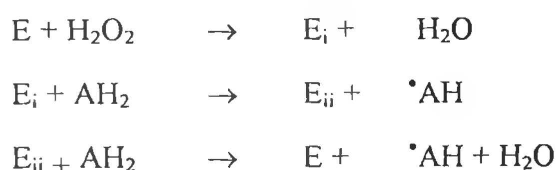
Other peroxidases besides HRP have been reported to polymerise phenols from water in the presence of H_2O_2. Carmichael *et al.* have compared the oxidation of phenols by HRP and a fungal chloroperoxidase. Both enzymes were equally effective in removing phenol and cresol; however, HRP appeared to be more capable of oxidising 2,3- and 2,6-dimethylphenols. The fungal chloroperoxidase displayed a greater affinity for 3-chlorophenol A fungal peroxidase isolated from *Coprinus macrorhizus* was studied for its ability to oxidise a selection of aromatic contaminants. The enzyme possessed the ability to catalyse the same reactions as HRP, removing 98.2% phenol with stirring when mixed with peroxide [104]. Spiker *et al.* reported on a *Streptomyces viridosporus* variant of lignin peroxidase with the ability to oxidise phenols but unable to remove non-phenolics. 4-aminoantipyrine was included to act as a marker for lignin peroxidase activity [105]. A recombinant peroxidase originally isolated from a basidiomycete and which closely resembles HRP was studied for its potential to degrade a wide range of aromatics. The enzyme is inexpensive to produce and can function in a wide range of environmental conditions. Side-products due its oxidative properties include CO_2, H_2O and native peroxidase [106]. The phenol-removing characteristics of other peroxidase-types have also been studied [107].

1.3.6 Strategies to enhance phenol removal by peroxidases

It is known that the removal efficiency of phenol from aqueous solution by a HRP/H_2O_2 system decreases with a reduction in the concentration of peroxidase [108]. However, a plausible explanation for the incomplete removal of phenol by low concentrations of peroxidase is the inactivation of the enzyme. It is thought that this inactivation takes place during the oxidation of the substrate, possibly due to the interactions of phenoxy radicals with the enzyme's active centre [109]. HRP in soluble form has a finite catalytic lifetime when used to precipitate phenolic substrates [110]. Nakamoto *et al.* have proposed incorporating gelatin or polyethyleneglycol into the HRP/H_2O_2/phenol reactor to alleviate such a problem [111]. Gelatin competitively suppresses HRP adhesion to polyaromatics thus reducing the amount of enzyme required. Also, phenol removal rates exhibited greater pH dependence using PEG instead of gelatin [112]. Removal efficiencies were in excess of 90% at pH 5-8 but decreased rapidly when the pH exceeded 9. It was estimated that the amount of HRP required was reduced 40- and 75- fold compared with the same process in the absence of PEG, for treating 1 and 10mM phenol solutions respectively [113]. Thus, it would appear that the use of additives can offer an option towards reducing costs of phenol removing processes involving soluble enzymes

1.3.7 Catalytic cycle of HRP

The one-electron oxidation of aromatic substrates (AH_2) catalysed by peroxidase is depicted in the following mechanism:



$$E + H_2O_2 \rightarrow E_i + H_2O$$
$$E_i + AH_2 \rightarrow E_{ii} + {}^{\bullet}AH$$
$$E_{ii} + AH_2 \rightarrow E + {}^{\bullet}AH + H_2O$$

The native enzyme (E) is oxidised by peroxide to the active intermediate called Compound I (E_i). This state of HRP will accept an aromatic compound into its active

site and catalyse its oxidation. The resulting free radical (•AH) is released back into solution leaving HRP in the Compound II (E_{ii}) state. The formation of free radicals by Compound I has been verified using electron paramagnetic resonance [114]. Compound II oxidises a second aromatic molecule and releases it into solution while the enzyme returns to its native state (E). It is known that the rate of phenol oxidation by Compound II is higher than that of Compound I. Oxidation of phenol by Compounds I and II is approximately 1000 times higher than that of aniline [115]. Free radicals diffusing from the enzyme into solution combine to form polyaromatic products which are usually less soluble than their monomeric precursors and will therefore precipitate from solution. Polymers failing to precipitate can return to the active site resulting in greater polymer formation with still reduced solubility in water [116]. Two free radicals are formed for every H_2O_2 molecule consumed (one molecule of peroxidase can remove 1000 molecules of phenol) [109]. However, insoluble dimers (free radicals) will react again at HRP's active site resulting in greater free radical formation. These radicals will also polymerise to form trimers, tetramers or larger polymers which readily precipitate out of solution. Thus, a stochiometric reaction ratio of peroxide to free radicals is usually 1:1. An important consideration is the concentration of peroxide neccessary for complete removal of aromatics. Increasing the concentration of H_2O_2 improves removal efficiencies up to a certain point [107]. Arnao *et al.* have demonstrated that a loss of peroxidase activity occurs with an apparent bi-exponential behaviour when HRP is stochiometrically deficient to H_2O_2 [117]. Thus it is preferable to use a ramped addition where greater amounts of peroxide are used at the beginning of the reaction when H_2O_2 is consumed rapidly and scaled down amounts are used in the later stages [118]. An attempt was made to identify the end-products of phenol oxidation by HRP/H_2O_2. GC-MS analysis confirmed the formation of both *o,o'*- and *p,p'*-biphenol. Spectral data indicated that the latter is further oxidised to *p*-diphenoquinone [82] as peroxidase is known to catalyse the oxidation of catechol to *o*-quinone [102].

1.3.8 Resistance of aromatic compounds to biodegradation

It is known that some phenolic compounds and aromatic amines have higher bio-removal efficiencies than others Compounds such as *o*-, *m*- and *p*-nitrophenol, *p*-cyanophenol and pyrogallol do not precipitate when exposed to a HRP/H_2O_2 system. Two possibilities could explain this First, the specific reactivity of the particular compound towards peroxidase and hydrogen peroxide, and second, compounds such as naphthols, for example, are more rapidly removed, as their polyaromatic products are more hydrophobic (and are therefore more water-insoluble), resulting in greater precipitation from solution [119] Compounds with high removal efficiencies can aid the removal of those that are not readily precipitated. Phenol has been shown to be more readily oxidised in the presence of such compounds as 3,3'-dimethoxybenzidine, o-dianisidine, benzidine or 8-hydroxyquinoline [119, 120] The explanation for this is that free radicals from benzidine, for example, attack and precipitate phenol. This mechanism is reported to work with non-phenolic and non-amine compounds. Also, ortho- and meta-substituted cresols [121] are removed at a greater rate than p-cresol at pH 4.0 [96, 120]. 4-chlorophenol has a high removal rate than 2- and 3-chlorophenols [120]. Naphthalene is easily removed from aqueous solution by adding phenol [98]. Carmichael *et al.* noted that removal rates of phenol and cresol were similar but monochlorophenols were most readily oxidised by HRP and a fungal chloroperoxidase [103].

On the other hand, Capestany *et al.* demonstrated phenol as being more susceptible to bio-induced oxidation than monochlorophenol, with pentachlorophenol the most difficult phenolic compound to degrade. The halogenation of the phenol nucleus reduces the availability of the molecule for oxidation [122]. Increased resistance to biological oxidation is related to increased substitution and position of Cl atoms on the aromatic ring [123] Nevertheless, Li *et al.* reported a manganese peroxidase which was capable of oxidising pentachlorophenol more efficiently than phenol. Another fungal peroxidase, lignin oxidase, displayed similar degradation capacities for various chlorinated phenolic compounds [124] A wide range of phenol

and chlorophenol contaminants have been enzymatically removed from wastewater [104].

1.3.9 Reactor configurations

An enzyme reactor is described as a vessel in which a reaction catalysed by free or immobilised enzymes takes place, together with all the equipment necessary for sampling and control. In industrial situations, the economic feasibility of the process will depend on the useful lifetime of the biocatalyst. Reactor systems (see Figure 1.5) must be developed which will maintain and extend the biocatalyst's efficiency by reducing its rate of inactivation through such mechanisms as free radical binding, Compound III formation, and the entrapment of the enzyme in formed precipitates [125]. Various types of reactors used in the removal of undesirable compounds from wastewater have been described by Harrison. The advantages and disadvantages of all systems must be considered in order to develop an industrial

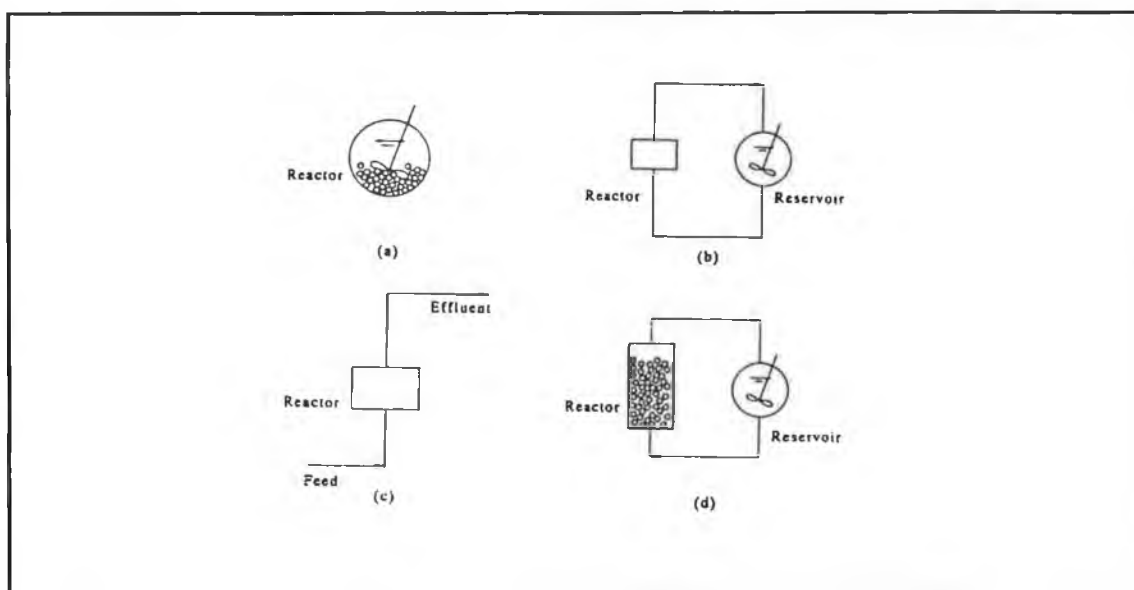


Figure 1.3: Examples of reactor systems for immobilised enzymes/cells. (a) batch reactor, (b) recycle batch reactor, (c) continuous flow reactor and (d) packed bed reactor with total recycle.

process which produces high product yields quickly with minimum cost [101]. Nicell *et al.* have compared the performance of batch and continous stirred tank reactors (CSTRs) for the peroxidase catalysed removal of phenol and chlorophenol from wastewater [116]. Removal of aromatics using the CSTR design operated in series was found to be preferable [126] when environmental conditions such as pH and temperature are optimised [125]. Removal efficiencies were best at 25^{0}C; however, the removal of phenol and amines from aqueous solution using immobilised peroxidase has been reported as working satisfactorily over the temperature range 0-95^{0}C [98]. Siddique and coworkers employed a recycle batch reactor for 4-chlorophenol degradation. HRP was immobilised in cellulose disks, nylon balls and tubing in the reaction vessel and removal efficiencies were in excess of 80% [96]. Other reactor designs such as packed-bedslime digestors for phenol degradation have been shown to be effective oxidisers of phenolics into soluble polymerised precipitates [127]. This precipitate may be recovered for use as a fuel [109].

1.3.10 Oxidation of phenolics in non-aqueous media

Enzyme catalysis in organic solvents has many applications in industrial processes. HRP catalyses the oxidative coupling of phenols in a wide range of aqueous-organic solvent mixtures [128]. A phenol resin has been produced in a 1,4-dioxane/phosphate buffer mixture (80:20%v/v) using H_2O_2 as an oxidising agent. The polyaromatic precipitate can be removed by filtration and washed with methanol to separate phenol, peroxidase and other contaminants [129]. It has been noted that increasing the percentage dioxane in the reaction mixture results in a proportional increase in the oxidation of *p*-cresol, also, the apparent K_m of phenols increases as the solvent polarity decreases [130]. Lignin *p*-cresol copolymers have been produced in 50-70% mixtures of dioxane (both lignin and *p*-cresol are highly soluble in dioxane) [131]. Use of organic solvents such as dioxane also plays a major role in controlling the molecular weight of the resin. Polymers in excess of 25kDa have been synthesised. These phenolic resins or polymers are useful as developer resins for carbonless recording

materials and as adhesives. Attempts to synthesise phenolic polymers in aqueous solution using HRP/H_2O_2 have not been successful as of yet due to the inability of the medium to dissolve the polymer compound [132].

1.3.11 General treatment of industrial effluents

The ability of HRP to catalyse colour removal from bleach plant extraction effluent is well known [133]. A HRP/H_2O_2 system initially produces a faster rate of decolourisation than mycelium from *Coriolus versicolor*; however, a greater proportion is removed by the latter activity over a two day period. Davis *et al.* have reported similar findings using soluble laccase, HRP and *Coriolus versicolor* [99]. Laccase phenol-oxidases secreted by *Trametes versicolor* can oxidise a wide variety of biological and synthetic phenolic compounds [100]. Such enzymes are functionally superior to lignin-peroxidase from *Penicillium chysosporium* for pollutant dechlorination and are also more stable and cheaper to produce [134].

High phenol and ammonia concentrations are typical of coal conversion process wastewaters. Numerous experiments have demonstrated that the concentration of phenol can be removed to achieve levels as low as 20ng cm^3. Other major constituents include sulphur compounds and cyanides. Biological oxidation shows a very high potential for applications to hydrocarbonization wastewater treatment. In bioreactors for phenol degradation, the phenolic content is reduced by greater than 99.5% using trace metal-phosphate-supplemented hydrocarbonization scrubbing water as a reactor. The concentrations of other organic compounds (polyaromatic hydrocarbons and xylenols) are also reduced [135].

1.4 BIOSENSORS

1.4.1 Introduction

Biosensors are sensing devices which incorporate biological material in their construction and were first described in 1962 by Clark and Lyons, where glucose was determined by measuring the consumption of dissolved oxygen [136]. A biosensor can be defined as "a probe that encorporates a bioactive substance that can specifically recognise a species of interest with a physiochemical transducer". Biosensor development attracts interest from such far reaching fields as pharmacology, biochemistry and electronics. An ideal biosensing device should exhibit a number of characteristics such as being relatively small in size, a fast response time, be highly selective and possess a high affinity for the analyte of interest [137]. However, the numerous practical problems that exist are partly due to the active biocatalytic layer. Problems also arise when the analyte of interest is present in a complex matrix, such as blood plasma. Detection of a particular analyte can be prone to interference or matrix effects. A biosensor is usually composed of two components, a means of recognition and a method of transduction. Biomolecular sensing may be defined as the detection of analytes of biological interest, using an enzyme or receptor which has a specific affinity for a target molecule. The occurence of a physical/chemical signal in response to this biorecognition, which is subsequently converted by the transducer into a secondary signal, is usually electrical in nature [138], with a transduction mode which can monitor either thermal, electrochemical, optical or mass changes.

1.4.2 Transduction

The purpose of the transducer is to convert the biochemical signal into an electronic signal that can be easily manipulated. A range of transduction techniques exist in biosensor technology such as electrochemical, optical, calorimetric and piezoelectric-type devices. There are two main methods in electrochemical detection; amperometry and potentiometry. The former involves the application of a constant potential or

voltage with respect to a reference electrode. This results in a non-spontaneous electron transfer. The current (produced between the working and auxillary electrodes) is due to the reduction or oxidation of an electrochemical species at the working electrode and is proportional to the concentration of the analyte [139]. Steady state is a condition at which the rates of diffusion of the electroactive surface are equal. Net sensor current is dependent on factors such as charge transfer, adsorption, chemical kinetics, diffusion, convection and substrate mass transfer. The Clark electrode was the first reported transducer associated with biocatalytic recognition. Transduction of the signal due to an enzyme-based sensor can be useful in a number of ways:

1. the electrode detects a naturally existing cofactor, e.g. oxygen, or a product of an enzymatic reaction e.g. hydrogen peroxide. The resulting current is then proportional to the concentration of analyte;
2. mediators or cofactors have been employed in biosensor systems that reduce potential interferences by operating at lower applied voltages;
3. the electrode itself is used as a mediator to aid direct electron transfer where the catalytic activity is reacting directly at the electrode surface.

1.4.3 Working electrodes

Glassy carbon electrodes have been commonly used as amperometric sensors. Stulik *et al.* reviewed a number of techniques for the activation of solid electrodes such as polishing, chemical and electrochemical pretreatment, application of heat and electromagnetic radiation [140]. Electrochemical pretreatment of solid surfaces activates surface groups which may enhance the electrode's response. A glassy carbon electrode with immobilised uricase modified with Nafion and methyl viologen was constucted for the detection of uric acid. The biosensor had the advantage of high sensitivity and fast response times as compared with previous sensors [141]. Glassy carbon type electrodes have also been successfully used in flow injection analysis (FIA) systems. A detection limit lower than others previously reported for H_2O_2 has been

obtained [142]. The electrode had similar deection limits in both static and flow systems; however, the incorporation of the electrode in an FIA system resulted in a wider dynamic range and a greater sampling frequency

The carbon paste electrode was invented over 35 years ago by Adams [143]. Amperometric biosensors based on mixed carbon paste redox enzymes have been of a source of considerable interest. Biocatalysts have been found to retain their cataltic ability for an unexpected length of time. Carbon in the form of graphite is particularly suitable for electrode fabrication since the working surface may be easily renewed. It may be necessary to add a mediator to the enzyme/carbon paste mixture to enhance electron transfer. A HRP modified carbon paste electrode useful for the determination of aniline at low ppb levels was reported [144]. HRP was dispersed in carbon paste and immobilised on a Nafion membrane. Addition of triethylamine to the carrier stream in an FIA system increased sensitivity and suppressed memory effects. Good recovery was obtained when samples of spiked oils containing different concentrations of aniline were analysed. The simultaneous incorporation of ferrocene [$Fe(Cp)_2$] and HRP into a carbon paste matrix resulted in an effective electrode for sensing hydrogen peroxide [145]. HRP catalyses the reduction of H_2O_2 in the presence of an electron transfer mediator according to the below reaction. The electrochemical reduction of the oxidised mediator provides the amperometric signal for the measurement of peroxide. FIA analysis of H_2O_2 had a high sampling frequency of approximately 70 per hour.

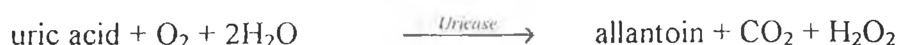
$$H_2O_2 + Med_{red} \rightarrow H_2O + Med_{ox}$$

Xanthine oxidase was adsorbed on carbon paste and physically entrapped in a semi-permeable membrane for the detection of hypoxanthine and xanthine. Measurement of the latter compound was based on the detection of enzymatically liberated uric acid which was electrochemically oxidised at 0.4 V vs. Ag/AgCl. The dynamic range of the electrode remained constant for 6 days, however, some compounds were found to interfere [146]. Carbon paste (without bioactivity) has proven to be of great benefit in

the voltammetric measurement of uric acid, a major constituent of body fluids. Urate rapidly adsorbs at carbon surfaces [147] A subsequent flow injection system with adsorptive accumulation of uric acid at the electrode surface showed good precision and compared favourably with other adsorbable species.

Cai *et al.* investigated the influence of electrochemical pretreatment of carbon paste electrodes on the oxidation of uric acid [148]. Anodic pretreatment at 1.4V SCE for a short period of time greatly enhanced the response to urate. The authors attributed the improved responses to the actual preanodization potential, the duration of the applied voltage and the degree of alkalinity of the supporting electrolyte.

The fabrication and characteristics of graphite epoxy modified electrodes have been reported [149]. Unmodified epoxy-bonded graphite electrodes possess desirable electrochemical, mechanical and chemical properties and have been used for *in vivo* voltammetry and microanalysis. Gilmartin *et al.* have described the preparation and analytical utility of chemically modified graphite-epoxy resin electrodes as amperometric sensors for uric acid [150]. The precision of surface-surface reproducibility was calculated as being 7.3% (n=5). Cobalt phthalocyanine (CoPC-) doped epoxy resin electrodes coated with cellulose acetate membranes appeared to act in reducing the overpotential required (<600 mV) for the oxidation of peroxide (liberated from the uricase catalysed oxidation of uric acid).



$$\text{uric acid} + O_2 + 2H_2O \xrightarrow{\text{Uricase}} \text{allantoin} + CO_2 + H_2O_2$$

This methodology was further developed for the evaluation of modified screen printed carbon electrodes (SPCEs) with a view to developing an amperometric sensor for uric acid [150, 151]. The system entailed a CoPC electrode for the electrocatalytic oxidation of H_2O_2 by means of a cellulose acetate (CA)-uricase bilayer. Amperometric calibrations were linear up to 1 mM, with a limit of detection of 13×10^{-6}M. Precision of electrode fabrication was 4.9% (n=6) with a deviation in signal intensity of 14% over a week.

Platinum has been widely used as an electrode material (based on hydrogen peroxide and oxygen consumption modes) because of its inherent superior catalytic response. Nanjo and Guilbault used a platinum electrode for the selective detection of uric acid in serum and urine [152]. The working surface area of the electrode was covered with immobilised uricase, acting as a thin reaction layer. The initial rate of disappearance of dissolved oxygen was proportional to the uric acid concentration present.

An amperometric platinum based sensor was able to enhance selectivity towards peroxide determination when pretreated with 4-aminophenol and phenol solutions [153]. This makes possible the elimination of interferences encountered in clinical monitoring. This approach has the advantage over a permselective barrier such as cellulose acetate in that it almost completely eliminates potential paracetamol interference from glucose measurements. The polymerisation of phenol at the electrode surface is dependent on the applied potential; however, this coating is rather unstable.

The reduction of electrode poisoning can be achieved by dispersing a Nafion film incorporating platinum particles on a glassy carbon electrode. This has been shown to act as a selective and sensitive surface area for monitoring glucose [154]. Tatsuma reported on the use of a tin(IV) oxide plate electrode for the amperometic detection of lactate, pyruvate, cholesterol and uric acid [155]. The tin(IV) oxide bilayer modified electrodes were prepared by firstly treating the surface with 10% aqueous (3-aminopropyl) triethoxysilane followed by 2.5% glutaraldehyde. HRP was immobilised onto the surface by passive adsorption. The respective oxidase activity (e.g. uricase) was immobilised after the further treatment of the electrode with glutaraldehyde solution. The system has the advantage of using the minimum amount of enzyme; however, response times were slow (approximately 5 minutes).

Potentiometric transducers measure the difference in potential between the working electrode and a second reference electrode while the current remains constant. The systems are based on the Nernst equation, where a logarithmic relationship exists between the potential and the activity of the ion in solution. Examples of potentiometric devices include ion selective electrodes (ISEs) and gas-sensing

electrodes [156]. Potentiometric enzyme electrodes have been fabricated by immobilising or covalently binding an enzyme or biological component onto or in the selective membrane of an ion/gas selective electrode [157]. The most common example of this type of device would be the ammonia sensor, based on the hydrolysis of urea, creatine and amides and the oxidation of amines and amino acids. These devices are based on the consumption or formation of substrate or product respectively, which causes a change in the activity, a_i, of the ion for which the membrane is selective.

1.4.4 Immobilisation of biological components onto electrode surfaces

Modification of an electrode is achieved by immobilising a biological molecule onto an electrode's working surface area. A good immobilisation technique is one that will be applicable to a wide range of surfaces. Many surfaces have hydroxyl groups attached to them whether adjacent to carbon, silicon or other atoms. Surface pretreatment with titanium tetrachloride (activates existing surface groups), can provide a stable titanium link between the surface and a designated biocomponent. This link is resistant to a wide range of physiological pH values and is also non-biodegradable. Titanium tetrachloride can also have a use in premodifying surfaces that possess $-NH_2$ groups. The most important consideration is that the biocatalyst exhibits maximum activity in its immobilised state, where the active centre of the component is facing towards the solution of interest. A bound bioactivity comparable to activity in free solution is the main goal of immobilisation techniques [158]. Important factors in immobilisation include:

1. ability of the biocomponent to function over a wider pH range than in solution;
2. the ability to achieve greater stability;
3. an ability to dispense with its coenzyme;
4. the possibility of co-immobilising more than one biocatalyst;

5. exhibition of maximum activity.

It is important that overloading of the solid support with the biocomponent be avoided. This can result in a decrease in the overall performance because of the restricted access of the analyte to the active centre. This problem may be overcome through the use of a porous surface on the solid support [158]. Taking this into account, it is important that the biocomponent does not leak into the analyte solution while the biosensor is in operation. This is generally not a problem except where the biocatalyst is a microbial cell.

1.4.4.1 Adsorption: A variety of substances are capable of adsorbing enzymes and other proteins onto their surfaces, e.g. alumina, charcoal, cellulose, silica gel, glass, collagen, hydroxyapatite and clay. This list may also include ion exchangers such as DEAE cellulose and a variety of phenolic resins. The advantage is that no reagents are required and only a minimum of activation or "clean-up" steps are necessary.The process is less disruptive to the protein than other immobilisation methods. Binding forces are due to hydrogen bonds, salt linkages and van der Waals forces. Even though it is a convenient immobilisation technique, it is not stable, as the aforementioned binding forces are susceptible to changes in pH, temperature, ionic strength and the presense of substrate [159, 160]. This method can be performed by dropping an aliquot of the protein solution on the carrier and allowing the solvent to evaporate. The preferred application is adsorption on graphite electrodes [161]. A hydrogen peroxide sensor has been prepared by adsorption of HRP onto a glassy carbon surface using hydroquinone as a mediator. When used in an FIA system, the electrode gives stable responses for at least two weeks without desorption effects [142]. Unlike the latter case, a glucose electrode was described where the mediator, Meldola Blue and glucose dehydrogenase were co-adsorbed at a carbon surface [162]. Porous carbon paper, acting as a supporting material for uricase, has been used as a basis for an amperometric sensor for uric acid. The concentration of uric acid is measured when the

sample solution is dropped onto the membrane and the resulting current measured at an applied potential of -0.6V. The thickness of the paper was considered as being extremely important in obtaining a rapid electrode response; the performance being dependent on the rate of oxygen transfer through the enzyme modified carbon felt [163].

1.4.4.2 Covalent binding: generally, stable attachment of proteins to a support is best achieved by covalent binding or attachment. Covalent binding between the enzyme and the support matrix is accomplished through suitable functional groups in the enzyme which are not essential for its catalytic activity (Figure 1.4) [164]. Amino acids essential for this purpose used in covalent linkage generally results in loss of enzyme activity. The affinity of the enzyme for the substrate may be affected by conformational changes brought about by covalent bonding. Nucleophilic functional groups present in amino acid side chains of proteins provide the target for coupling. These range from amino, carboxylic acid, hydroxyl, phenolic, imidazole and thiol groups. Coupling preferably occurs at low temperature, low ionic strength and within the physiological pH range. Coupling is often carried out in the presence of the enzyme's substrate to protect the active site. Covalent binding of enzymes to electrode surfaces has been achieved on metals, graphite and conducting polymers. These surfaces require pretreatment (oxidation) prior to binding. Such surface hydroxy groups are reacted with silanizing reagents such as (aminopropyl)triethoxy silane to produce up to three bonds on the electrode surface [165, 166]. Bifunctional reagents, such as glutaraldehyde, can be used to form intermolecular cross-links, which results in the binding of the protein to a solid support [167]; however, a large amount of biological sample is somtimes required [158]. Moreover, the degree of oxidation of graphite electrodes can be increased by electrochemical oxidation resulting in active carboxylic groups on the surface. These groups can be activated for protein coupling using dicyclohexylcarbodiimide. Such techniques, if carried out correctly, can result in a monolayer of enzyme permitting rapid substrate diffusion to the active site. Yao *et al.*

immobilised a number oxidases on glass particles using 4% glutaraldehyde for FIA analysis of glucose, cholesterol and uric acid in blood serum [168]. Uricase was loaded

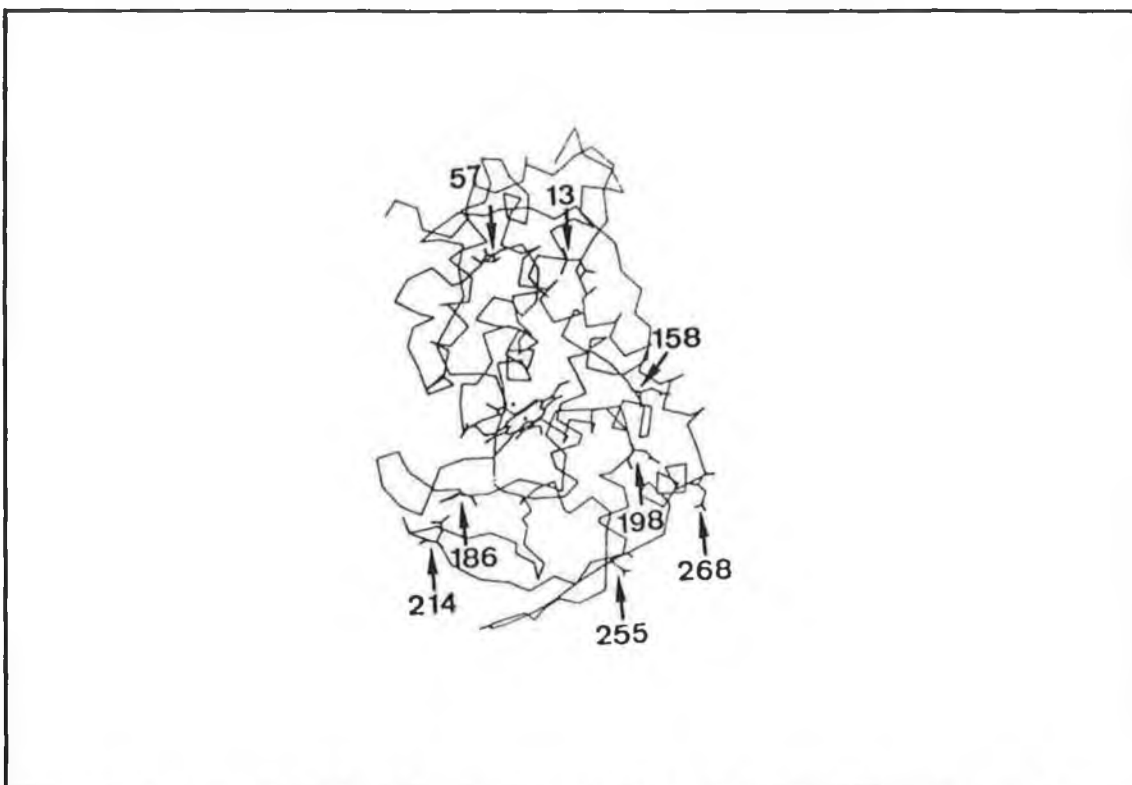


Figure 1.4: Predicted backbone structure of HRP-C. Glycosylation sites are shown as bold numbers.

on the glass particle column for two hours at room temperature by circulating 0.1M enzyme-borate solution. Enzymatically generated H_2O_2 was detected at a poly(1,2-diaminobenzene) film coated platinum electrode at a constant potential of 0.6V vs. Ag/AgCl. Estimated sample throughput was 38 per hour.

1.4.4.3 Entrapment in a matrix: the entrapment of biomolecules behind or within a polymer film has a number of advantages such as providing a stable environment and enhanced porosity, allowing for both movement of substrate and electrolyte. This method of preparation can be applied to any enzyme since the protein molecule is trapped in a three-dimensional lattice. Physical entrapment in a gel matrix e.g. gelatin [169], involves casting the gel over the electrode surface and holding it in place with a dialysis membrane such as cellulose acetate [170, 171]. Immobilisation is as simple as

physical adsorption: the enzyme need only be mixed with the paste or prepolymer and applied to the solid surface. Kinoshita *et al.* described the application of a plastic carbon disk electrode covered with a dialysis membrane for assaying inorganic phosphate and adenosine deaminase in serum based on the measurement of uric acid at 0.45V against a saturated calomel electrode [172]. The dialysis membrane (20 μM thick) was fixed by covering with a nylon net. The membrane covering the electrode provided a stable diffusion layer for uric acid, resulting in a reproducible steady state current. It also acted as a barrier to high molecular weight proteins in serum, preventing contamination. This method does, however, suffer from two major drawbacks: (a) large diffusional barriers to the transport of substrate and product, leading to reaction retardation with high molecular weight substrates such as trypsin, ribonuclease and dextranase, and (b) continuous loss of enzyme as some pore sizes permit escape; however crosslinking the protein with glutaraldehyde can alleviate this problem. The main advantage of this technique is its compatibility with mass production methodologies such as screen-printing [173].

1.4.4.4. Electropolymerisation: membranes can be produced at the working surface area of electrodes using this technique. Conducting polymers represent a class of new materials that have electronic conductivity. Polyacetylene was the first polymer that could be made electronically conductive. The first experiments were made in the mid 1970's and since then a number of conducting polymers have been synthesised. The most important polymers are polyacetylene, polypyrrole, polythiophene, poly-3-alkylthiophenes, poly(phenylene) and polyaniline [174]. Such polymer films are prepared by chemical or electrochemical polymerisation from a monomer solution containing an electrolyte salt. In the latter case, a three electrode voltammetric cell is normally used. Reduction of the polymer film can be accomplished either electrochemically where the potential is kept at the reduction potential of the polymer or chemically, where the polymer film is washed with a reducing solution such as ammonia. Polymer films are self-regulatory with uniform thickness. The chemical and

physical properties of such films can be easily controlled by the polymerisation conditions [164].

The properties of polypyrrole-modified amperometric cholesterol electrodes have been discussed [175]. Polypyrrole layers show electrical conductivity, this has been used in designing mediator-based biosensors. The polymer can only be used at low potentials as it loses its conductivity properties when higher potentials are applied [176]. Results have demonstrated that polypyrrole cholesterol sensors remain stable in organic solvent systems. Thus, they would have applications in cholesterol determination in foodstuffs such as butter and oil [175]. Incorporation of glucose oxidase into polypyrrole has attracted most attention. It has been possible to directly incorporate glucose oxidase and an electron transfer mediator, ferrocene carboxylic acid, simultaneously into a polypyrrole film by electropolymerisation of pyrrole. The sensor's behaviour is determined by the balance of the diffusion of reactants and products within the film and the kinetics of the immobilised activity [177]. Foulds *et al.* reported on the electrochemical deposition of a redox enzyme, glucose oxidase, in polypyrrole. Ferrocene was incorporated into the polymer to create a reagentless glucose sensor. A number of different types of polymer were examined for their compatibility with the redox activity, showing faster enzymatic regeneration times than others, however, all polymer films regained their initial cyclic voltammetric behaviour when rinsed in protein-free electrolyte solution [178].

Some workers have directly incorporated glucose oxidase into polyaniline. This was carried using microelectrode technology, for which resistance problems (due to the growth of a less conductive polymer) were not a problem [179]. Other workers have successfully incorporated polysaccharide matrices such as cellulose and dinitrocelluose into polyaniline conducting composites [180]. The physical properties of the composites can be varied by changing the polysaccharide matrix material, thus allowing the material to be tailored to meet specific requirements. 10% glycerol was found to increase the polymer composite lifetimes. Thiophene-based polymers, polythiophene and its derivative, poly-3-methylthiophene have shown to have

enhanced conductivity properties after electrochemical doping in aqueous media. The undoped polymer appeared to be oxidised by by-products derived from H_2O_2 oxidation. Such ion exchange can be used to incorporate active species [181]. King *et al.* successfully copolymerised [Ni(cyclanN-CH_2CH_2-thiophene)]$^{2+}$ with 3-methylthiophene and achieved in more adherent stable films. The solubility of the oligomers was reduced. The Ni(II) redox behaviour was unaffected by incorporation into the conducting polymer matrix [182]. An amperometric biosensor for hypoxanthine was constructed by forming a layer of gluataraldehyde cross-linked xanthine oxidase on a platinum electrode followed by electropolymerisation of a submonolayer film of resorcinol and 1,3-diaminobenzene in aqueous solution. Electrodes were stable for up to 60 days and could be used for over 6 hours without any adverse effects [183]. Geise and colleagues reported on a sensor for serum glucose determination by FIA. The authors used a poly(1,3-DAB/resorcinol) matrix. Glucose oxidase was immobilised at the electrode surface with glutaraldehyde. The biosensor was found to be effective for 5 months. The electropolymerised film was found to effectively block non-electroactive species commonly found in blood serum, thus preventing electrode fouling, while minimising diffusional problems [184]. The electrochemical properties of various heteroaromatic compounds used as conducting polymers have been discussed. Advantages include the existence of a three-dimensional reaction zone at the electrode surface permitting a greater rate of electron transfer. However, disadvantages in certain cases include poor adherence of polymerised films and incompatibility with some enzyme immobilisation procedures [164]. However, electropolymerisation appears to be one of the most promising avenues for the mass production of biosensors.

1.4.5 Kinetics of amperometric electrodes

Amperometric detection has found wide application to selective measurements in biological media. Such sensors combine the specificity of the enzyme for its particular substrate with the direct transduction of reaction rate into a current. First generation

devices, for example bienzyme electrodes [136, 186], involved directly measuring the consumption of dissolved oxygen or the enzymatic production of hydrogen peroxide. Such responses were somewhat affected by ambient O_2. These biosensors were generally prepared by physical entrapment of soluble enzymes behind membranes or by attaching such membranes to a transducer Second generation systems have been developed where the enzyme performs the first redox reaction with its substrate but is then reoxidised by a mediator instead of O_2, the mediator is subsequently oxidised by the electrode (Figure 1.5) [187, 188]. Various ferrocene/ferrocinium couples are efficient mediators. Other species, such as $[Fe(CN)_6]^{3-}$ and N-methylphenazinium (NMP^+) can also be used. A simpler method is where no mediator is applied, but where the reduced enzyme can be direactly oxidised at the electrode surface [189]. The feasibility of using conducting organic salts such as NMP^+ for oxidising reduced forms of enzyme complexes has been demonstrated. A large range of salt have been shown to be electrochemically active with glucose oxidase [190] and indeed with a number of different flavoproteins.

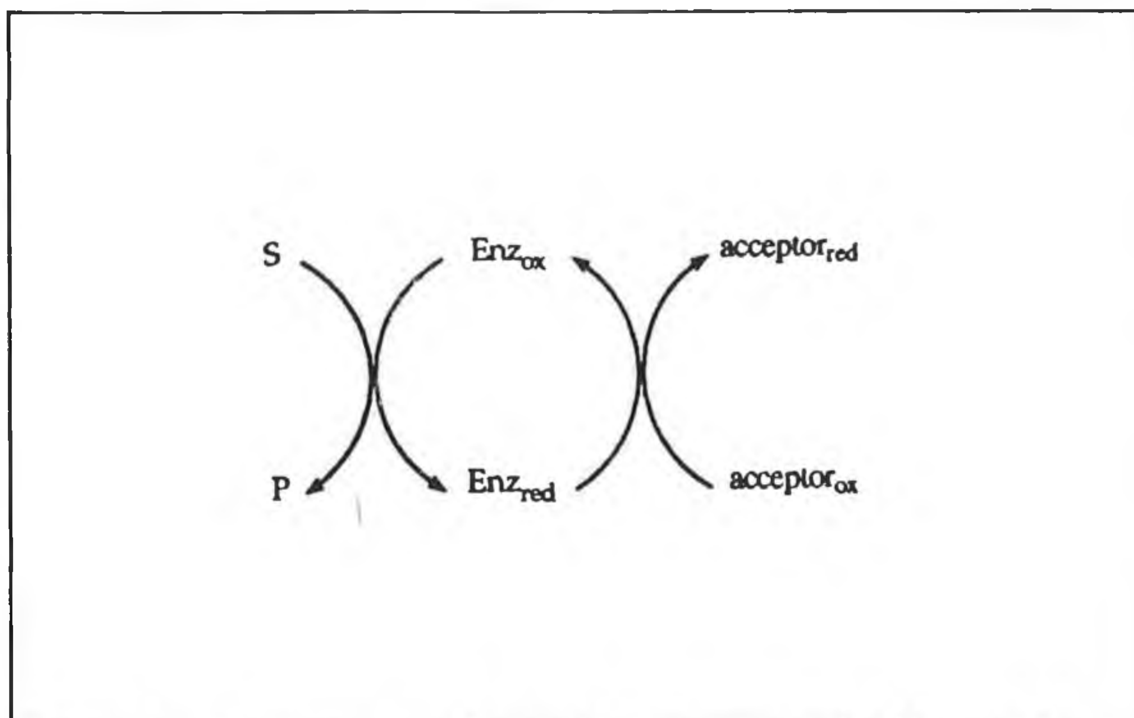


Figure 1.5: Reaction sequence for an oxidation reaction catalysed by an oxidase using an artificial electron acceptor.

In optimising an electrode's performance, it is important to understand the kinetic behaviour of the immobilised enzyme along with other factors affecting stability, dynamic range and response time If the catalytic activity of the enzyme is high, the overall reaction rate may be limited by mass transfer to the catalytic layer. A parameter which defines the former situation is referred to as the Damkoehler number, where V_{max}

$$D_a = \frac{V_{max}}{\frac{D}{\delta} K_m}$$

is the maximum rate of homogenous enzymatic reaction and K_m the Michaelis-Menten constant. For a D_a value ≤ 0.1, the reaction will be catalysis-controlled whilst for values ≥ 10, the reaction will exhibit mass transfer characteristics Conversely, catalysis-limited rates will be observed if the opposite occurs. A non-linear Lineweaver-Burk or Eadie-Hofstee plot would suggest a mass transfer limited process [191]. Mass transfer limitations cause K_m values to increase, if the rate of enzymatic catalysis is fast compared to the rate of substrate transportation to the biocatalytic layer. It must be noted that in glucose-based systems, the presence of oxygen can increase K_m values [192]; however, more rapid oxidation by the oxidase could alleviate this problem. Under kinetically-controlled conditions, the current/concentration-relationship would be non-linear and the dynamic range of the electrode narrowed. Such sensor configurations usually incorporate a membrane between the enzyme layer and solution and a current response proportional to the diffusional flux which is not limited by the enzyme's kinetics unless the activity becomes too low. As long as the enzyme's activity is high, the response of the sensor will be unaffected [193]. However, the response itself will become kinetically controlled and unsteady when the activity decays. An important point to note is that the kinetic parameters of immobilised activities are not

similar to those in solution because of conformational changes, steric effects and diffusional constraints [194]

1.4.6 Applications

Biosensors have been the focus of considerable attention in recent years as potential successors to a wide range of analytical techniques in the food and cosmetics industries, clinical chemistry and environmental monitoring. Even though the potential for biosensor development in an area such as human health is limited, the actual cost of reagents and the sensor element would be minimal compared to that of a more established analytical technique [195]. Nevertheless, a biosensor must be practical and have analytical utility if it is to be economically feasible. It is apparent which technologies (including membrane fabrication, screen printing and thin-film methods) are, in theory, suitable for the mass production of enzyme electrodes. This knowledge has led to the commercialisation of enzyme analysers that are used mainly for glucose or lactic acid determination [196]

1.4.6.1 Medical applications: the clinical chemistry laboratory has often favoured the use of more sophisticated and automated analytical devices. However, the necessity for more rapid results in the treatment of patients has led to the development of dedicated single specimen devices, for measuring important blood analytes and capable of giving rapid results. It would be an advantage if some types of investigations were performed in a doctor's office, hospital out-patient clinics or in the ward itself to provide on-the-spot data and necessary aid in diagnosing a medical problem. Blood gas (O_2 and CO_2) and electrolyte (Na^+ and K^+) estimation will presumably continue to be by chemical sensors; however, there is a requirement in clinical situations for accurate continuous monitoring. Approximately 20 self-contained analysers based on enzyme electrodes for the determination of 15 analytes are commercially available [197]. The ExacTech is a pen shaped amperometric device for the selective determination of glucose in blood. Glucose estimations were carried out by placing an aliquot of sample on the working

electrode surface and measurements were referred to a Ag/AgCl reference electrode. The device is marketed by MediSense, Abingdon, U.K. and Cambridge in the U.S.A. [158]. A bienzyme amperometric glucose electrode coupled with a pH probe has been used for short term *in vivo* monitoring of glucose based on the liberation of hydrogen peroxide [198]. Glucose oxidase and catalase were immobilised in a hydrogel bilayer. The most important aspect of such *in vivo*-type sensors is their susceptibility to intereferences from physiological components. Encapsulation of needle-shaped glucose sensors with silanised membranes reduced the adsorption of proteins at the membrane surface [199]. Silanizing reagents such as (aminopropyl) triethoxysilane can react with surface hydroxy or oxide groups. Yoshino and Osawa coupled uricase at a H_2O_2 permselective membrane which is applied to the commercial uric acid analyser, UA 300A by Fuji Electric, Japan. The system is based on the liberation of peroxide from the oxidation of urate by uric oxidase [200]. Commercial biosensors are also available for other clinically important analytes such as lactic acid [201], cholesterol [202] and urea [203].

The development of miniaturised voltammetric sensors for *in vivo* study is a focus of great interest. Unlike *in vitro* analysis, where the electrode sensitivity depends on the enzyme's reaction rate, diffusion of analyte and the efficiency of the electrochemical response, *in vivo* applications are influenced by such considerations as analyte diffusion in tissue, physiological reactions and the presence of metabolites. Thus, the sensor's activity and sensitivity are at risk. Miniaturisation of biosensors can offer greater *in vivo* biocompatibility and the chance of devising multisensor arrays and probes [204]. *In vivo* voltammetry has provided a novel route towards neurological studies. Neurotransmitter movement between sensory neurons can be studied by implanting a probe in the brain for a fixed duration. The sensor only has a fixed lifetime due to biological matrix effects [205]. Future work will undoubtedly focus on the development of probes for the selective determination of organic drugs and the monitoring of *in vivo* enzymatic and immunochemical reactions.

1.4.6.2. Food industry: biosensors have applications in the food industry at both the manufacturing and processing stages. A fibre optic sensor for the chemiluminescent FIA analysis of glucose in soft drinks has been developed. Glucose oxidase and peroxidase were covalently attached to polyamide and polyvinylidene difluoride-based membranes. Results compared favourably with a standard spectrophotometric method and the authors reported on the use of this technology in analysis of other analytes by coimmobilising the suitable oxidase with peroxidase [206]. Jawad *et al.* have described the electrochemical quantitative analysis of uric acid in milk. The peak in the urate oxidation wave at 0.8V was exploited. This method proved a highly accurate and rapid method without the necessity of milk pretreatment prior to analysis [207]. Adenosine-5'-monophosphate (AMP) concentrations in fish have been determined using a xanthine oxidase-based enzyme electrode. AMP was oxidised to uric acid and the disappearance of dissolved oxygen monitored. The sensor was stable for greater than 30 days [208]. Specific enzyme based sensors have been developed for sugars [209]. The determination of L-glutamate is important in fermentation control as many food products contain it as a flavouring agent [210]. A synopsis of the state of electroanalytical techniques in the food sector has been given by Mannino and Wang [211].

1.4.6.3. Environmental monitoring: biosensors are used in the monitoring of pollution. Kalvoda has reviewed current methods [212]. Monitoring of pesticides and herbicides can be carried out using enzyme electrodes. An amperometric sensor for the detection of phenols has been reported. Tyrosinase was dispersed in a ruthenium-doped carbon paste mixture and used in an FIA system which facilitated the determination of phenolics in a complex sample [188]. Another approach involved immobilising the same enzyme in a nylon membrane [213]. Hall *et al.* described an electrode selective for *p*-cresol in chloroform [214]. The requirement for reliable and robust devices capable of measuring acid rain or industrial pollutants in the soil or water will pave the way for future large scale development.

1.3.6.4 Industrial applications: biosensors have applications in process and quality control. However, many processes require sensors at remote parts of a manufacturing system where it is difficult to gain easy access to replace a sensor component. Many food processes require high temperature sterilisation procedures, thus making biosensors incompatible. Future work could be directed at these problems in order to make such devices more robust for industrial applications.

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CHAPTER 2

CHEMICAL MODIFICATION OF HORSERADISH PEROXIDASE

2.1 INTRODUCTION

Proteins such as horseradish peroxidase (HRP) play important roles in many processes encountered throughout biotechnology. Production of easily detectable compounds promotes the enzyme's use in clinical, analytical and industrial situations; it has applications in enzyme immunoassay [1], DNA probes [2] and in biosensor construction [3], as well as organic synthesis [4]. HRP has good stability characteristics which contribute to its widespread use [5]. However, a stabilised form of the enzyme is desirable, which could function more efficiently in adverse conditions, e.g. extremes of temperature [6], high pressure, pH values, intensive irradiation, high concentrations of organic solvents [7], highly concentrated salt solutions etc. Frequently, the literature stresses that the art of enzyme stabilisation should be learned from nature (from studying microorganisms that exist under extreme conditions) [8].

The general principle of protein stabilisation has been defined in terms of "the inactivation of the enzyme due to unfolding of its molecular structure under a certain denaturing action may be sharply retarded provided the protein globule is rigidified by a chemical modification or immobilisation" [9]. Denaturation refers to a conformational change that results in the loss of molecular function, which may be reversible or irreversible. By definition, an immobilised enzyme is one that is physically localised in a certain position, or converted from a water soluble mobile state to a water insoluble immobile one [10].

Protein structural and sequence studies have depended heavily on specially designed chemical compounds designed to modify specific functional groups of proteins. Different chemical modification strategies used to stabilise enzyme activity have been reviewed [11]. The chemistry of such reactions is well understood allowing

for specific side chains to be targetted. Such compounds can be further classed as bifunctional reagents, which may be further subdivided into homobifunctional (where both functional groups are identical) and heterobifunctional (two different functional groups are located on the same molecule) and zero-length crosslinkers [12]. Bifunctionally reactive compounds include bis-imidates, bis-succinimides and bis-maleimides. The latter type reagents react specifically with thiols, whilst the first two crosslink amino groups. Such reagents have been shown to induce crosslinking (both intra- and inter- molecular links), especially within membranes [13].

Bifunctionally-induced inter- and intra- molecular crosslinks act by reinforcing the active conformation of the protein, decreasing the entropy and reducing the rate of denaturation [14]. The maximum or minimum cross-linkable distance of a reagent naturally has a significant effect on the success of crosslink formation. For example, a bis-imidate shorter than 5Å usually yields few or no crosslinks, whereas extensive crosslinking can be achieved when the length is between 11-22Å [15]. Beyond this range, greater crosslink length may not be considerably advantageous. Heterobifunctional reagents, as previously stated, incorporate two dissimilar functional groups, one photosensitive (e.g. azide) and one conventional (e.g. imidate). Crosslinking in this case can be controlled both selectively and sequentially. The major application of hetero- reagents is in macromolecular photoaffinity labelling which involves the binding of polypeptide ligands to a reagent and specific receptors [13]. Zero-length crosslinkers induce direct conjugation between two chemical groups, without introducing extrinsic material. Examples include carbodiimides and carbonyliimidazole, which induce condensation of a carboxyl and amine group, forming an amide bond [12].

In crosslinking reactions, the protection of the active site residues by substrate or reversible inhibitors is important and also to ensure that functional groups targetted for modification are not involved in biocatalysis. Potential target sites must be distant from the enzyme's active centre and not buried in the folded protein structure. Acylating and alkylating agents are the most commonly used functional groups [12]. Acylating agents are considered to be amino-specific while alkylating agents are thiol-specific. Homobifunctional reagents contain either type, whereas heterobifunctional reagents may contain a combination of both The specificity of these chemicals for a specific amino acid side chain depends on the relative reactivity of the nucleophile; however, several side chains may react with the same bifunctional reagent [12].

This chapter describes the use of bis-succinimides to modify free ε-amino groups of commercial HRP under mild conditions of pH and temperature. Reaction of HRP with the bifunctional reagents, ethylene glycol bis-succinimidyl succinate (EG-NHS) and suberic acid bis[N-hydroxysuccinimide ester] (SA-NHS) have yielded derivatives which display greater thermostability and organo-tolerance. The thermal stability of HRP has also been enhanced by acetylation using the monofunctional modifier, acetic acid N-hydroxysuccinimide ester (AA-NHS). These modifications preserve the carbohydrate moiety for subsequent reaction and/or immobilisation.

N-hydroxysuccinimide esters were initially developed by Anderson *et al.* [16]. NHS esters are stable for several months at 4-25^{0}C under anhydrous conditions. Reaction of NHS esters occurs at pH 6-9, with a high degree of reactivity (to most accessible protein groups) attained within 10-20 minutes [12]. The reaction of HRP with an NHS ester involves the nucleophilic attack of an amine on the acid carboxyl of an N-hydroxysuccinimide ester to form an amide, releasing the N-hydroxysuccinimide.

2.2 EXPERIMENTAL

2.2.1. Materials

Peroxidase (E.C.1.11.1.7.) Type VI from horseradish was obtained from Sigma Chemicals, Dorset, U.K. and Boehringer Mannheim, Germany.

Sigma also supplied 3,3', 5, 5'-tetramethylbenzidine dihydrochloride (TMB) in powder and tablet form, *o*-phenylenediamine (OPD), borax, ethylenediaminetetraacetic acid (EDTA), albumin (bovine), tris (hydroxymethyl) aminomethane hydrochloride, Tween 20, sodium hydroxide, citric acid, guanidine hydrochloride and all crosslinking and chemical modification reagents.

Dimethylsulphoxide (DMSO) and analytical grade hydrogen peroxide came from BDH Ltd., U.K.

Methanol, dimethylformamide and tetrahydrofuran were purchased from Labscan, Dublin, Ireland.

Bicinchoninic acid protein assay reagent was obtained from Pierce Chemical Co., Illinois, U.S.A.

96-well flat bottomed microtitre plates were obtained from Greiner, Germany.

ECL luminescent reagent was supplied by Amersham International plc., U.K.

DEAE-Sepharose was obtained from Pharmacia, Sweden.

0.20μm filters and all Corning glassware was obtained from Corning, New York, U.S.A.

All other reagents were of analytical grade and obtained from Merck, Germany; BDH Ltd., Poole, Dorset, U.K. or from Riedel de Haen, Germany.

Enzfitter software was obtained from Biosoft, U.K.

2.2.2. Equipment

A Morgan Grundy (Crowley, Middlesex, U.K.) waterbath with a Techne Tempette Junior TE-8J heating unit was used in denaturation studies.

A Titertek Twinreader type 381 (Flow Laboratories Ltd., Scotland) was used to read absorbances on microtitre plates.

An Amerlite plate reader supplied by Amersham International plc., was employed in luminescence assays.

A Perkin Elmer luminescence spectrometer was used for all fluorescence studies.

A Heraeus Christ Labofuge 6000 centrifuge was employed to centrifuge samples in universal tubes (1-20ml volumes).

A Shimadzu UV/Visible spectrophotometer was used for all spectral determinations.

2.3 METHODS

2.3.1. Horseradish peroxidase microassay

Horseradish peroxidase (HRP) activity was measured using a method similar to that of Ryan *et al.* [17] and based on that of Bos *et al.* [18] and Gerber *et al.* [19]. HRP was prepared at a concentration of 1mg ml^{-1} in 0.1M phosphate buffer, pH 7.0. Serial dilutions from stock HRP were made in 0.01M phosphate, pH 7.0 containing 0.002% Tween 20 to a final concentration of 80μg L^{-1}. The hydrogen donor, tetramethylbenzidine dihydrochloride (TMB), was used in powder form at a concentration of 0.1g L^{-1}.

Dimethylsulphoxide, at 2% final volume, was used to initially dissolve powder TMB. The buffer employed was 0.1M sodium citrate, pH 5.5. Prior to assay, 30% v/v hydrogen peroxide (H_2O_2) was added to (0.1g L^{-1}) TMB solution to give a final concentration of 0.03%, i.e., 3μl peroxide per 10ml TMB.

All assays for HRP activity were carried out in quadruplicate in 96 well flat-bottomed microtitre plates. Fifty microlitre aliquots of 80μg L^{-1} HRP samples were pippetted into wells and the plate was allowed to achieve thermal equilibrium (25^{0}C) in a Titertek plate reader. 150μl of buffered substrate (TMB and H_2O_2) was dispensed into each well to initiate the reaction. The plate was then gently shaken to ensure the formation of a homogenous mixture in each well. Reaction mixtures turned blue with time, indicating catalytic activity Control wells contained 50μl 0.01M phosphate buffer, pH 7.0. Reactions were allowed proceed for 2 minutes before A_{620} values were determined. An alternative would be to add 50μl 2M sulphuric acid to each well and measure absorbances at 450nm.

HRP catalytic activities were also determined using the ECL luminescence assay. 100μl enzyme fractions, prepared in 0.01M phosphate buffer, pH 7.0, were dispensed into wells and 100μl Amerlite luminescence reagent added to start the reaction. Luminescence intensity values were measured after 2 minutes.

2.3.2. Thermal inactivation of HRP

Thermal inactivation of HRP samples was carried out at 65^0C and 72.5^0C for periods of up to 60 minutes. All samples were 80μg L^{-1} (standard assay concentration) in 0.01M phosphate buffer, pH 7.0 + 0.002% Tween 20. Aliquots of each sample were withdrawn onto ice at various time intervals throughout the hour. Each was assayed under the standard assay conditions as described in the previous section. Percent relative catalytic activity (% RCA) was determined for each time point (the activity at time t as a percentage of that at time zero). The apparent or pseudo half-lives, defined as the time required for HRP activity to be reduced to 50% of its initial value, were estimated by inspection of plots of percent activity versus time. Rate constants (k) were calculated from fits of data points to a single exponential decay (Enzfitter programme; also, $\frac{0.693}{k}$ = true half-life).

2.3.3. Determination of protein concentration

Protein concentration was determined using the bicinchoninic (BCA) method of Smith *et al.* [20]. In this method, protein reacts with Cu^{2+} in an alkaline environment producing Cu^+. BCA is a sensitive, stable and highly specific reagent for Cu^+, forming a water soluble product which exhibits strong absorbance at 562nm. The BCA assay, supplied in kit form by Pierce Chemical Co., contains two reagents.

Reagent A: an alkaline buffer containing sodium carbonate, sodium bicarbonate, sodium tartrate and BCA reagent.

Reagent B: 4% (v/v) copper sulphate solution.

The working solution was prepared by mixing 50 parts of Reagent A with 1 part Reagent B. A range of protein standards were prepared from a 2mg ml^{-1} solution of BSA using PBS as diluent. 10μl of each standard or unknown protein sample was pippetted into quadruplicate wells of a 96-well microtitre plate. Controls, consisting of 10μl of diluent, were also included. 200μl of working reagent was added to solutions and then gently mixed. The plates were covered and incubated at 37^0C for 30 minutes. A_{560} values were determined on a Titertek Twinreader Plus.

2.3.4. Extended storage studies

Studies were performed on HRP samples diluted in 0.01M phosphate buffer, pH 7.0 Samples were aliquoted into sterile microtubes and stored at 4^0C. At certain time intervals, samples were withdrawn from storage and then diluted in 0.01M phosphate buffer, pH 7.0. Thermal inactivation studies were carried out as described in Section 2.3.2.

2.3.5. Chemical modification of HRP

The protocol for the N-hydroxysuccinimide modification of HRP was similar to that of Ryan *et al.* [17] and based on that of Ji [12], Partis *et al.* [21] and Massague *et al.* [22]. N-hydroxysuccinimide and bifunctional N-hydroxysuccinimide esters [suberic acid bis(N-hydroxysuccimimide ester) and ethylene glycol bis(succinic acid N-hydroxysuccinimide ester)] and the monofunctional control, acetic acid N-

hydroxysuccinimide ester, were initially dissolved in 5% v/v DMSO and added to 1mg ml^{-1} solutions (as determined by BCA reagent) of HRP in 0.1M phosphate buffer, pH 7.0. Prior to use, esters were stored at 4^0C (AA-NHS at -18^0C) under anhydrous conditions. Reactions proceeded for approximately 60 minutes at room temperature, unless otherwise stated, and were terminated by adding an equal volume (1050μl) of cold 0.1 M Tris-HCl, pH 7.0. Enzyme samples were diluted to the standard assay concentration in 0.01M phosphate, pH 7.0, and assayed for initial recoveries and % RCA as described previously (Section 2.3.1.)

The chemical modification protocol was optimised with respect to the; concentration of modifier used in the reaction (0.1-5.0mg ml^{-1}) and duration time of the reaction (10-90 minutes). Reaction temperature (25^0C) and pH of the reaction medium (7.0) were kept constant. All reactions were terminated with cold 0.1M Tris-HCl, pH 7.0

2.3.6. Comparison of fresh and aged succinimides

Native HRP (Boehringer source) was modified using the N-hydroxysuccinimide (NHS) esters, SA-NHS and EG-NHS. The aged NHS esters had been stored in a freezer at -18^0C in a dessicator for over 3 years while the fresh reagents had been purchased a short time before use. The modification reaction was carried out as described in Section 2.3.5. Catalytic activities were measured using the TMB method.

2.3.7. Organic solvent profiles

Organic solvent profiles of various HRP samples were determined at 25 and 60^0C with exposure times of 60 and 10 minutes, respectively. Native HRP, acting as a control,

was included in all experiments. Methanol (MeOH), dimethylformamide (DMF) and tetrahydrofuran (THF) were selected for investigation as they possess respectively low, medium and high denaturation capacities [23]. Reaction mixtures were set up with increasing 10% volumes of organic solvent in 0.01M phosphate buffer, pH 7.0. Four 50μl aliquots were withdrawn from each reaction mixture onto ice and assayed under the standard conditions. Replicate solvent tolerance experiments had a relative standard deviation of 3.6% (n=3).

2.3.8. pH activity profiles

HRP samples investigated were (a) Native, (b) SA-NHS, (c) EG-NHS and (d) AA-NHS HRPs. The pH range examined was 6.0-9.0. At each pH value, the succinimide modification reaction was carried out at a HRP protein concentration of 1mg ml^{-1} in 0.1M phosphate buffer of the appropriate pH, length of exposure to the particular modifier was 20 minutes. Reactions were terminated by addition of 0.1M Tris-HCl of the appropriate pH. Subsequent enzyme dilutions for assay to a final concentration of 20μg L^{-1} were carried out in 0.01M phosphate, of the suitable pH. Activity was assessed by both TMB and ECL assays. The experiment was repeated using 0.05 M borate as buffer in place of 0.1M phosphate.

2.3.9. Determination of free amino groups

Free amino group estimation in various HRP samples was carried out using a method similar to that of Fields [24]. This method is useful for checking the extent of blocking or unblocking of amino groups in proteins and peptides. HRP was added to 0.5ml borate buffer (0.1M $Na_2B_4O_7$ in 0.1M NaOH, pH 9.5) and the volume made up to

1.0ml with ultra-pure water. 20μl of a 1.8 M TNBS (trinitrobenzenesulphonate) solution was added to the sample and mixed thoroughly. The reaction was allowed proceed for 5 minutes at room temperature before being terminated with 2.0ml 0.1 M NaH_2PO_4/1.5mM sodium sulphite solution. A_{420} values were determined (phosphate acting as a control). Free lysine content was estimated from an N-acetyl-L-lysine standard curve in the range 0.1-10.0mM. All HRP samples, native and NHS derivatives, were dialysed against 0.05M phosphate buffer, pH 7.0, for approximately 18 hours at 4^0C prior to TNBS estimation.

2.3.10 Stability towards denaturing and reducing agents

12M guanidine hydrochloride (GnCl), a protein denaturant, was prepared in 0.01M EDTA [25]. Equal volumes of 12M GnCl and HRP were added together to give a final denaturant concentration of 6M. Exposure time was approximately 10 hours at 25^0C. The protein content of each sample was 0.1mg ml^{-1}. Test and control samples were diluted to the optimum assay concentration (80μg L^{-1}) and their activities assayed under the standard conditions.

2-Mercaptoethanol, a reducing agent, was added to GnCl-treated stocks to a final concentration of 25mM [26]. Treatment time was 60 minutes at room temperature. Samples were then diluted and assayed as described previously, to assess the effect of a denaturant combined with that of a reducing agent. Native and modified samples were exposed solely to 6M GnCl/EDTA. The effects of the denaturing and chelating agents on the thermostability of HRP at 65^0C were investigated. Control samples were included throughout.

2.3.11 Fluorescence studies

All fluorescence spectra were recorded using a Perkin Elmer LS-50 fluorimeter in the spectral range 300-400nm, with a 1cm light path cell. Excitation and emission slit widths were set at 10.0nm [27]. The fluorimetric emission of the tryptophan residue in HRP was measured at λ_{max} (335nm) by exciting at 280nm. The concentration of peroxidase in all fluorimetric experiments was 0.4μM. HRP samples were incubated at 65^0C and aliquots withdrawn onto ice at regular intervals (10 minutes). Samples were allowed cool prior to spectral analysis. Fluorimetric emission of buffers were substrated from sample intensities. A correlation between HRP fluorimetric emission and % relative activity at 65^0C was carried out by assaying the above samples for activity (TMB).

2.3.12 UV/Visible spectrophotometric analysis

Spectra of all HRP samples (concentration: 0.4g L^{-1}) were determined using the spectrum mode on the Shimadzu recording spectrophotometer. Absorbances were read over the wavelength range 200-500nm. The effect of elevated temperature on native HRP was investigated by monitoring the absorbance of the characteristic Soret band at 403nm.

2.4 RESULTS AND DISCUSSION

2.4.1. TMB assay

A microassay for HRP activity was used as previously described by Ryan *et al.* [17]. TMB was employed as the hydrogen donor. The HRP-TMB reaction was optimised with respect to enzyme concentration only, as the optimisation of other relevant experimental parameters had been previously addressed.

Many hydrogen donors form coloured products on oxidation and this is particularly useful in the development of a colorimetric assay. Other commonly used hydrogen donors, often refered to as chromogenic substrates, include *o*-phenylenediamine (OPD) and 2,2'-azino-di-(3-ethyl-benzthiazoline-sulphonate). HRP catalyses the peroxide oxidation of such substrates by transferring electrons from the hydrogen donor to peroxide to yield a coloured product.

The optimum working pH range of HRP is 4.0-8.0 [28]. 1mg ml^{-1} HRP stock solutions were prepared in 0.1M phosphate buffer, pH 7.0 and stored at $4^{0}C$. Serial dilutions of stock were made up in 0.01M phosphate, pH 7.0. HRP can be inactivated by polystyrene microtitre plates if Tween 20 is omitted from the diluent (0.01M phosphate) [29]. Plates containing HRP samples only were allowed to achieve thermal equilibrium ($25^{0}C$) for 5 minutes in a Titertek Twinreader Plus prior to initiating the TMB reaction [19]. 50μl HRP and 150μl TMB buffered substrate solution were the assay volumes (1:3 ratio) [18]. HRP activity can be measured indirectly by following the rate of transformation of the hydrogen donor (TMB). Enzyme activity was represented by a colour change in the wells (colourless to blue). The intensity of the blue colour was proportional to the concentration of HRP present. A range of HRP concentrations ranging from 1.0 to 90.0μg L^{-1} were assayed at various time intervals at

a wavelength of 620nm (Figures 2.1 and 2.2). Although TMB has an absorption peak at 650nm, no filter was available for this wavelength. The absorption at 450nm can be measured if the HRP-TMB reaction reaction is terminated using 2M H_2SO_4 [18]. The colour of the reaction turns from blue to bright yellow due to the loss of two electrons (from the substrate) under acidic conditions. The use of acid is said to increase the sensitivity of the assay 2-4 fold, however, A_{620} readings were regarded as being adequate.

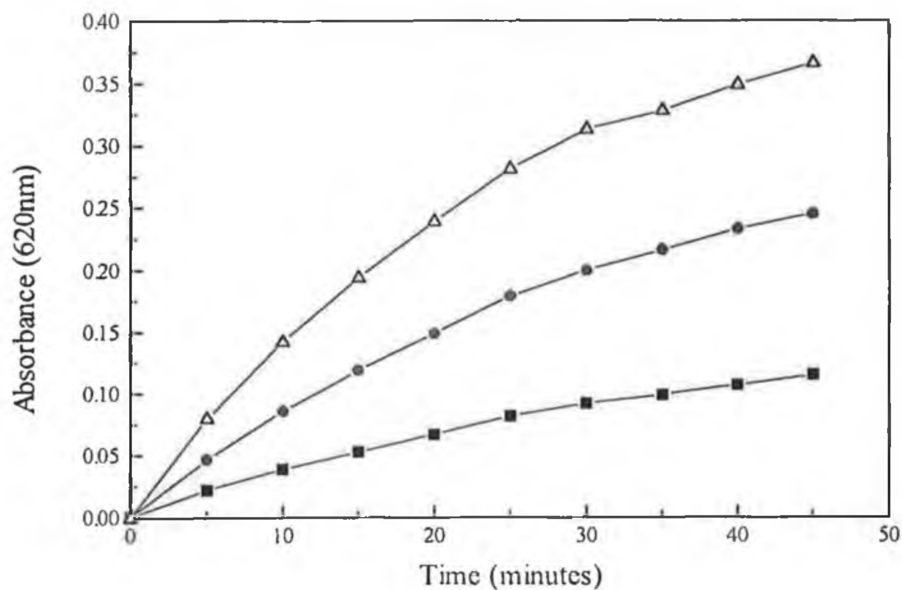


Figure 2.1: Absorbance (620nm) of TMB as a function of HRP concentration. ■ 1.0μg L^{-1}; ● 2.0μg L^{-1}; ▲ 3.0μg L^{-1}

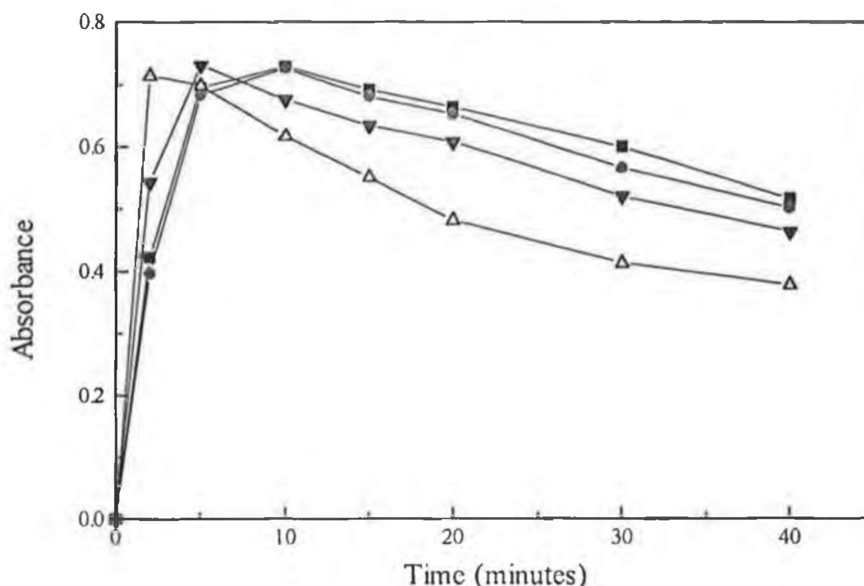


Figure 2.2: Absorbance (620nm) as a function of HRP concentration
■ 60μg L^{-1}; ● 70μg L^{-1}; ▲ 80μg L^{-1}; ▼ 90μg L^{-1};

The optimum assay conditions were determined as being: 80μg L^{-1} HRP read after 2 minutes at 620nm at 25^0C. 80μg L^{-1} was chosen as a linear increase in absorbance with time is desirable [19]. At this HRP concentration, the reaction displayed a linear response for 2 minutes only, followed by a rapid decline in absorbance, which suggested that the rate of reaction was extremely fast. Assays for HRP using other hydrogen donors have been reported where the reaction response is linear for 2 minutes [30]. A high absorbance value is desirable in standard assays as it is then possible to observe small differences in enzyme activity. Assay results (in quadruplicate) were found to be quite reproducible (% R.S.D. < 4.0%).

TMB in the dihydrochloride form is described as being water/buffer soluble. However, problems were encountered in dissolving TMB even at low water

concentrations. Bos *et al.* used various organic solvents to dissolve TMB free base, which is insoluble in water [18], where as the suppliers recommended DMSO. It was found necessary to dissolve the dihydrochloride form of TMB in DMSO (at 2% of the final buffer volume) before adding to citrate buffer, pH 5.5. TMB tablets were found not to fully dissolve in the same buffer. The only problem encountered in the use of TMB as a hydrogen donor was that care was required in its preparation as the substrate is susceptible to photo- and thermal-oxidation. All glassware used in TMB preparation was thoroughly washed and rinsed in ultra-pure water. TMB solution was stored at 4^0C in a sealed, covered glass beaker after preparation. The hydrogen donor was found to spontaneously oxidise (turn blue) in the absence of HRP system. The concentration of H_2O_2 used in the microplate assay was 0.03%. Greater concentrations could cause protein inactivation[19]

To summarise, a microassay for HRP using the highly sensitive, non-mutagenic and non-carcinogenic hydrogen donor 3, 3', 5, 5'-tetramethylbenzidine dihydrochloride was used as the standard assay in the following chemical modification studies. TMB, which has an absorption spectrum showing three peaks at 370, 655 and 450nm, is superior to some of the other commercially available hydrogen donors [18, 31]. It has a rapid reaction rate and is ideal for use in kinetic enzyme assays and immunoassays. The advantages of this assay include high sensitivity, lower enzyme concentration, absence of toxic effects from reagents and short incubation and assay times.

2.4.2. Optimisation of HRP modification protocol

As chemical modification of HRP's lysine residues has previously yielded derivatives with greater thermostability [17], it was decided to optimise the modification procedure, based on previous methods, employing ethylene glycol bis succinimidyl succinate (EG-NHS) only, which gave better stabilisation than the SA-NHS reagent. Preliminary results confirmed that succinimides are best used at neutral to mildly alkaline pH values [12]. Thus, 0.1M phosphate buffer pH 7.0, was used in all modification trials. Native HRP acted as a control in all experiments. 1.0mg ml^{-1} HRP was modified with EG-NHS dissolved in 5% v/v DMSO.

The effect of increasing the concentration of EG-NHS modifier is observed in Figure 2.3 where modified HRPs were incubated at 72.5^{0}C for 60 minutes. The reaction time of EG-NHS ester on native HRP was 60 minutes.

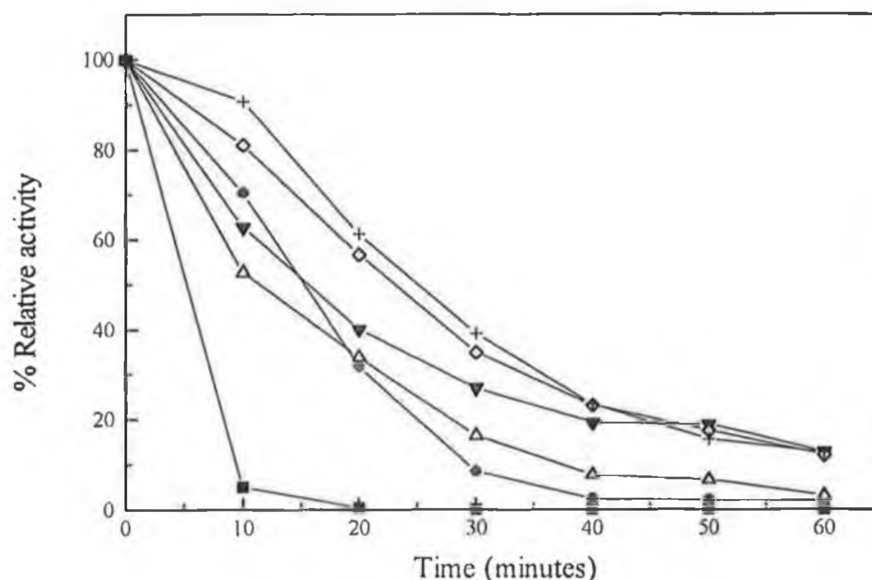


Figure 2.3: The effect of ester concentration on HRP thermostability at 72.5^{0}C. + 5.0mg ml^{-1}; ◆ 4.0mg ml^{-1}; ● 0.1mg ml^{-1}; ▲ 0.5mg ml^{-1}; ▼ 1.0mg ml^{-1}; ■ 0.0mg ml^{-1}

Assay for initial recoveries after the crosslinking reaction yielded 100% activity for all samples. The thermostability of the various HRP derivatives appeared to be directly dependent on the concentration of ester used. 4.0 and 5.0mg ml^{-1} NHS samples yielded less than optimal stabilisation despite their molar excess over the available target lysine residues of HRP. The 0.1mg ml^{-1} concentration was limiting at all exposure times while 0.5mg ml^{-1} became so at 40 minutes and longer. Therefore, the 1.0mg ml^{-1} NHS concentration was selected for use in the time variable experiment.

Figure 2.4 illustrates the effect of exposure time of 1.0mg ml^{-1} EG-NHS ester on 1mg ml^{-1} HRP in 0.1M phosphate buffer pH 7.0. Thermal inactivation curves for 1.0mg ml^{-1} HRP solutions modified with EG-NHS for 10, 20, 40, 60 and 90 minutes are shown in Figure 2.4.

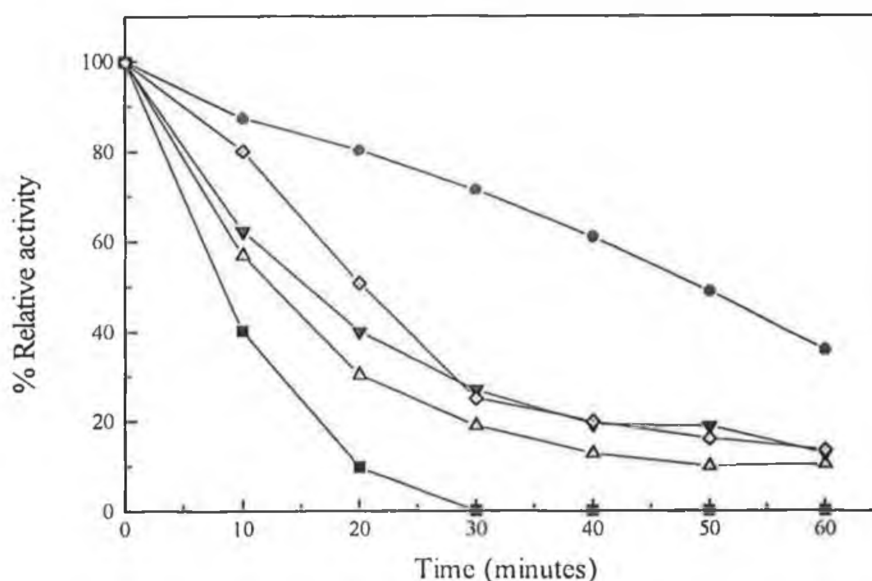


Figure 2.4: Dependence of ester exposure time on HRP thermostability at 72.5^{0}C
● 20min; ◆ 90min; ▼ 60min; ▲ 40min; ■ 10min;

It appeared, up to a certain point, that greater exposure times yield more stabilised derivatives of the enzyme. However, a variation in thermal stabilities with increasing ester exposure time was increasingly apparent with longer reaction times (at 72.5^{0}C). Ten minutes was clearly too short, while ninety minutes went beyond the optimal time for maximum stabilisation. A period of 20-60 minutes was found to be adequate.

These results suggested that succinimide stabilisation of HRP was best achieved at pH 7.0, using an ester concentration of 1-2mg ml^{-1} with a reaction time of 20-60 minutes.

2.4.3. Effect of pH on enzyme modification and activity

The pH range investigated was 6.0-9.0. At each pH value, modification was carried out at a HRP concentration of 1mg ml^{-1} in 0.1M sodium phosphate of the appropriate pH. HRP samples investigated were:

1. Native; 2. SA- and EG-NHS derivatives and 3. AA-NHS derivative

The results obtained are depicted in Figures 2.5 and 2.6. Variation of activity with pH in the range 6.0-9.0 was minor when using the TMB method of detection. However, Figure 2.5 shows the pH profile of the various HRP fractions in phosphate buffer using the ECL detection system. Greater fluctuations were noted, especially in the case of SA-NHS HRP where a distinct bell-shaped curve with a peak in catalytic activity at pH 8.0 was apparent. This probably reflects the greater sensitivity of the ECL assay and its ability to detect relatively minor differences between HRP concentrations and activities. Activity at pH 6.0 was by far the lowest within the pH range studied. Activities of the EG-NHS and AA-NHS forms showed less variation in

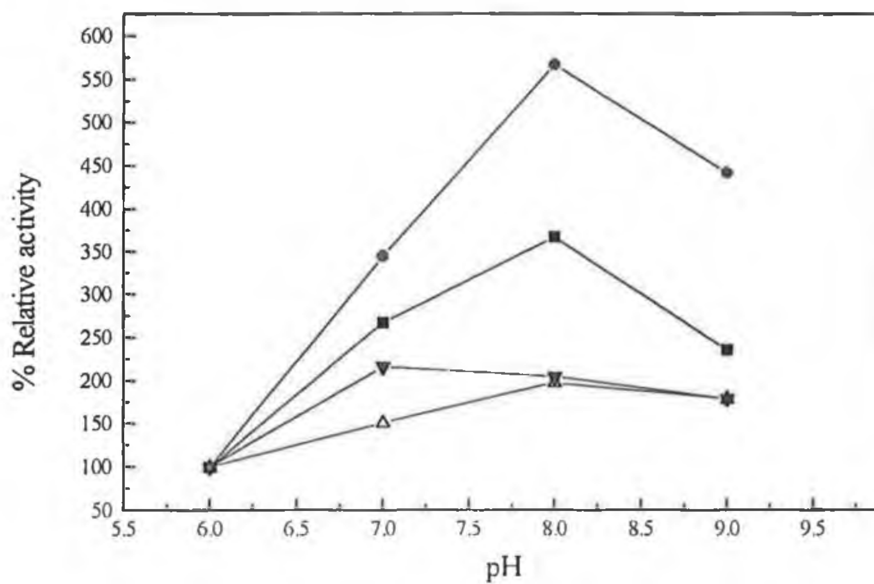


Figure 2.5: pH dependence of HRP preparations in phosphate buffer.
■ Native; ● SA-NHS; ▲ EG-NHS; ▼ AA-NHS;

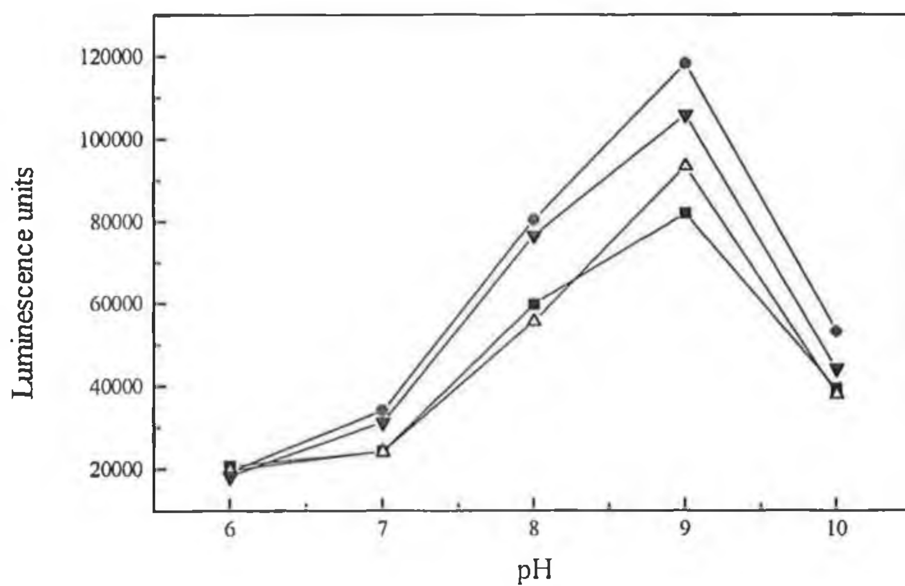


Figure 2.6: pH dependence of HRP preparations in borate buffer
■ Native; ● SA-NHS; ▲ EG-NHS; ▼ AA-NHS;

pH than SA-NHS HRP. Responses were almost constant in the 8.0-9.0 range for acetylated HRP and 7.0-9.0 for EG-NHS HRP. As borax buffer has been reported to have a stabilising effect on HRP [32], all HRP preparations were made up in 0.05M borate. Activities determined in borax by the TMB method displayed a flat pH profile over the range 6.0-9.0 but with a sharp decline in activity at pH 10.0 for all enzyme forms. Using ECL detection, the lowest activity occured at pH 6.0 (Figure 2.6). However, activities of all HRP preparations increased with a corresponding increase in pH up to pH 9.0, before a sharp decline at pH 10.0. The pH of a working medium is a factor that affects catalytic activity when the enzyme's active site is controlled by ionising groups.

Overall, these results agree with literature assertions that succinimide compounds are best used at neutral to mildly alkaline pH values. The best results arise from the use of phosphate buffer at pH 7.0-8.0 or with borate made up at pH 9.0.

2.4.4. Thermal studies on Native HRP

The thermal inactivation of native HRP was carried out at 65 and 72.5^{0}C (Figure 2.7). It was observed that after a 10 minute exposure at the higher temperature (72.5^{0}C), % relative catalytic activity (% RCA) dropped to 15.13% and HRP activity was practically zero at 40 minutes. Incubation at 65^{0}C produced a slightly greater level of enzyme activity; a % RCA of approximately 35% after 10 minutes. Also, some residual activity existed after one hour. An enzyme loses activity at high temperatures due to unfolding of the protein backbone. Inactivation is often caused by the destruction of one or two "weak points", such as hydrolysable peptide bonds, easily oxidised functional groups, etc. [33].

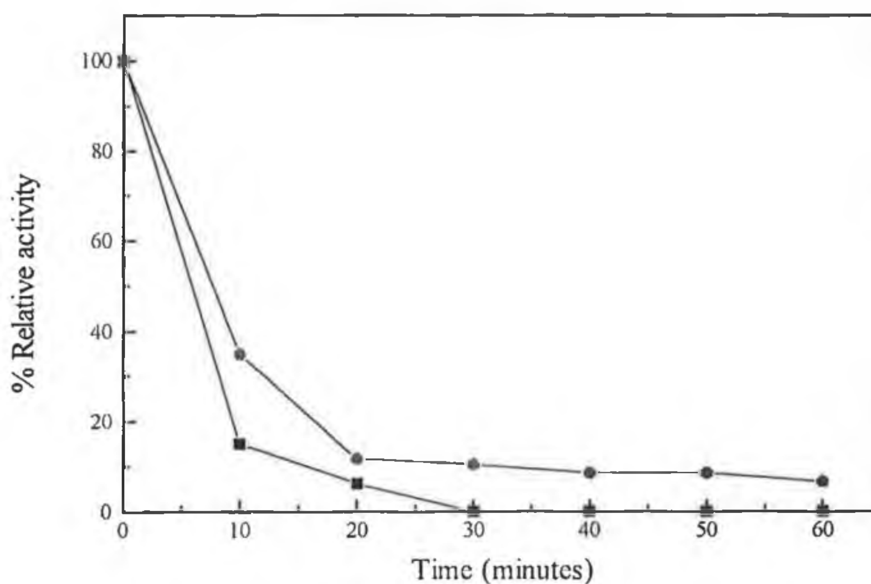


Figure 2.7: Thermostability profiles of native HRP at 65 and 72.5^{0}C.
● 65^{0}C; ■ 72.5^{0}C;

Activity is lost if exposure to high temperatures is prolonged, as unfolding disrupts the active site to an extent that is irreversible. Thermal inactivation is the process that is responsible for the gradual loss of enzyme activity with time at an elevated temperature. Irreversible thermoinactivation is treated as a two step process

$$N \xleftrightarrow{K} D \xrightarrow{k} I$$

where N, D and I are the native, reversibly denatured and irreversibly inactivated forms of a protein, respectively. Inactivation usually begins with a reversible unfolding step (N↔D) characterised by K, an equilibrium constant, and is followed by the D→I transition which is controlled by a rate constant, k. Irreversible inactivation may be due

to a number of factors including chemical processes [34], protein aggregation [33], or incomplete/incorrect protein refolding or renaturation. This implies that the irreversible status of an inactivated protein may in fact be due to the conformation of the tertiary structure [35]. The rate of inactivation (V_{IN}) and the apparent rate constant (k) are governed by both the reversible and irreversible steps, according to the following equation (2.1):

$$V_{IN} = \frac{-d[N]}{dt} = k_{IN}[N_O] \text{ and } k_{IN} = \frac{k.K}{1+K} \qquad (2.1)$$

where $[N_O]$ and [N] are the initial and current concentrations of the native enzyme [36]. Inhibition of the irreversible steps (by decreasing k) can be achieved by site-directed mutagenesis [37]. Chemical modification or immobilisation strategies [11] can be used to reduce the extent of initial unfolding, i.e., to decrease the equilibrium constant, K.

Thermal inactivation of commercial native HRP preparations deviated from a first order decay at 65 and 72.5^{0}C during a 60 minute incubation period (k-value at 65^{0}C was calculated to be 0.098 ± 0.012). Chang *et al.* observed that soluble HRP's thermal inactivation characteristics did not follow first order kinetics over the temperature range 60-94^{0}C [38]. The process was estimated as being of the order of 1.5. Hendrickx *et al.* noted a biphasic inactivation process for solid-state HRP in the range 140-160^{0}C; this phenomenon possibly being due to the presence of two individual HRP fractions with first-order kinetic properties [39]. Ugarova and colleagues observed first-order thermal decay patterns for HRP at 56^{0}C [6].

2.4.5. Thermal studies on HRP derivatives

Treatment with succinimides led to noticeable increases in the thermal stability of HRP at 65^{0}C (Figure 2.8). All derivatives displayed approximate 400% increases in apparent half-life.

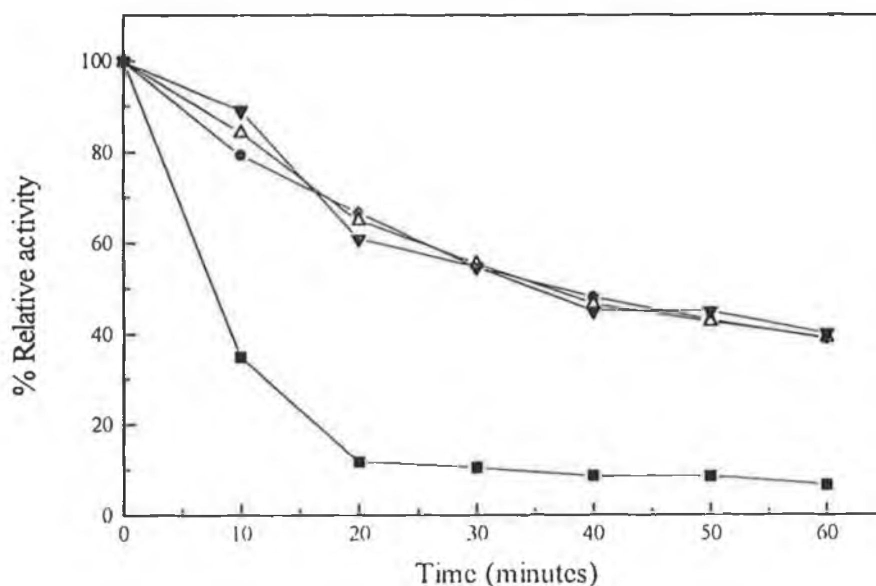


Figure 2.8: Thermostability profiles of native and modified forms of HRP at 65^{0}C ■ Native; ● SA-NHS; ▲ EG-NHS; ▼ AA-NHS;

HRP-treated with AA-NHS showed greater heat tolerance despite this reagent's inability to form crosslinks. The succinimide modification protocol did not result in any loss of HRP activity. Single exponential fits of thermal inactivation data indicated a five-fold stabilisation at 65^{0}C with apparent half-lives of 7 and 39 minutes for native and acetylated (AA-NHS) HRPs, respectively: k-values were calculated to be 0.098 $\pm$ 0.012 and 0.018 $\pm$ 0.002 min^{-1}, respectively. These values are for comparing stability

only; they may not represent true half-lives since HRP's thermal inactivation is not first order [38]. Stabilisations persisted on storage at 4^0C.

Table 2.1 shows the apparent half-lives (in minutes) for NHS-modified samples at 72.5^0C.

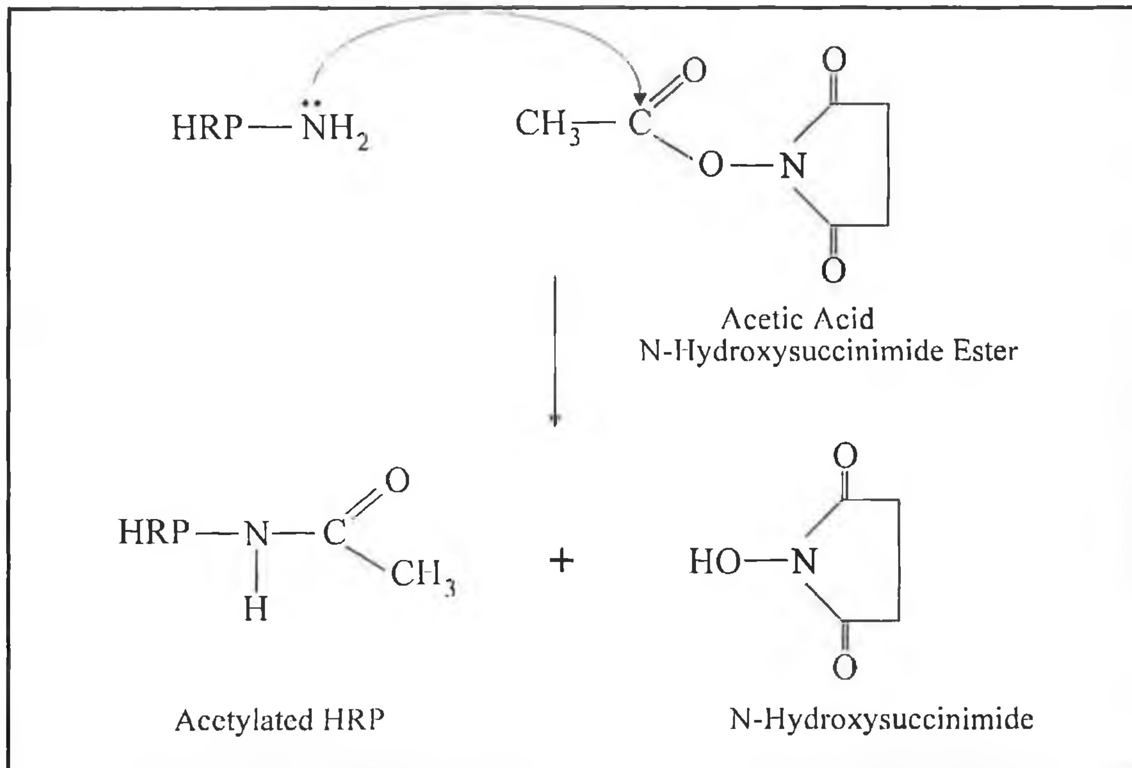
TABLE 2.1

Apparent half lives at 72.5^0C of HRP fractions stored at 4^0C

Time (days)	1	7	14	21
Native HRP	8.80	5.89	5.09	5.08
SA-NHS HRP	12.30	7.50	7.85	6.89
EG-NHS HRP	13.09	10.45	8.54	6.94
AA-NHS HRP	8.16	7.33	6.94	6.27

In this regard, it is interesting to note that modification of HRP with AA-NHS enhanced thermostability, although not to the same degree as bis-succinimide modification. At neutral pH, the amine functions of lysine residues are mostly protonated and thus positively charged. However, unprotonated lysines will exist in equilibrium with the changed form. Such unprotonated lysines react nucleophilically with the ester carbonyl group of the N-hydroxysuccinimide ester to form an amide, with the release of the N-hydroxysuccinimide. An amide cannot carry a charge and the positive charge of the original amino group is lost [12]. Although one would expect more alkaline conditions to favour such a reaction, rapid hydrolysis of the succinimide ester occurs in basic mixtures. Hydrolysis inactivates the NHS ester and so decreases

the efficiency of the reaction. Hydrolysis is favoured in dilute protein solutions and acylation in more concentrated samples.



Modification of HRP with the monofunctional reagent enhanced thermostability of the enzyme, possibly due to charge neutralisation in the absence of any crosslinking (see above). The acetyl group introduced by the acetylation reaction is small and electrically neutral. It cannot bring about charge reversal and is too small to provide significant shielding of close-lying groups. Neutralisation of like charges' repulsion may be responsible for the observed stabilisation: a decreased number of like charges will lessen the protein's tendency to unfold at high temperatures. There exists an alternative explanation. HRP's 6 lysine residues are located at positions 65, 84, 149, 174, 232 and 241 [40]. Lysine (Lys) 174 is thought to interact with the heme prosthetic group: it is

therefore unlikely to react with the acetylating agent, as any such reaction would likely cause inactivation. Of the 5 available lysines, at least 3 occur in regions of positive hydropathic character, indicating hydrophobicity (titration of AA-NHS HRP's free amino groups suggests alteration of 3 lysines; see Section 2.4.8.). Only lysine 65 and the heme-associated 174 are in hydrophilic portions of the enzyme. Neutralisation of positive charges within such hydrophobic sequences may well have a thermostabilising effect. This interpretation may be relevant to the increased "organotolerance" observed for acetylated HRP (see Section 2.4.6.).

Acetylated HRP did not confer the same degree of thermostability on HRP at 72.5^{0}C as did SA- and EG-NHS HRPs, even though all three reagents displayed similar effects at the lower temperature of 65^{0}C (Figure 2.8). It is probable that the AA-NHS ester is bound to HRP in a one-point modification (as this chemical modifier does not possess the ability to form protein crosslinks) and this charge neutralisation is not sufficient to withstand a temperature of 72.5^{0}C for long periods. The lack of a chemical crosslink gives only limited protection against high temperature. EG-NHS derivatives appeared to be more thermostable than SA-NHS HRPs at both temperatures (65 and 72.5^{0}C)

Native and modified forms of HRP were exposed to temperatures in the range 25-80^{0}C for periods of 30 minutes (Figure 2.9). Native HRP appeared to have an optimum working temperature of 35^{0}C, however its activity decreased rapidly at temperatures greater than 50^{0}C. In the range 60-80^{0}C, NHS derivatives exhibit greater stability, particularly noticeable around the 60^{0}C mark. In some cases, EG-NHS forms appeared more thermotolerant than acetylated HRPs and vice-versa. The thermal profile of EG-NHS HRP showed a gradual decline in relative activity between 40 and

80^0C, unlike that of acetylated HRP, where catalytic activity was lost more rapidly. Both bifunctional forms displayed similar activity profiles over the range investigated.

Apparent thermal activation, as opposed to thermal inactivation, was noted for all HRP forms at 42^0C (Figure 2.10). As the temperature of incubation increases, the extent of thermal activation decreases. This phenomenon has been reported for some immobilised and chemically modified enzymes [41].

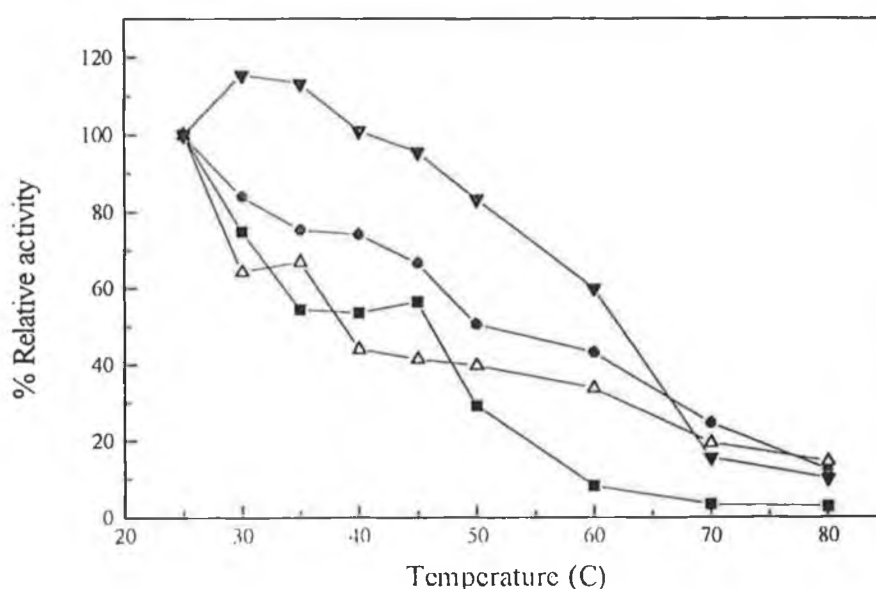


Figure 2.9: Activity of native and modified HRPs as a function of temperature. ■ Native; ● SA-NHS; ▲ EG-NHS; ▼ AA-NHS;

Ryan *et al.* previously reported a 6- to 23-fold thermostabilisation following reaction of bis-succinimides with HRP's lysine residues [17]. Another class of crosslinking reagents, bis-imidates, resulted in little or no stabilisation despite earlier successes in crosslinking and stabilising alanine aminotransferase [42]. Overall, bifunctional succinimide forms of HRP displayed greater thermostability (compared with the

monofunctional derivative). Bis-succinimides are capable of forming protein crosslinks [12, 16] unlike the AA-NHS compound. The SA-NHS compound spans a distance of 11Å, whereas the EG-NHS can form a link 14Å long. It was apparent that EG-NHS stabilised HRP to a greater extent than SA-NHS. It is known that imidoesters shorter than 11Å failed to stabilise HRP [17]. These results would suggest that this may be the minimum distance to obtain a stable crosslink. Unambiguous demonstration of such molecular links is still awaited.

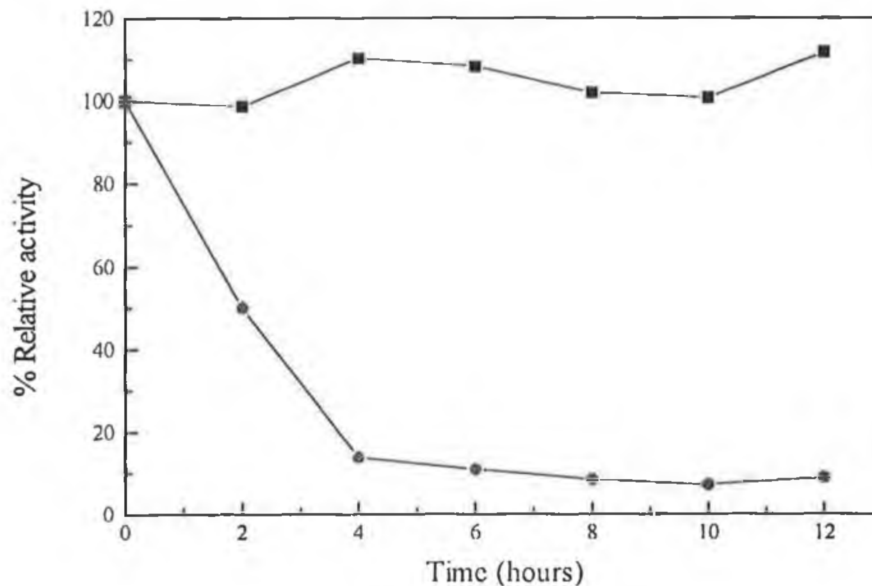


Figure 2.10: Activity of native and bifunctional HRPs at 42^{0}C.
● Native HRP; ■ EG-NHS HRP;

2.4.6. Exposure to water-miscible organic solvents

As previously stated, methanol, dimethylformamide and tetrahydrofuran were selected for investigation as they possess respectively low, medium and high denaturation capacities as defined by Khmelnitsky *et al.* [23]. In most cases, native HRP was inactivated as the percentage volume of organic solvent was increased. Overall, modified forms of peroxidase were found to be more organo-tolerant. The stability profiles of all derivatives in MeOH at 25^{0}C closely resemble that of the native enzyme; indeed, the native form displayed marginally better catalysis. At the 60^{0}C exposure to MeOH (Figure 2.11), all modified HRPs underwent an activation up to 20% v/v MeOH, whilst activities fell to zero at the 40% level of the organic solvent. Acetylated HRP, for example, was more active in 20% v/v MeOH than in aqueous solution (% RCA of 217% at 20% MeOH). Complete loss of native HRP activity was observed at all MeOH concentrations tested. DMF had similar adverse effects on modified HRPs. In a 1:1 ratio of phosphate buffer and DMF at 25^{0}C (Figure 2.12), SA- and EG-NHS each retained only 4% of their relative activities in aqueous solution. The acetylated form appeared to be able to withstand the effects of DMF to a greater extent. Native HRP was catalytically inactive under the given experimental conditions. At 60^{0}C, activities were practically zero at 30% DMF (Figure 2.13). However, modified HRPs were much more tolerant of DMF than was native HRP, which again was found to be inactivated at all concentrations tested. THF appeared to be less detrimental to HRP activity than DMF, despite the former having the higher denaturation capacity [23]. The bifunctional derivatives were found to be slightly more active in 10% THF than in aqueous solution at room temperature (Figure 2.14). At this level, acetylated HRP

activity was below 80% of that in aqueous solution, however, its relative activity in 20-30% THF was similar to that in 100% 0.1M phosphate buffer, pH 7.0.

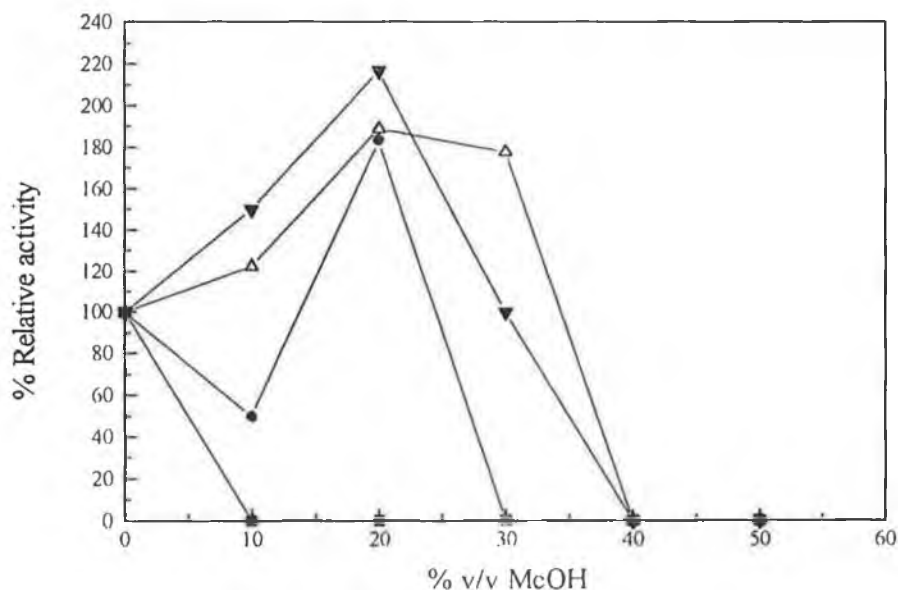


Figure 2.11: Effect of methanol on native and HRP derivatives at 60^{0}C.
■ Native; ▲ SA-NHS; ● EG-NHS; ▼ AA-NHS;

Increasing the level of THF to 50% reduced the % RCA to approximately 38%. Both SA- and EG-NHS forms possessed marginally better catalysis at 50% v/v THF, % RCAs of 44 and 51% respectively. Native HRP retained some activity at all THF concentrations investigated (% RCA of 14% at 50% v/v THF), in contrast to its characteristics in MeOH and DMF. No increases in catalytic activity were noted in the presence of THF at 60^{0}C (Figure 2.15). Steep decreases in activities occured at THF volumes greater than 10%. However, derivatives were slightly more tolerant of THF than the native enzyme. Tables 2.2 and 2.3 summarise the findings of all organo-tolerance experiments.

TABLE 2.2

The % volume solvent required to reduce catalytic activity by 50% at 25^0C

Solvent	Native HRP	SA-NHS HRP	EG-NHS HRP	AA-NHS HRP
MeOH	6	8	9	9
DMF	9-10	9	15	16
THF	16	44	49-50	46

TABLE 2.3

The % volume solvent required to reduce catalytic activity by 50% at 60^0C

Solvent	Native HRP	SA-NHS HRP	EG-NHS HRP	AA-NHS HRP
MeOH	9-10	10	39-40	39-40
DMF	9-10	15	13	13
THF	6	13	11	11

The stability properties of native and modified peroxidases in various water-organic solvent homogeneous mixtures have been examined. Each sample was diluted into the optimal assay mix after exposure to solvent to assess catalytic activity under the standard conditions and to avoid any solvent effects on the TMB assay. Native and modified HRPs behaved quite differently in mixtures of water and organic co-solvents. The native enzyme was inactive in MeOH volumes of 10% or greater. Over a two-fold activation in 20% v/v MeOH at 60^0C was observed for acetylated HRP, whilst both bis-succinimide derivatives showed similar levels of activation.

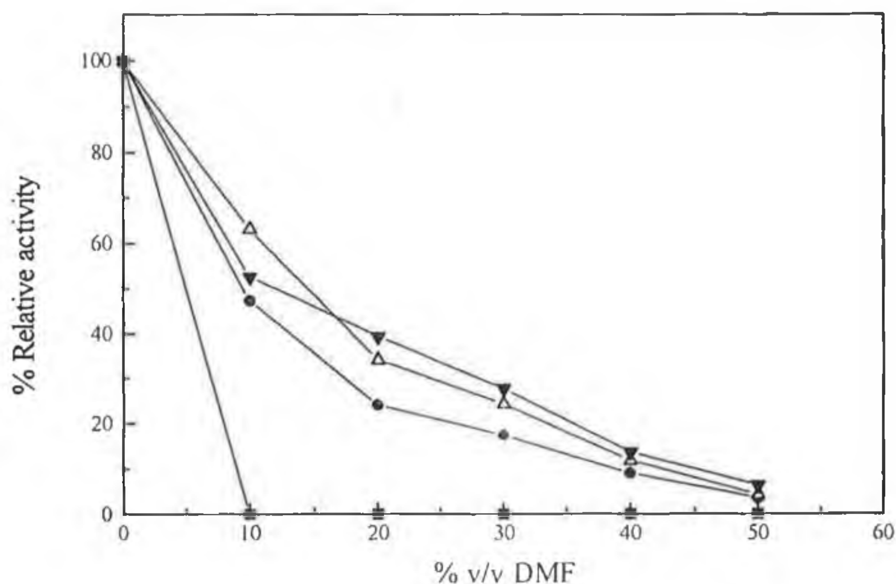


Figure 2.12: Effect of Dimethylformamide on native and HRP derivatives at 25^{0}C
■ Native; ▲ SA-NHS; ● EG-NHS; ▼ AA-NHS;

This activation effect may be solvent specific Khmelnitsky *et al.* have noted numerous examples of enzyme activation by moderate concentrations (10-30% v/v) of organic solvents. In some cases, the activation effect reached values "as high as tenfold" [43]. MeOH more than doubled the calcium dependent adenosine triphosphatase (Ca^{2+}-ATPase) activity of spinach chloroplast coupling factor 1 [EC 3.6.1.3]. The K_m for ATP was halved in the presence of 20% MeOH and optimum activity occured at a 30% level [44]. Batra and Gupta studied the effects of four solvents on the kinetic parameters of four enzymes, including HRP, at 10% v/v acetonitrile or THF; HRP activity was 20% greater than in aqueous buffer and the K_m for *o*-dianisidine was notably reduced. The same report noted a 216% activation of polyphenol oxidase in 20% DMF [45]. Vazquez-Duhalt *et al.*, studying polyethylene glycol-modified HRP in

methanol, noted a four-fold decrease in apparent K_m for guaiacol compared with its value in buffer (although k_{cat} also decreased significantly) [46]. A similar reduction of HRP's K_m for TMB has possibly occurred in the presence of MeOH. This may account for the activation that was observed here (Figure 2.11). Alternatively, the activation may have been due to "conformational changes in the enzyme molecule caused by the introduction of the organic solvent into the system" [43]. HRP derivatives may adopt an altered, more active conformation in the methanol-aqueous system while the native enzyme simply denatures and loses activity

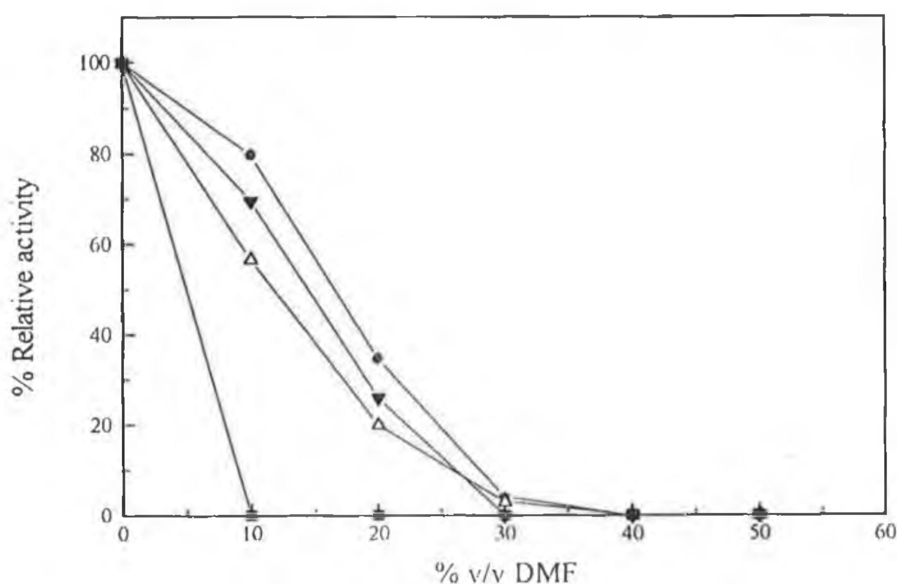


Figure 2.13: Effect of Dimethylformamide on native and HRP derivatives at 60^0C
■ Native; ▲ SA-NHS; ● EG-NHS; ▼ AA-NHS;

In each of the systems tested, increasing solvent concentrations resulted in loss of peroxidase activity. However, chemical modification of HRP with NHS esters greatly improves tolerance in water-organic co-solvent mixtures. The effect on native HRP

was much more detrimental than on modified forms (Figures 2.12 and 2.13). However, all HRP derivatives were completely inactivated by approximately 30% v/v DMF at 60^0C (Figure 2.13) and by >50% v/v DMF at 25^0C (Figure 2.12). Nevertheless, reaction with succinimides (by either forming lysine crosslinks and/or altering protein surface charges) brought about significant improvement in peroxidase stability in DMF. Native and modified forms appeared to be more stable in THF than in DMF at room temperature, even though THF was classed as having the higher denaturation capacity [23]. HRP derivatives were marginally more active in 10-20% THF than in aqueous solution (Figure 2.14); however, this catalytic activity was not sustained at the elevated temperature (Figure 2.15).

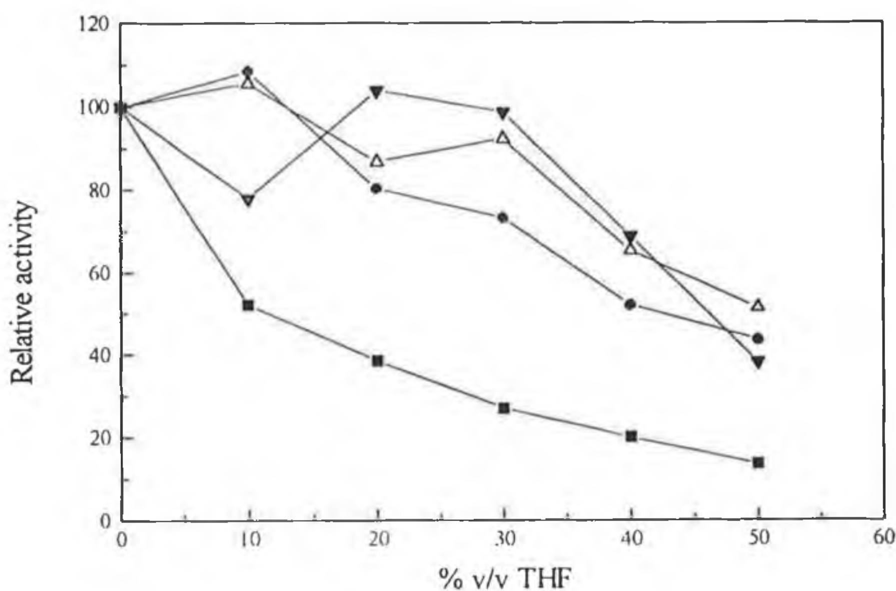


Figure 2.14: Effect of Tetrahydrofuran on native and HRP derivatives at 25^0C.
■ Native; ▲ SA-NHS; ● EG-NHS; ▼ AA-NHS;

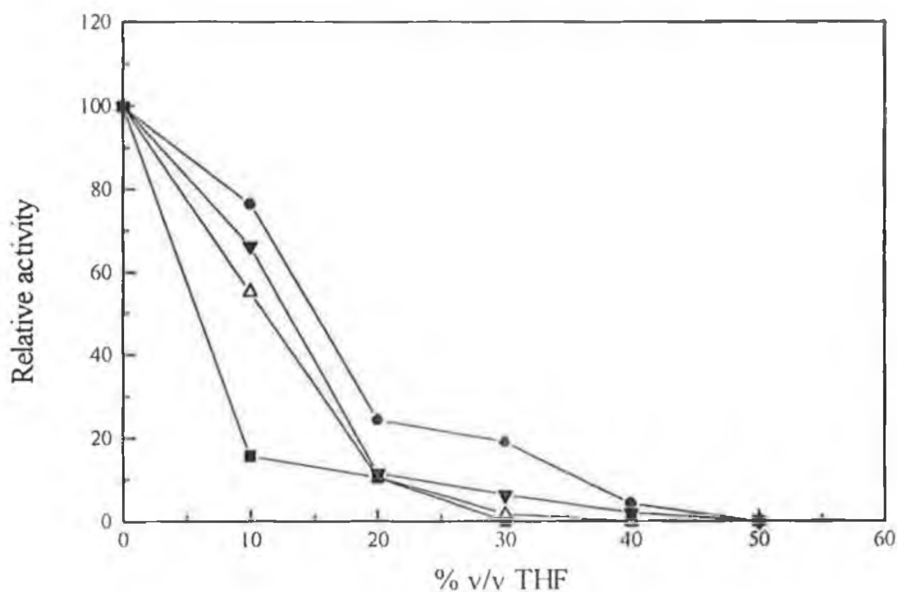


Figure 2.15: Effect of Tetrahydrofuran on native and HRP derivatives at 60^0C
■ Native; ▲ SA-NHS; ● EG-NHS; ▼ AA-NHS;

Even though enzymes can function in anhydrous organic solvents such as hexane or toluene [47], water miscible organic solvents often lead to protein inactivation. Gorman and Dordick described the desorption of tritiated water (T_2O) bound to HRP in a range of organic media [48]. The fractions of bound T_2O desorbed from HRP by MeOH, DMF and THF were 0.56, 0.40 and 0.37, respectively. Peroxidase was shown to retain less T_2O in solvents of moderate polarity (DMF and THF) than, for example, chymotrypsin. However, the requirement of some water for peroxidase activity in a range of organic solvents has been well documented [48,49]. Although DMF has the higher dielectric constant, it desorbs significantly less bound H_2O from the enzyme surface than MeOH. It was suggested that as methanol's structure is closer to that of water than DMF, MeOH could replace water as well as strip it away [48]. This theory

of "water stripping" has been previously reported for anhydrous organic solvents [50]. It is questionable whether water stripping is relevant to the present findings. Here, substantial inactivation has taken place at solvent concentrations <50%, i.e., where the concentration of water in the system is greater than 28M. Thus, there is still adequate water available to solvate the HRP molecules. Gorman and Dordick have opined that MeOH may replace the water around the HRP molecule [48]. It is possible that all three solvents have a greater affinity than H_2O for the surface of HRP and that they can replace water bound to the enzyme molecule, even when they are present at low concentrations. No direct evidence for this hypothesis exists, but it is interesting to recall the work of Arakawa and Timasheff in understanding the stabilising and destabilising effects of certain solutes on proteins. Stabilising solutes such as ammonium sulphate, are preferentially excluded from the immediate neighbourhood of protein molecules relative to their concentration in the bulk solvent. In contrast, destabilising additives such as thiocyanate bind preferentially to proteins. Their concentration tends to be higher in the vicinity of the protein molecules than in the bulk solvent [51]. The solvents used here may act in a similar manner to destabilising ions, disrupting the water "shell" surrounding the HRP molecule and leading to unfolding and loss of function. The bifunctionally modified HRPs resist this solvent-induced activity loss much more successfully than does the native enzyme, due to the probable presence of one or two intramolecular crosslinks. Neutralisation of positive charges on lysines in hydrophobic sequences may offer an explanation for acetylated HRP's organotolerance. It would be interesting to ascertain whether these activity losses in solvents might be reversible (due to denaturation) or irreversible (due to inactivation).

2.4.7. Long and short term stability studies

HRP has a complex thermal inactivation profile which cannot be described in terms of a "normal" half-life, since this convention applies to first-order processes (such as radioactive decay) only. The apparent half-lives referred to here are empirically determined from thermal inactivation profiles and correspond to the time at which 50% of the initial HRP activity remains.

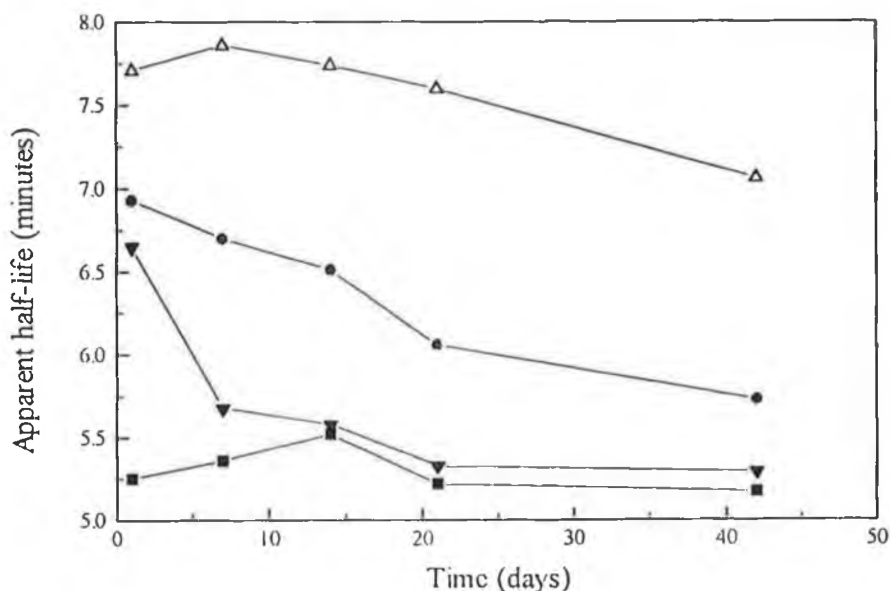


Figure 2.16: Apparent half-lives calculated at 72.5^{0}C over a 42-day period
■ Native (Sigma); ▼ Native (Boehringer); ▲ EG-NHS (Sigma);
● SA-NHS (Sigma)

All modified forms were observed to have longer half-lives when incubated at 72.5^{0}C than the native enzyme and also maintained these values on extended storage at 4^{0}C for up to 42 days. Results are depicted in Figure 2.16. It was observed that the Boehringer source of HRP was more catalytically active than the Sigma source. After one day (T1), native Sigma HRP had a half-life ($t_{½}$) value of 5.26 minutes compared

with 6.65 minutes for native Boehringer HRP. No loss of activity was observed after chemical modification. Furthermore, EG-NHS HRP possessed a greater $t_{½}$ value than SA-NHS HRP. Stability factors ($\frac{Mod.}{Nat.}[t_{½}]$) of respectively 1.30 and 1.10 were calculated for both derivatives. It was clear that the thermostability of even the most tolerant of the modified HRPs declined with longer and longer storage periods (0.75 minutes after a 42 day storage at 4^0C; however, modified form half-life values were consistently better than the native enzyme (Figure 2.16). This could have implications for use of such derivatives in a commercially manufactured product.

2.4.8. Determination of free amino groups

Various HRP forms were reacted with trinitrobenzenesulphonate (TNBS) to determine their unmodified lysine contents. N-hydroxysuccinimide esters react specifically with the ε-amino groups located on lysine amino acids. Native HRP has 6 free or unaltered lysines and a blocked N-terminus [40]. This method is useful for checking the extent of blocking or unblocking of amino groups in proteins and peptides, owing to the short time required for reaction (5 minutes at 25^0C). An N-acetyl-L-lysine standard curve was set up (see Section 2.3.9.) and the concentration of free lysine in each HRP sample was estimated (Table 2.4). Data suggested that up to 80% of lysine amino groups had been altered by the addition of bifunctional reagents, i.e., 4-5 lysines were chemically modified. This would imply the formation of two molecular crosslinks on the polypeptide backbone. Acetylated HRP's free amino content was calculated at 0.41mM, which indicated that 3 of the available lysines had been acylated by the AA-NHS reagent. However, neutralisation of the positive charges of lysine amino groups has a stabilising effect even in the absence of crosslinking. This result compared

favourably with stabilisation of up to 23-fold obtained when bis-succinimides were employed (where up to 5 residues were modified) [17]. Obviously, bis-succinimides are more successful stabilisers of HRP.

TABLE 2.4

TNBS estimation of free lysine content in native and modified HRP samples

HRP Sample	Free lysine (mM)	% Relative to Native
Native	0.90	100
SA-NHS	0.22	24
EG-NHS	0.19	21
AA-NHS	0.41	46

Therefore, the difference may be due to the likely formation of an intramolecular crosslink by the bifunctional reagents or to the greater proportion of lysine residues chemically altered: note that Ugarova *et al.* concluded that stabilisation resulted from the degree of modification rather than from the nature of the modifier [6]. TNBS reacted with all 6 HRP lysines only at 40^0C; the same reaction at 4^0C modified only 3. Reaction at 0^0C with anhydrides modified between 3 and 4 amino groups.

It is known that non-crosslinking chemical modifications can benefit the stability of enzymes other than HRP. Tuengler and Pfleiderer acetamidinated 17 of the 24 lysines of pig heart lactate dehydrogenase, converting them to arginine-like structures. Modification increased the enzyme's tolerance of heat, alkali and tryptic digestion [52]. Melik-Nubarov and colleagues used glyoxylic acid and sodium cyanoborohydride to perform reductive alkylation of up to 10 amino groups in α-

chymotrypsin. The enzyme was dramatically stabilised against heat: modified forms tolerated 60^{0}C 1000-fold better than did the native enzyme. The effect was ascribed to hydrophilisation of the protein surface, resulting in decreased contact between water and non-polar clusters [36, 53]. This was a remarkable result from such simple chemical alterations with low molecular weight compounds. Using the same enzyme, Mozhaev *et al.* later carried out acylation (using carboxylic acid anhydrides) and reductive alkylation (with aliphatic aldehydes) of the protein. These compounds could not form crosslinks. They obtained a wide range of "hydrophilised" and "hydrophobised" derivatives. The stabilised enzyme forms had identical fluorescence emission spectra to the native chymotrypsin. Increasing stability correlated with increasing hydrophilisation, whether this was due to the nature of the modifier itself or to the number of lysines modified by the compound in question (up to a limiting value) [36].

To conclude, a five-fold increase in HRP's apparent half-life at 65^{0}C resulted from modification with AA-NHS. 3 of the enzyme's 6 lysine amino acid residues were altered. This level of thermostabilty at 65^{0}C was comparable to bis-succinimide modification of HRP. The difference (enhanced stability of EG-NHS) may be due to the presence of molecular crosslinks, although thermostabilisation is known to be due to the nature of the chemical modifier [6]. These results have confirmed that one need not crosslink HRP to achieve enhanced thermostability.

2.4.9. Effects of denaturing and reducing agents

HRP is a metalloprotein where calcium aids its stability. It is reported to contain two bound calcium ions [54]. The removal of protein-bound calcium by EDTA and unfolding of the protein backbone with guanidine hydrochloride (GnCl) has been reported by Haschke and Friedhoff [54]. It was attempted to ascertain whether reaction of HRP with succinimides could confer resistance to GnCl-EDTA denaturation. All HRP samples (protein content of 0.1mg ml^{-1}) were exposed to 6M GnCl-0.01M EDTA for 10 hours and their relative catalytic activities assessed. The EG-NHS derivative possessed a relative activity of 45% and appeared to be able to withstand the effects of the denaturant better than the native enzyme (% RCA of 23). GnCl-EDTA appeared to have an even more detrimental effect on acetylated HRP (% RCA of 15%). 0.025M 2-mercaptoethanol was added to GnCl-treated stocks for 60 minutes at 25^0C. An activity check (by TMB) revealed complete inactivation of all HRP forms. The reducing agent alone had no effect on HRP. GnCl-EDTA-treated HRP samples were incubated at 65^0C. Activities were calculated as a percentage of initial activity (zero time) in their respective control samples and are depicted in Figure 2.17.

Guanidine hydrochloride was chosen as a test denaturant in preference to urea as the latter has been shown to have a limited effect on HRP [55]. EG-NHS HRP retained more activity than the native enzyme. This suggests that the bifunctional reagent crosslinks HRP and this theory may be reinforced when looking at the effect of GnCl-EDTA on acetylated HRP. Modification of lysines without crosslinking appeared therefore not to counteract the denaturing effects of GnCl. Addition of

mercaptoethanol to GnCl-treated samples eliminates catalytic activity irrespective of whether peroxidase is crosslinked or not

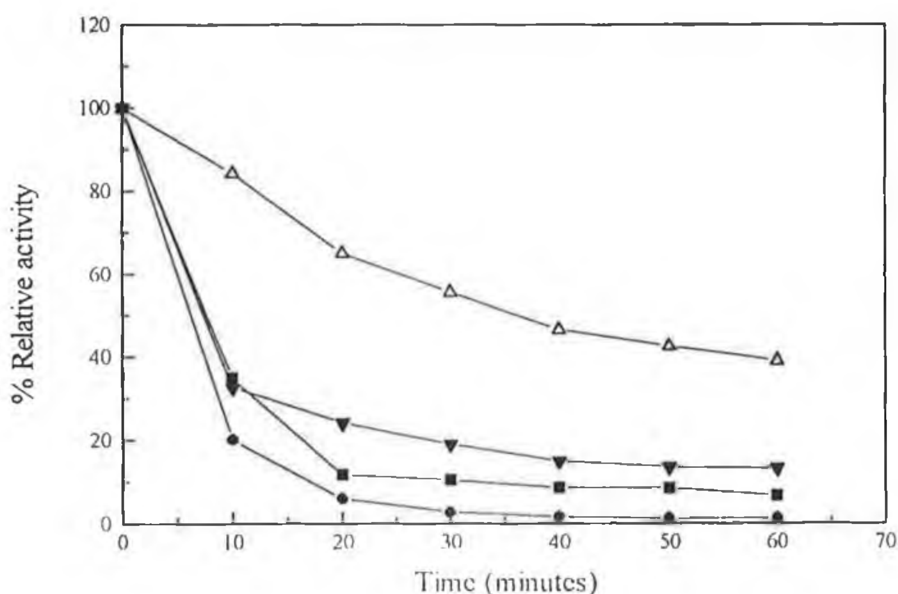


Figure 2.17: Effect of 6 M guanidine hydrochloride/10mM EDTA on Native and EG-NHS HRPs thermostability at 65^{0}C.
▲ EG-NHS, no GnCl; ▼ EG-NHS + GnCl; ■ Native, no GnCl; ● Native + GnCl;

The effect of EDTA alone on HRP was investigated (Figure 2.18). It was seen that longer exposure times and increasing concentrations of EDTA reduced native HRP activity at 45^{0}C. SA-NHS HRP withstood the effects of the chelating agent better than native HRP. The effects of EDTA were not as detrimental when the incubation temperature was 25^{0}C (results not shown). For a 3-hour exposure at 45^{0}C, native HRP was rapidly inactivated to a level of 28% whereas SA-NHS HRP had a % RCA nearly double that of the native enzyme Thus EDTA effects appeared to depend on the length and temperature of incubation

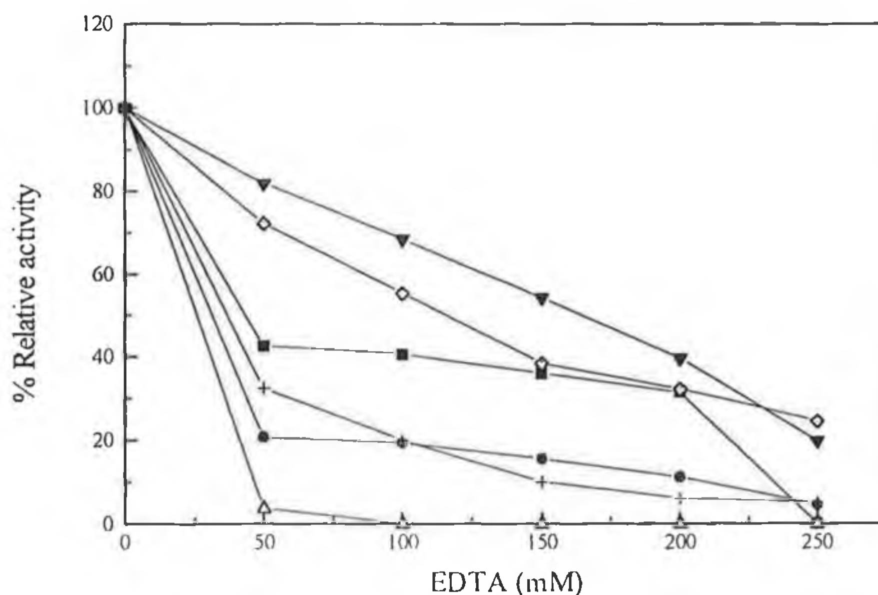


Figure 2.18: Effect of EDTA on HRP activity at 45^{0}C as a function of concentration & time. ▼ SA-NHS (60 mins.); ♦ SA-NHS (120 mins.); + SA-NHS (180 mins.) ■ Native (60 mins.); ● Native (120 mins.); ▲ Native (180 mins.);

Mercaptoethanol, in the absence of GnCl, does not affect HRP catalytic activity. This illustrates the importance of the enzyme's four disulphide bridges to its overall molecular stability and shows that modification with EG-NHS does not compensate for loss of the -S-S- links. It would be interesting to observe the renaturation patterns of the three peroxidase forms as unfolding has been shown to be reversible when the denaturant (GnCl) is removed [55] Pappa and Cass reported an increase in tryptophan fluorimetric emission on exposure of HRP to guanidine hydrochloride concentrations greater than 1M. Denaturation of holo-HRP was complex, with two distinct steps. These were attributed to unfolding of the polypeptide and to the loss of the heme prosthetic group. Such fluorescence increases have been thought to be due to an increase in the tryptophan (Trp)-heme distance (usually > 2.2nm) [55]. The sole Trp

residue in holo-HRP C seems to lie in a hydrophobic region of the protein, also, it does not react with Koshland's reagent (2-hydroxy-5-nitrobenzylbromide) but is prone to modification in the apoprotein following removal of the heme [56]. The lysine residue at position 174 also interacts with the heme group [57]. It is possible that acylation of this residue has occurred, perhaps altering the lysine-heme interaction and therefore delaying the loss of the heme group during the inactivation process. It is more likely, however, that the observed stabilisations arose from decreased unfolding of the polypeptide chain. In any case, action of bis-succinimides with HRP lysines increased the functional stability of the protein in the presence of a denaturant, by preventing either gross unfolding or loss of the heme The effects of mercaptoethanol (alone) were investigated (Figure 2.19).

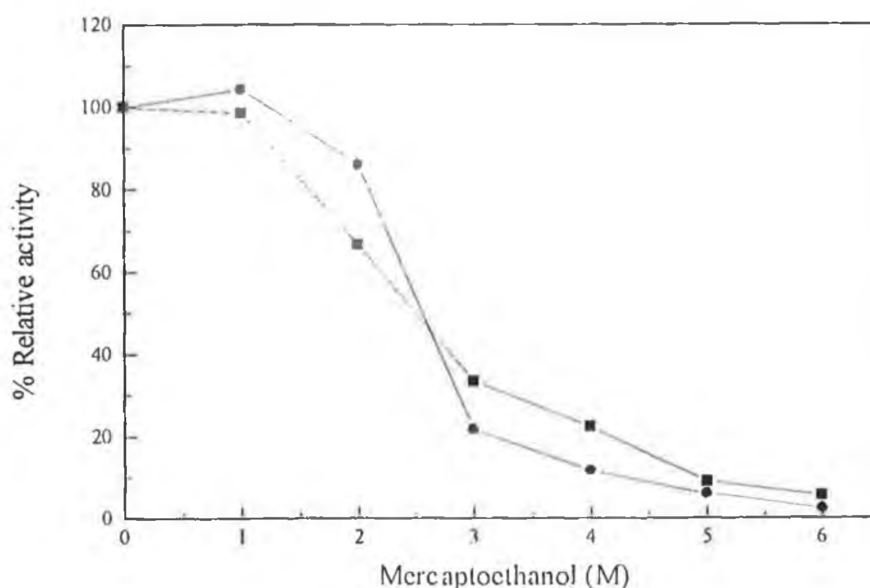


Figure 2.19: Effect of mercaptoethanol on native and acetylated HRPs
● Native HRP; ■ AA-NHS HRP;

Native and AA-NHS HRPs were incubated at various concentrations of the reducing agent up to 6M. Up to a 2M level, native HRP had a better tolerance of mercaptoethanol than did acetylated HRP A slight activation was noted at 1M. In the 3.0-6.0 M range, the native enzyme was less stable than modified forms. Acetylated possessed the greatest catalytic activity in this range The results suggest that charge neutralisation as opposed to crosslink formation conferred the best resistance to the reducing agent.

2.4 10 UV/Visible spectrophotometric analysis

Spectral methods can be used to determine and characterise protein conformational changes. Such methods are sensitive and only require small amounts of protein. Another feature is that they are non-destructive as samples can be recovered after analysis. The UV/Visible absorbance spectra of native and modified HRPs were recorded in the range 200-500nm Greater absorbance in the spectral range 200-250nm was noted for derivatives, possibly due to excess NHS ester and DMSO remaining in solution. The characteristic absorption spectrum of native HRP shows a major Soret band at 403nm (Figure 2.20) [58]. Changes in the microenvironment of the heme present in the active site of HRP can shift the intensity and position of this band. For modified HRP samples, this peak at 403nm seemed to decrease slightly. This may have in fact been due to conformational changes brought about by bis-succinimide modification. Changes in the heme environment of native HRP at 65^0C were studied by monitoring the relative absorbance of the Soret band (Figure 2.21). The heme site remained intact for 20 minutes, followed by a decrease in the heme absorbance with prolonged exposure. These findings were similar to that of Ryu and Dordick [58].

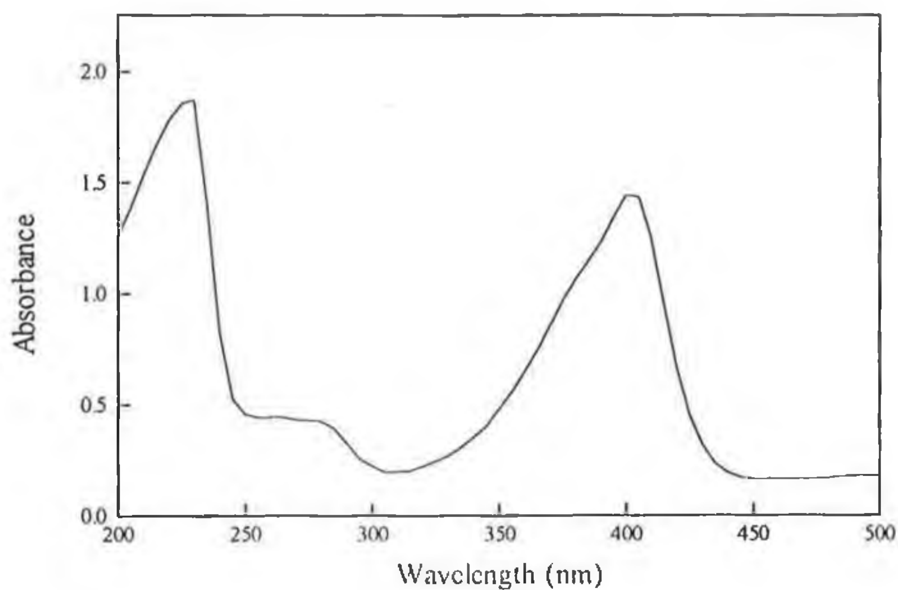


Figure 2.20: Characteristic UV/Visible spectrum of native HRP displaying Soret band.

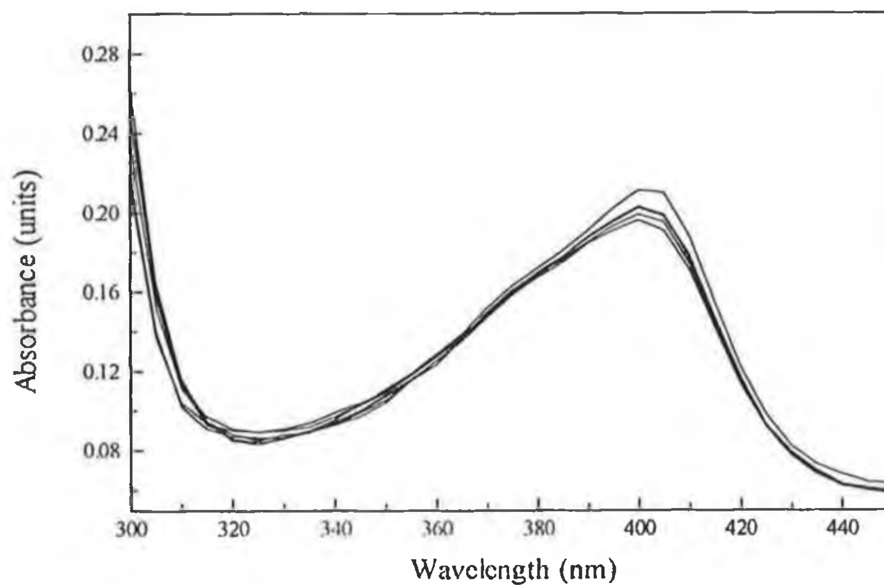


Figure 2.21: Effect of high temperature on the characteristic Soret band at 403nm. (Spectra of decreasing intensity correspond to longer exposure times).

2.4.11. Fluorescence studies

Fluorimetry was used to investigate the effects of thermal inactivation on the microenvironment of the single tryptophan residue in native and bifunctionally modified HRPs. Changing emissions from the fluorescent residue during the inactivation suggested alterations in the tertiary structure of the protein. Ugarova *et al.* deduced that the aromatic amino acid (which is not located in the same domain as the active site [40]) is a fluorescent group in HRP which is sensitive to the overall conformation of the protein moiety [59] Thus, it was attempted to correlate changes in fluorimetric emission with loss of catalytic activity at 65^{0}C for native and EG-NHS HRPs. The native enzyme was completely inactivated after 50 minutes (Figure 2.22). A gradual increase in fluorescence occurred which levelled off between 40 and 60 minutes. The greatest increase occurred between 20 and 30 minutes while most of the catalytic activity had already been lost by 20 minutes. In contrast, EG-NHS HRP demonstrated greater catalytic thermostability and little net change in fluorimetric emission (Figure 2.23). Modification with the EG-NHS ester resulted in partial quenching of tryptophan fluorescence (zero time reading) relative to that of the native enzyme; also, greater emission fluctuations occurred over the 60 minute incubation. Fluorescence measurements revealed differences between native and EG-NHS HRPs upon thermal inactivation at 65^{0}C In proteins such as peroxidase that contain both tyrosine and tryptophan aromatic amino acids, fluorescence is usually dominated by the contribution of tryptophan. Changes in protein structure, due to environmental factors such as extreme temperature, often lead to changes in fluorimetric emission. Intensities at λ_{max} for example, generally decrease by 1% per degree increase in temperature [27].

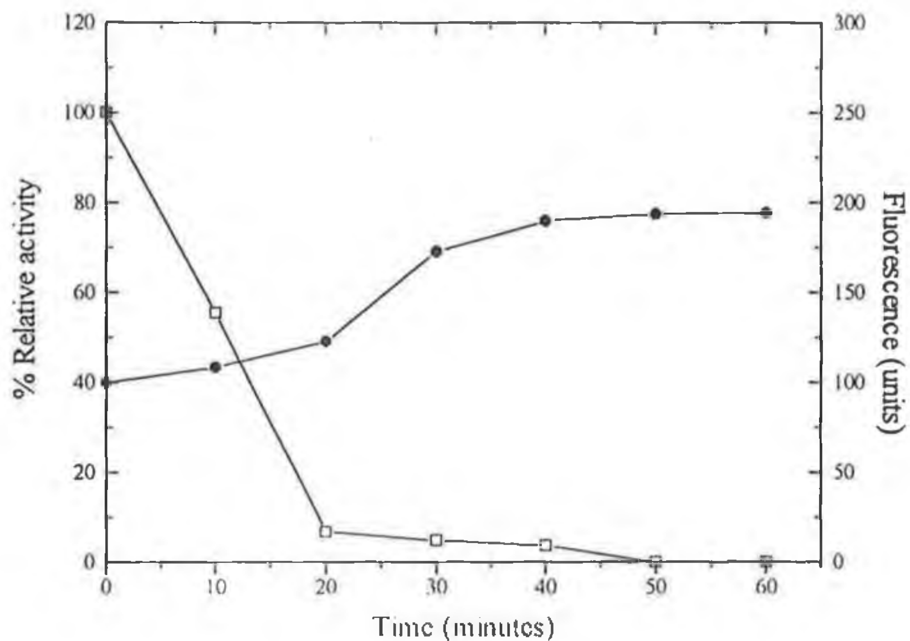


Figure 2.22: Remaining Activity/Fluorescence profile of Native HRP at 65^{0}C.
□ HRP activity; ● Fluorimetric emission.

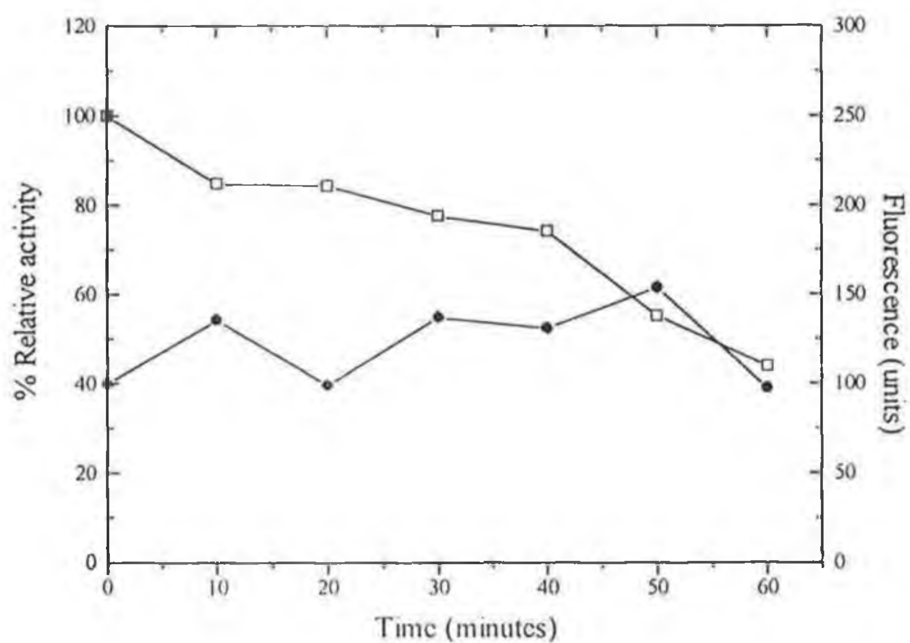


Figure 2.23: Remaining Activity/Fluorescence profile of EG-NHS HRP at 65^{0}C
□ HRP activity; ● Fluorimetric emission.

Peak emission wavelengths can shift toward that of free tryptophan in solution [59]. However, the exact location of this maximum can also depend on the choice of buffer used [27]. Thus, it is advisable that buffers employed should not fluoresce at wavelengths greater than 250nm. Ugarova *et al.* recorded that the fluorescent properties of HRP's Trp residue changed under the same conditions as those causing conformational changes [59] and concluded that the Trp amino acid acted as a fluorescent label sensitive to the overall conformation of the protein. In the present studies, samples taken at various time intervals from a HRP solution (0.4μM in concentration) undergoing thermoinactivation at 65^0C were cooled and stored on ice (for approximately 20 minutes) until fluorimetric determination and assay of catalytic activity measurements were carried out.

The modified peroxidase form showed greater thermostability at 65^0C than did the native enzyme. Minimal changes in fluorescence occurred with the native enzyme below 20 minutes exposure, but most activity was lost in this time. Possibly, a relatively small conformational change results in the loss of the heme, resulting in an inactive but still-folded apoenzyme. This sharp increase in intensity after 20 minutes suggests that native peroxidase undergoes thermal denaturation, resulting in the Trp residue becoming more accessible to the medium, i.e. a loss of activity before unfolding [55]. After 40 minutes at 65^0C, no fluorimetric intensity changes occurred which would indicate that HRP's Trp residue remained in contact with the reaction medium. No change in λ_{max} was observed. The sigmoidal shape noted (Figure 2.22) is characteristic of a simple two-state transition between the native (N) and unfolded (U) forms of peroxidase, where inactivation is reversible [55].

$$N \Leftrightarrow U$$

The fluorescence emission profile of EG-NHS HRP was different to that of the native form (Figure 2.23). Fluorimetric emission intensities of the EG-NHS derivative were less than those of the native at 65^{0}C.

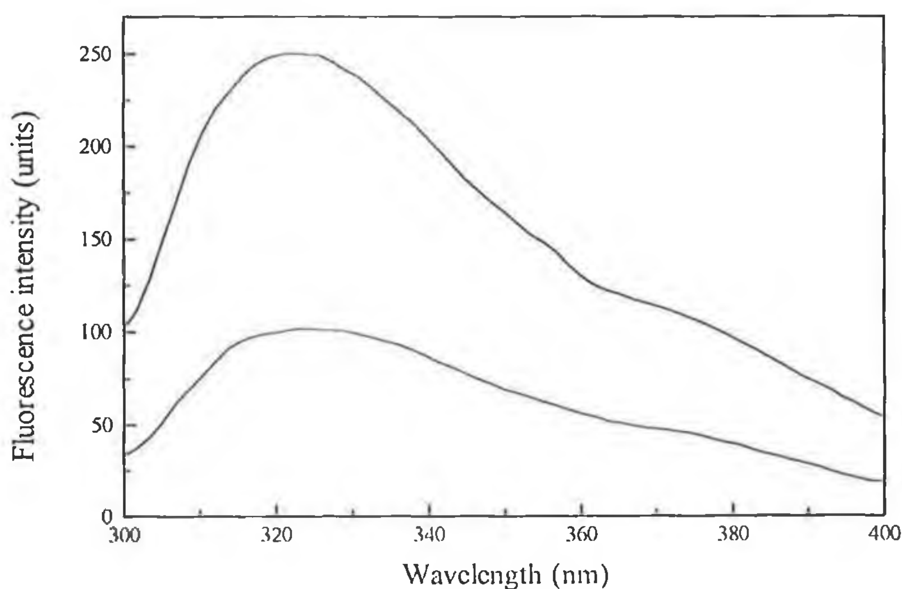


Figure 2.24: Fluorimetric emission profiles of Native and EG-NHS HRPs. (280nm excitation)

Changes in emission were more pronounced at various time points (Figure 2.23); however, the net fluorimetric changes were of much less magnitude than that of native HRP. Schmid noted that increases and decreases in fluorimetric emission can occur upon protein unfolding [27]. Data indicated that EG-NHS HRP's fluorescent label was not exposed to the reaction medium to the same extent as that of native peroxidase and remained buried in a more intact, chemically stabilised protein conformation. Fluorescence emission is observed when an excited electron returns from the first

excited state back to the ground state. As some energy is always lost by non-radiative processes such as vibrational transitions, the energy of the emitted light is always less than that of the absorbed light; hence, the fluorimetric emission is always shifted to longer wavelengths. Fluorimetric emission is much more sensitive to changes in the environment of the chromophore than is light absorption. Fluorescence is thus an excellent method for investigating conformational changes in proteins.

A great deal of information can be obtained about protein aromatic side chains when the solvent composition around these residues is varied. The location of such residues can be studied by using probes such as ethylene glycol, dimethyl sulphoxide and sucrose. This technique is known as solvent perturbation.

The fluorometric emission of proteins originates from phenylalanine, tyrosine and tryptophan residues. In proteins that contain all three amino acids, emission is usually dominated by the contribution of the tryptophan residues (HRP has a single Trp). This is because both their absorbance at the wavelength of excitation and their quantum yield of emission are considerably greater than the respective values for tyrosine and phenylalanine. The latter's fluorescence is not observed in native proteins because its sensitivity is very low. The other factor in fluorescence analysis is transfer of energy between residues. Phenylalanine emission is barely observed because it is practically quenched by energy transfer to the other two aromatic amino acids. Tyrosine and tryptophan absorb strongly around 280nm, where phenylalanine emits. In proteins that contain both tyrosine and tryptophan, emission of the former is barely detectable, for a number of reasons

1. Tryptophan emission is too strong

2. In folded proteins, Trp emission is frequently shifted to shorter wavelengths towards tyrosine

3. Non-radiative energy transfer can occur from tyrosine to tryptophan residues in the compact native protein structure. In a hydrophobic environment (the interior of a folded protein), Trp emission occurs at shorter wavelengths.

To conclude, HRP contains a single tryptophan residue at position 117 on the polypeptide backbone, thus simplifying the interpretation of any fluorimetric results. It location in the three-dimensional structure has not been identified due to the absence of an X-ray crystallographic structure, but previous spectroscopic and chemical modification studies have suggested that it is not a part of the catalytic site [56]. A marked difference was observed in the tryptophan fluorescence of native and bifunctional HRPs upon thermal inactivation at 65^0C. Native HRP underwent a sharp increase in fluorimetric emission after 20 minutes, at which point % RCA was practically zero EG-NHS HRP, in contrast, demonstrated greater thermostability and little net change in emission Chemical modification of up to 5 lysines resulted in partial quenching of Trp emission relative to that of the native enzyme, also greater emission fluctuations occurred over the 60 minute incubation at 65^0C.

2.4.12 Conclusion

It has been demonstrated that simple chemical modifications can greatly stabilise HRP activity in a range of adverse environmental conditions. These beneficial reactions appeared to be equally effective on the Boehringer as well as on the Sigma enzyme source. Reaction of N-hydroxysuccinimide esters with HRP's lysine side chains resulted in derivatives with increased heat resistance and a greater tolerance of water-miscible organic solvents. The increased tolerance of organic solvents may be just as important as the enhanced thermostability for commercial applications.

Derivatives also showed greater stability in the presence of denaturing and reducing agents. These observations may be attributed to the introduction of molecular crosslinks between adjacent lysine amino acids (in the case of bis-succinimides). Evidence supporting the existence of such crosslinks may lie with the fluorescence data, where emissions from bifunctional preparations were less in magnitude than those of their native counterparts at ambient and high temperatures.

The behaviour of acetylated HRP illustrated that "point" modification of lysine residues benefits overall enzyme stability, despite this reagents' inability to form protein crosslinks. This would suggest that the formation of "molecular bridges" is not critical for increased thermal or organic solvent stability, although the AA-NHS reagent is limited to lysines on the polypeptide chain. The improved stability may have arisen from the addition to the free amino groups of the lysines or perhaps from neutralisation of the amino groups' positive charge.

SA- and EG-NHS esters are bifunctional compounds which are capable of crosslink formation. SA-NHS spans a distance of 11Å while EG-NHS bridges a 14Å gap. The latter-type reagent consistently yielded a more stable HRP derivative. This

could be related to the difference in crosslink length formed by the two compounds, assuming that a crosslink has indeed formed. SA-NHS is a non-cleavable crosslinker while EG-NHS may be cleaved with hydroxylamine. However, this is unlikely to present a problem in practice. Hydroxylamine at concentrations greater than 1mM acts as an inhibitor of HRP and so is unlikely to occur in any HRP-based system.

It is quite apparent that reaction has occurred with the HRP lysine residues. The number of free amino groups decreased after succinimide treatment. Post-modification TNBS values indicated that 4-5 of HRP's lysine amino acids were chemically altered (3 with AA-NHS). This would suggest that two crosslinks at most could have formed in the HRP molecule since any crosslink will of course involve two adjacent lysines

HRP is extensively glycosylated and while the carbohydrate side chains (18% of the enzyme) contain the amino sugar glucosamine, these are invariably acetylated [60] and therefore blocked from reaction with either the succinimides or TNBS. Note that the carbohydrate side chains remain available for reaction with periodate or other agents for immobilisation procedures

The work presented here has future potential in many areas including industrial applications, biosensors, clinical assays and reagents. Indeed, the application of these succinimide derivatives in waste-water treatment is the subject of Chapter 3 in this thesis. Stabilised enzymes have the advantages of longer life and a wider range of applications than other less stable activities. These experiments have clearly demonstrated a quantitative difference between native and chemically modified forms of HRP which could be considered for a wide number of future developments.

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CHAPTER 3

PHENOL REMOVAL FROM AQUEOUS SYSTEMS AT HIGH TEMPERATURES BY CHEMICALLY MODIFIED HORSERADISH PEROXIDASES

3.1 INTRODUCTION

In 1746, Benjamin Franklin wrote "when the well's dry, we know the worth of water". At that time people were primarily concerned with finding a reliable source of water. Its quality was often taken for granted. The quantity of pollutants contaminating water resources was relatively small and those existing pollutants were removed through natural processes. Increasing population, growing industry and rapidly developing technology since the industrial revolution have increasingly tested nature's capacity for maintaining clean water. Increased water use and wastewater discharge have added impurities to water which overload natural cleansing processes, either because of the amount or the chemical complexity of the impurities. Hence, we are compelled to turn to technology to protect our water supply.

Aromatic compounds such as phenols are present in wastewater of a number of industries including high temperature coal conversion, petroleum refining, resins and plastics. The term "phenols" in this context includes not only parent phenol (C_6H_5OH) but an assortment of organic compounds containing one or more hydroxyl (OH) groups attached to an aromatic ring. Phenols in water sources have special adverse effects. Such aromatic hydroxy compounds can be toxic at elevated levels and are known or suspected to be carcinogens [1]. As little as 0.005mg L^{-1} of phenol will impart objectionable tastes and odours to drinking water when it combines with chlorine to form chlorophenols. Thus, the removal of such chemicals from water or industrial effluents is of great practical significance.

Current methods for removing phenolics from wastewater include microbial degradation, adsorption on activated carbon, chemical oxidation (using agents such as

ozone, hydrogen peroxide or chlorine dioxide), incineration, solvent extraction and irradiation [2-5]. The choice depends on economic and other factors.

Klibanov *et al.* first proposed a horseradish peroxidase (HRP) method for the removal of toxic aromatics from aqueous solution [6]. HRP catalyses the oxidation of a variety of phenols and aromatic amines in the presence of H_2O_2, generating phenoxy radicals. These free radicals diffuse from the active centre of the enzyme into solution [7] where they can form dimers, trimers, etc. which eventually result in higher oligomers and polymers which are nearly water-insoluble (formation of polyaromatic products). Such polymers have been produced by Saunders and colleagues from several phenols and aromatic amines [8]. One molecule of peroxide can remove approximately 10^3 molecules of phenol [9]. Moreover, two free radicals are generated for every molecule of peroxide consumed (reaction 3.1)

$$H_2O_2 + 2AH_2 \rightarrow 2^{\bullet}AH + 2H_2O \qquad (3.1)$$

This enzymatic approach is suitable for the treatment of wastewater containing aromatic contaminants. A significant feature of these apparently non-toxic polyaromatic products is that, in contrast to their monomeric precursors, they are practically insoluble in water [10]. Therefore, peroxidase-catalysed oxidation reactions transform phenols from water-soluble compounds into water-insoluble ones. This phenomenon, if general, could be used for the removal of pollutants from water because insoluble chemicals may be easily separated from the water by simple filtration or sedimentation procedures [11, 12]. This enzymatic method has many advantages over conventional procedures [9, 12]. Although current methods such as solvent

extraction and adsorption onto activated carbon are effective, they suffer from such shortcomings as high cost, incomplete purification, formation of hazardous by-products and applicability to only a limited concentration range. In order to achieve a high degree of phenol removal, large amounts of enzyme are required to counteract the effects of enzyme inactivation, thus limiting the industrial applicability of the method [13, 14]. It has been postulated that this inactivation most likely occurs as a result of the interactions of phenoxy radicals with the enzyme's active site [9]. If this is the case, it would be extremely difficult or near impossible to reduce the amount of enzyme required. Nakamoto *et al.* have pointed out that enzyme inactivation may in fact be attributed to the adsorption of enzyme molecules onto the end product polymer, thus limiting diffusion of substrate to the active centre [15]. Among other strategies, additives such as polyethylene glycol (PEG) and gelatin can exert a significant protective effect on HRP by suppressing enzyme adsorption without changing the reaction stoichiometry between H_2O_2 and phenol [16]. It was noted that the higher the concentration of phenol, the greater the effect of PEG. Immobilisation techniques where the enzyme is suitably attached (e.g. periodate linkage) to an insoluble carrier, offer the possibility of delayed protein inactivation by helping to prevent unfolding [17-19]. However, mass transfer limitations can evolve whereby the formation of reaction products on the carrier matrix can reduce the capacity for enzyme-catalysed polymerisation reactions [20].

At temperatures greater than 50^0C, peroxidase-catalysed decolorisation of bleached kraft mill effluent significantly decreases [21] whereas no apparent activity loss is observed for phenol removal in the range 5-35^{0}C [16]. HRP loses catalytic activity at elevated temperatures due to unfolding of the protein backbone. Inactivation

in this instance is often caused by the destruction of one or two "weak points" such as hydrolysable peptide bonds or easily oxidised functional groups [22]. However, thermostability of HRP has been significantly enhanced by chemical modification with a range of amino-specific bis-succinimides [23].

The purpose of the following work was to investigate the effects of monofunctional and bifunctional succinimides on the HRP-catalysed removal of parent phenol and other phenolic compounds (including chlorinated compounds) at high temperatures (>50^{0}C). The nature of the specific chemical modification (reactivity towards the ε-amino groups on HRP's lysine amino acid residues) leaves the carbohydrate moiety (18% of the enzyme) intact for further chemical reactions and/or immobilisation procedures.

3.2 EXPERIMENTAL

3.2.1. Materials

Horseradish peroxidase (EC 1.11 1 7, type I, RZ - 1.9, 190 purpurogallin units.mg^{-1} solid) and catalase (EC 1.11.6, 7,080 units.mg^{-1} protein) were purchased from Sigma. Phenol, 2-, 3- and 4-chlorophenol (99% + loose crystals), 2-naphthol, guaiacol, 2- and 4-cresol and pyrogallol were also obtained from Sigma Chemicals.

Sigma also supplied the bifunctional reagent ethylene glycol bis-succinimidyl succinate (EG-NHS) and the monofunctional modifier acetic acid N-hydroxysuccinimide ester (AA-NHS).

Tween 20, citric acid, tris (hydroxymethyl) aminomethanehydrochloride, sodium hydroxide (30% solution in water), dimethylsulphoxide (DMSO) and analytical grade hydrogen peroxide came from BDH Ltd , Poole, Dorset, U.K.

Bicinchoninic acid protein assay reagent was obtained from Pierce Chemical Co., Illinois, U.S.A.

96-well flat bottomed microtitre plates were obtained from Greiner, Germany.

Acetonitrile, methanol and all other chemicals used were of analytical grade and were obtained from Labscan Ltd., Dublin, Ireland or from Aldrich Chemicals.

3.2.2. Equipment

The disappearance of phenolic substrates was monitored using a System Gold™ High Performance Liquid Chromatograph from Beckman Instruments linked up to an Elonex PC-466 computer. The system incorporated a programmable solvent module 126 and a detection module 166 at absorbance units full scale (AUFS) 0.05, with detection at 280nm.

A μBondapak™ C18 column (3.9mm i.d. × 300mm) from Waters allowed separation of the compounds of interest.

Mobile phases were filtered with 0.22μM membrane filters (Millex-GV, Millipore Corporation) and placed in an ultrasonic bath for 30 minute periods.

UV/Visible spectra of phenols were carried out using a Shimadzu spectrophotometer.

A Heraeus Christ Labofuge 6000 centrifuge was used in phenol clearance experiments.

A Titertek Twinreader type 381 (Flow Laboratories Ltd., Scotland) was used to read absorbances on microtitre plates.

3.3 METHODS

3.3.1. Chemical modication of Horseradish peroxidase

The method for succinimide modification was similar to that of Ryan *et al.* [23] (full details in Chapter 2). Equal volumes of HRP (prepared in 0.1M phosphate buffer, pH 7.0) and N-hydroxysuccinimide ester (each typically 1mg ml^{-1}) were mixed together and the reaction allowed to proceed for approximately 20 minutes at room tempertaure before being terminated with an equal volume of cold 0.1M Tris-HCl, pH 7.0. As these succinimides (EG- and AA-NHS) possess limited solubility in water, they were initially dissolved in an organic solvent such as DMSO. The ester-solvent formed an emulsion which allowed the reaction to proceed

3.3.2. Determination of protein concentration

Protein estimation of HRP samples was previously described in Chapter 2. 10μl of each standard or protein sample was pipetted into quadruplicate wells of a 96-well microtitre plate. Controls consisting of 10μl diluent were also included. 200μl of working reagent was added to solutions and gently mixed. The plates were covered and incubated at 37^{0}C for 30 minutes. A_{560} values were determined on a Titertek Twinreader Plus.

3.3.3. Determination of HRP activity

A method based on that of the Sigma Bulletin and similar to that of Pokora *et al.* was employed to estimate HRP activity/concentration [24]. A "unit" of peroxidase is defined as the amount of enzyme which produces a change of 12 absorbance units measured at a 1cm pathlength in one minute at 420nm when HRP is added to a

solution containing 100mM potassium phosphate, 44mM pyrogallol and 8mM hydrogen peroxide and having a pH of 6.0. Purpurogallin, the oxidation product of pyrogallol (1,2,3-trihydroxybenzene) was measured at 420nm at room temperature.

3.3.4. Phenol precipitation reactions

Phenol precipitation reactions were carried out in triplicate (% relative standard deviation of less than 4.5%) in 30ml vials. Reaction solutions were allowed to achieve thermal equilibrium in a water bath (accuracy $\pm$ 0.5^{0}C). Mixtures were prepared by adding measured amounts of phenolic compound, HRP enzyme and hydrogen peroxide individually into buffer solutions of varying pH values. Polymerisation reactions were initiated by adding H_2O_2 The reacting solution was agitated by a magnetic stirrer and Teflon coated stir bars. Reactions were terminated by the addition of large doses of catalase enzyme (a final concentration of 30nmol dm^{-3}) solution to periodically withdrawn 1.0ml samples. (Catalase rapidly converts peroxide to oxygen and water, the consumption of peroxide thus halting the peroxidase-catalysed reaction) . Samples were then treated with a 40g L^{-1} solution of alum [$Al_2(SO_4)_3.14H_2O$] to enhance colloidal particle coagulation. Reaction solutions of varying pH were adjusted to approximately 6.3 (using either hydrochloric acid or sodium hydroxide) to optimise floc formation. After 20 minutes, samples were centrifuged at 3,000 $\times$ g for 40 minutes at room temperature. Residual phenol and 4-chlorophenol concentrations in the clear supernatant were detected by direct spectrophotometric measurement of absorbance at 280nm (ε- 1400M^{-1} cm^{-1} for both) [25]. Peroxide, HRP, catalase or alum did not interfere with absorbance measurements at this wavelength.

The concentrations of phenols used in this investigation were expressed in terms of molar quantities for convenience. A 1mM concentration corresponds to 94.1 and 128.6 mg L^{-1} for phenol and 4-chlorophenol, respectively.

3.3.5. HPLC analysis of residual phenols

The removal of phenols was also monitored using a System Gold ™ High Performance Liquid Chromatograph (supplied by Beckman Instruments) linked up to an Elonex PC-466 computer. Reversed-phase chromatography was achieved using a µBondapak™ C18 column (3.9mm × 300mm) from Waters. A number of mobile phase compositions were examined for their ability to resolve mixtures. Acetonitrile and methanol mobile phases were examined for their respective abilities to separate the compounds of interest. The effect of flow rate on resolution efficiency was also investigated. All mobile phases were filtered with 0.22µM membrane filters (Millex-GV, Millipore Corporation) and placed in an ultrasonic bath for approximately 30 minutes.

3.4 RESULTS AND DISCUSSION

3.4.1. Optimisation of HPLC analysis

All phenol removal experiments were analysed by HPLC as described in Section 3.3.5. The initial aim was to elucidate optimum working conditions. Regardless of its composition, an acetonitrile (CH_3CN)-water mobile phase failed to resolve a mixture of phenolic compounds such as parent phenol and 4-chlorophenol. Retention times for both phenolics were practically identical; 2.95 minutes for phenol and 3.11 for 4-chlorophenol. Addition of methanol to the CH_3CN-based carrier stream (70% CH_3CN, 10% MeOH and 20% H_2O) had little impact on phenol resolution. With a MeOH based mobile phase, however, retention times were significantly changed. It was apparent that as the % v/v of MeOH in the mobile phase was increased, the respective retention times of both compounds on the C18 column was reduced. Parent phenol had shorter retention times in the 20-70% v/v range; however, both compounds have similar elution patterns when using an 80% v/v MeOH phase. 4-Chlorophenol was extremely difficult to elute when using <40% v/v MeOH. For example, when a 20% v/v MeOH phase was employed, 4-chlorophenol was unable to elute; a prolonged washing with 100% v/v was necessary to regenerate the column. The effect of MeOH composition in the mobile phase is depicted in Figure 3.1. As expected, the efficiency of separation was enhanced with an increasing water content in the mobile phase (buffer-based phases were not used as water was found to be sufficient). 60-80% v/v water-containing mixtures offered similar resolution; less than 60% v/v delivered adequate separation. Retention times of phenolics were shown to be dependent on the flow rate of the system (Figure 3.2). An increase in the flow rate resulted in shorter

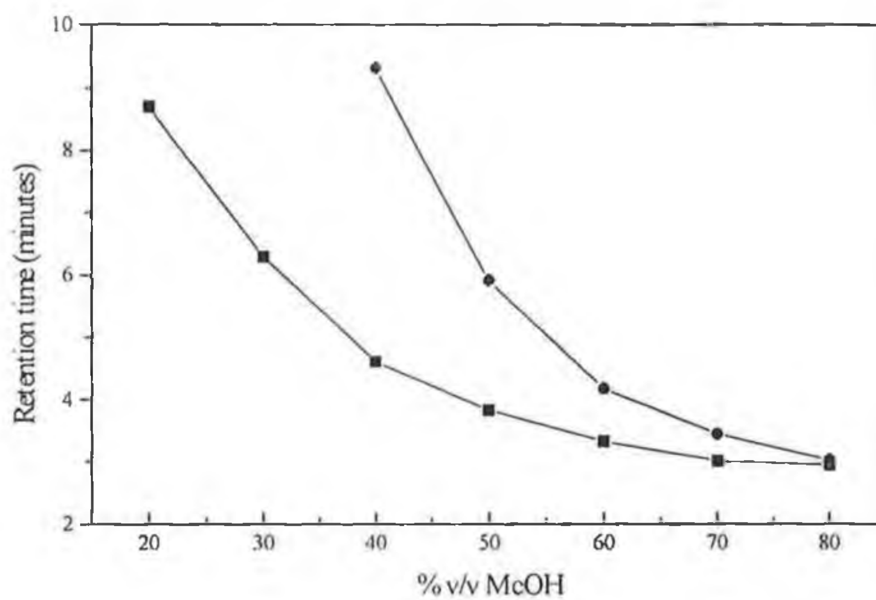


Figure 3.1: Effect of methanol composition in mobile phase on retention time.
■ phenol; ● 4-chlorophenol, detection - 280nm, flow rate - 1.4ml min^{-1}.

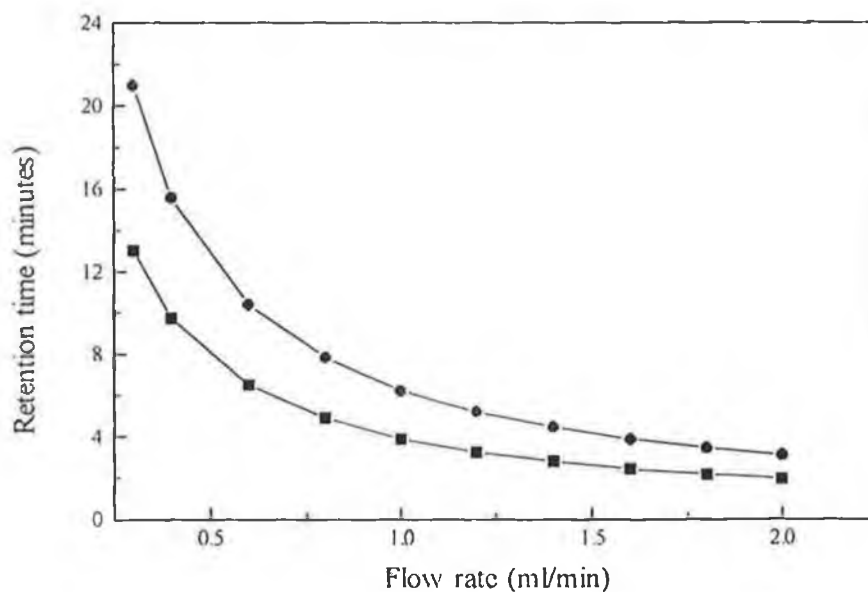


Figure 3.2: Effect of flow rate on retention. ■ phenol; ● 4-chlorophenol.

retention times for both phenol and 4-chlorophenol, with phenol itself having the shorter retention time (when using a 50:50 MeOH-water mobile phase). Rapid decreases in retention times were especially apparent when employing flow rates in the range 0.3-0.8ml min^{-1} (4-chlorophenol had a retention time of 20.99 minutes when the system was operated at 0.3ml min^{-1}, the time was reduced to 7.84 minutes when operated at 0.8ml min^{-1}). Table 3.1 summarises the retention times of a range of phenolic compounds that were used in this study. The mobile phase used was 50:50 MeOH-water and the flow rate was 1.4ml min^{-1}. 1mM standards of phenols were prepared in 0.01M phosphate buffer, pH 7.0 and diluted 1:5 with mobile phase. Samples were filtered and thoroughly degassed prior to analysis. 20µl of each sample was run on a C18 column and the concentration of aromatics were determined by monitoring the absorbance at 280nm.

Table 3.1

Retention times of a range of phenols. Experimental conditions (section 3.4.1.).

Phenol compound	Retention time (minutes)
Phenol	2.90
2-Chlorophenol	3.95
3-Chlorophenol	5.28
4-Chlorophenol	5.08
2-Naphthol	6.41
Resorcinol	2.28
Guaiacol	3.04
2-Cresol	3.90
4-Cresol	3.78

3.4.2 HRP-catalysed oxidation of phenol

HRP can remove of a wide variety of aromatic compounds from aqueous solution and is effective over a wide range of concentrations [9]. Even though the enzyme has good stability characteristics [26], an economically feasible treatment method at high temperatures would require a substantial increase in biocatalytic efficiency and lifetime. To describe quantitatively the degree of water purification achieved, the parameter "removal efficiency" is used, defined as the percentage of pollutant removed from solution under the given experimental conditions. Upon addition of HRP and H_2O_2 to a 1mM solution of phenol (made up in 0.01M borate buffer, pH 9.0), the solution immediately turns dark followed by separation of a brown precipitate (which can be easily removed by centrifugation). A colourless solution is obtained after centrifugation. To determine the relative removal efficiency by this treatment, a solution of phenol is analysed before and after addition of the HRP system (HRP-H_2O_2). Under these conditions, treatment for 24 hours resulted in near-complete removal of parent phenol (>98%). Reduction in the treatment time led to less overall removal of the aromatic. Removal efficiencies were greater when the reacting solution was agitated, rather than being in a quiescent state. It should be pointed out that treatment with peroxidase or peroxide alone did not result in any aromatic removal from aqueous solution

Figure 3.3 summarises the treatment accomplished during the removal of 1mM phenol in batch reactors as a function of enzyme dose. In the case of doses of 0.25 and 0.50U ml^{-1}, the amount of enzyme supplied to the reactor was limiting, as indicated by the significant residual concentrations of phenol at all time intervals (residual concentrations determined as described in Section 3.3.4.). In contrast, the 1.0U ml^{-1}

dose of HRP resulted in a greater degree of removal. Under actual treatment conditions, care must be taken to make full use of enzyme lifetime in order to avoid waste of catalyst and to improve the economic feasibility of the method.

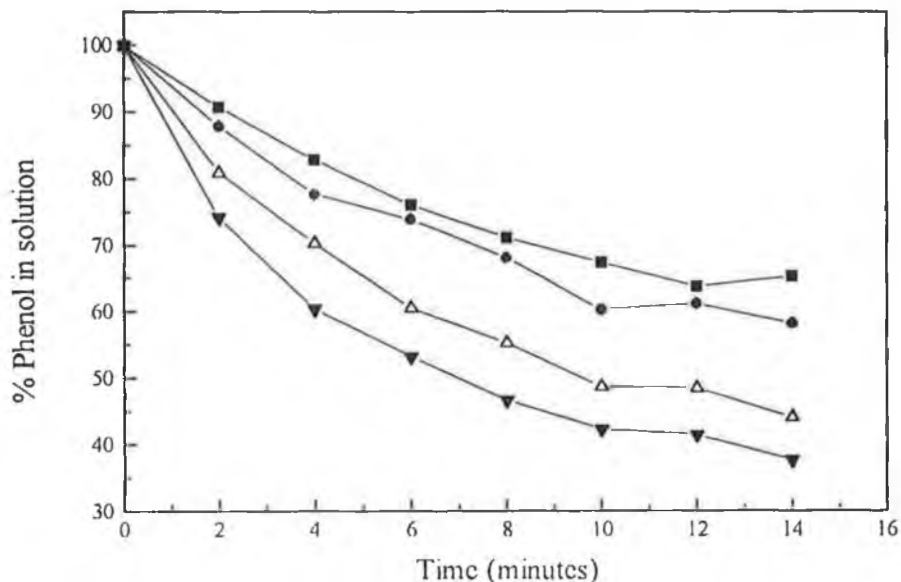


Figure 3.3: Phenol removal as a function of initial enzyme dose.
■ 0.25U; ● 0.50U; ▲ 0.75U; ▼ 1.00U ml^{-1}

In the past, peroxidase enzymes have not been available at a cost and in a purity amenable to many biocatalytic processes, such as the treatment of contaminated waters and, particularly, wastewater treatment. For example, horseradish roots, the main source of HRP, are cultivated generally in small quantities and are propagated through root cuttings, thus making it difficult to scale up production. The limited availability of horseradish root extract, coupled with the shortage of alternative sources of enzyme, has created a very expensive market for such enzymes. Accordingly there is a need for

relatively inexpensive methods for treating contaminated waters, and particularly for use in wastewater treatment to remove hazardous or toxic materials.

3.4.3. Effect of pH

Since the pH of industrial effluents may vary, the pH dependence of the enzymatic removal of phenol was examined. H_2O_2 was equimolar with phenol (1mM) in all tests. The percentage phenol remaining in solution after a 10 minute exposure to the HRP system was determined. HRP precipitated phenol in the pH range 4.0-11.0. Particularly good removal was achieved between pH 6.0 and 9.0, with an optimum at pH 9.0 [9]. Virtually no catalytic activity was observed below 2.0 or above 11.0. Activity increased substantially in the range 4.0-8.0. Boric acid buffer was employed at pH 9.0 and phosphate was used at pH 8.0.

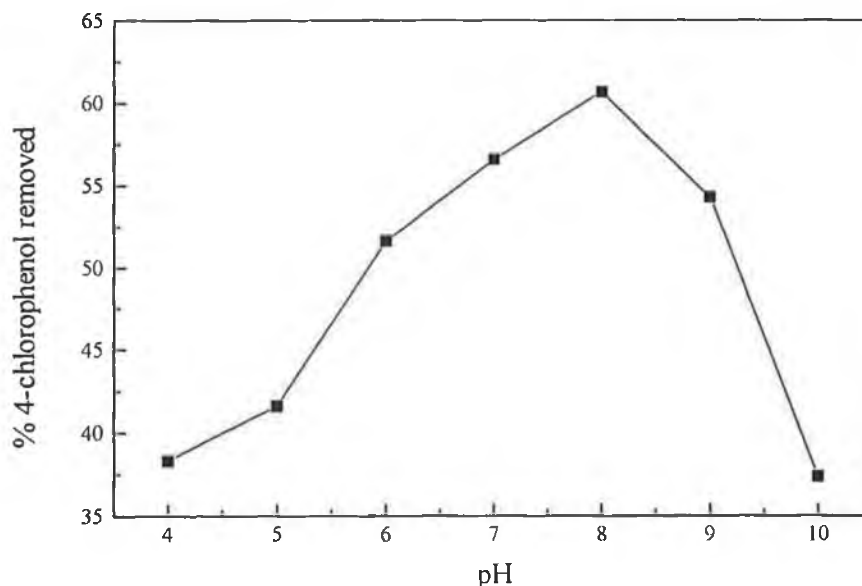


Figure 3.4: Dependence of 4-chlorophenol removal on pH of reaction medium.

It has been reported that borate has a protective effect on HRP activity [15]. This fact may go some way towards explaining the observed profile. The pH profile for 4-chlorophenol (4-CP) removal had a similar trend to that of phenol except that the pH for optimum removal was found to be 8.0 (Figure 3.4).

Nicell *et al.* pointed out that in the presence of excess HRP, greatest removal of 4-CP was achieved in a solution that had a pH of 3.9. This was attributed to the reduced solubilities of polyaromatic products in media of lower pH values [31]. Use of an excess amount of HRP represented a loss in catalytic efficiency . The optimum was defined as the pH at which the greatest number of substrate molecules were polymerised per amount of enzyme provided. They concluded that phenol oxidation was best at pH 8.1.

The activity of HRP was found to be pH-dependent (Figure 3.4). Oxidase reactions are typically carried out in the pH range 3.0-10.0 and particularly between 4.0 to 9.0 [6]. A pH value may be selected at which the enzyme is highly active for economic and environmental reasons; however, the pH of the aqueous composition may not lie in this pH range. Soils or sludges could be modified (with potassium phosphate) to bring their pH into working pH ranges, but because of HRP's broad activity, it would be possible to treat soil or sludge as they exist.

3.4.4. Temperature studies

Inactivation of HRP as a function of time at temperatures between 5 and 65^0C has been reported [12]. Native HRP was subject to rapid inactivation at temperatures above 65^0C during phenol removal (Figure 3.5). The temperature maximum of this catalytic effect was around 50^0C with over 50% of phenol removed within 10 minutes. Thus, more than twice the concentration of phenol was oxidised at 50^0C than at 80^0C. A similar degree of removal can be achieved at all temperatures when sufficient enzyme is supplied to the batch reactor initially; however, this represents a loss in catalytic efficiency and a corresponding increase in cost. A number of reasons may account for reduced phenol removal at temperatures greater than 65^0C. Klibanov *et al.* noted that neither phenol nor peroxide alone inactivate the peroxidative reaction [9]. They suggested the possibility of interactions between enzymatically generated pheno-

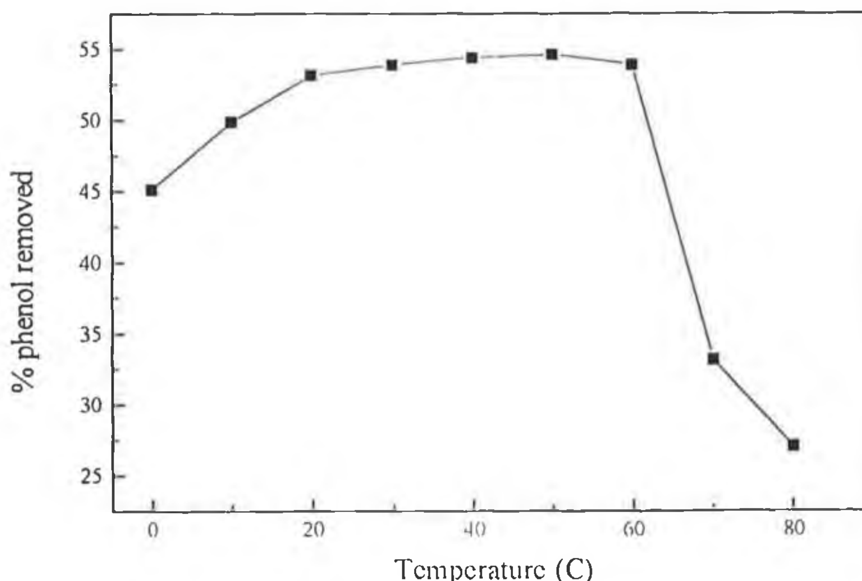
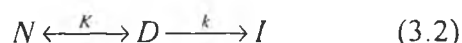


Figure 3.5: The effect of temperature on native HRP-catalysed removal of phenol from aqueous

-xy radicals and the enzyme's active centre. Alternatively, inactivation may be due to the adsorption of protein molecules onto polyaromatic products. This would adversely affect aromatic substrate diffusion to the active site [15]. Also, peroxidase activity is lost if the enzyme is exposed to high temperatures for prolonged periods, as unfolding disrupts the active site to an irreversible extent. Irreversible enzyme thermoinactivation is responsible for the gradual loss of enzyme activity with time at an elevated temperature. It may be treated as a two-step process, where N, D and I are the native, reversibly denatured and irreversibly inactivated forms of a protein, respectively (equation 3.2). K and k represent the equilibrium and rate constants. Various aspects of thermal inactivation have been addressed in Chapter 2 (Section 2.4.3).



$$N \xleftrightarrow{K} D \xrightarrow{k} I \qquad (3.2)$$

Nicell *et al.* reported that the extent of 4-CP removal from aqueous solution in a batch reactor was dependent on temperature, except where HRP was present in an excess amount and exposure temperatures were below 50^{0}C [12]. Significant improvement in the efficiency of the process occurred when carrying out the reaction below 35^{0}C. HRP activity was stable for long periods when stored at low temperatures [23]. It is possible that the catalyst's longer lifetime at lower temperatures was due to a slower reaction rate [27] rather than to decreased thermal denaturation of the enzyme. Lower reaction rates would suggest a lower concentration of free radicals during the polymerisation process at any given time; the rate of enzyme adsorption onto polymer products [16] and/or interactions with the active centre [6] would be minimised. HRP-catalysed colour removal from bleach plant effluent was severely hampered when carried out at

temperatures greater than 60^{0}C. The thermal inactivation characteristics of native HRP at elevated temperatures in this range have been reported [28]. Results here have demonstrated that the efficiency of phenol removal from aqueous solution decreased as the enzyme was exposed to a temperature of 70^{0}C for long periods. Catalytic activity of a 1U ml^{-1} enzyme fraction was practically zero after 60 minutes. Thus a strategy is desirable that in some way improves catalytic efficiency under these conditions.

3.4.5. Effect of succinimides on phenol removal

Treatment of HRP with the amino-specific reagent, acetic acid N-hydroxysuccinimide ester (AA-NHS) led to noticeable increases in phenol precipitation at 70^{0}C (Figure 3.6). Note that modification of HRP with higher concentratons of the acetylating agent (2.0 and 3.0mg ml^{-1}) led to increased phenol removal, i.e., to a more thermostable form of the enzyme. The optimum ester concentration was approximately 1.5mg ml^{-1}. Concentrations above this did not lead to any further increases in removal efficiency. These stabilisations persisted on storage at 4^{0}C. Also, modification with the agent did not alter the enzyme's pH profile. It is important to note that the succinimide modification protocol did not result in any loss of HRP's ability to catalyse the oxidation of phenols. However, chemical modification of HRP did not result in any improvement in phenol removal at room temperature.

A 6- to 23-fold thermostabilisation of HRP at 75^{0}C following reaction of bis-succinimides with 5 of the available 6 lysine residues has been reported [23]. Analysis of acetylated HRP's free amino groups indicated that the modifying agent altered three lysines [28]. Thermal inactivation of these commercial preparations deviated from a first-order decay at this temperature. Similar observations were made by Chang *et al.*

who estimated the process to be 1.5-order [29] when the thermal inactivation profile of HRP deviated from first-order kinetics in the range 60 - 94°C.

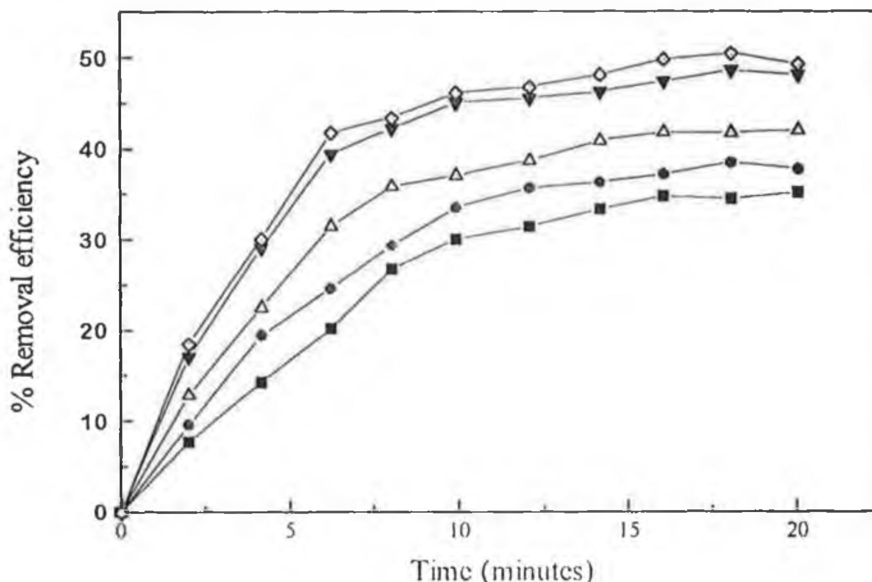


Figure 3.6: Removal efficiency (%) of phenol as a function of AA-NHS concentration at 70°C. ■ 0.0mg ml^{-1}; ● 0.2mg ml^{-1}; ▲ 0.6mg ml^{-1}; ▼ 0.8mg ml^{-1}; ◆ 1.4mg ml^{-1}.

Ugarova and colleagues studied the thermostability of the enzyme following modification of its lysine amino groups with a variety of carboxylic acid anhydrides and with trinitrobenzenesulphonate (TNBS). Some of these compounds reversed the positive charge on the lysines. These chemical treatments led to restricted conformational mobility and to increased thermostability. Stabilisation was due to the extent of modification, i.e., the number of lysines modified (not the nature of the modifier). The authors reported first-order thermal decay kinetics of HRP derivatives at 56°C [30].

Urrutigoity and Souppe reported on the attachment of polyethylene glycol (PEG) to periodate-oxidised carbohydrate chains of HRP, enhancing the enzyme's solubility and activity in chloroform and toluene [31] Oxidised PEGs of differing molecular weights have been attached to free amino groups (as opposed to the carbohydrate moiety) of HRP, increasing organotolerance [32]. Wu *et al.* have demonstrated that the addition of PEG had a significant protective effect on the HRP activity. The amounts of HRP required to remove 1 0 and 10 0mM phenol from solution were reduced 40- and 75-fold, respectively, in the presence of PEG [16] Apparent enzyme inactivation during phenol oxidation processes is thought more likely to be due to adsorption of enzyme molecules by the end-product polymer [15, 16]. By adding proteins or hydrophilic synthetic polymers, enzyme adsorption is competitively suppressed; inactivation is therefore alleviated and this in turn reduces the overall cost of the process. The extent of PEG's inhibitory effects are thought to be primarily dependent on PEG molecular weight [15] In a similar fashion, maximum removal of phenol occurs at pH 9.0, as borate possesses a protective effect on the enzyme's activity.

Chemical modification can dramatically increase enzyme stability [33]. In this study, amino-specific succinimides have been employed to modify HRP's lysine residues with the aim of increasing thermostability, thus reducing the amount of peroxidase neeeded to catalyse the removal of phenolics from aqueous solution at high temperatures. By specifically targetting the lysine amino acids [34], the carbohydrate portion has been left intact for further modification This could be exploited, in that additives such as PEG or gelatin are capable of protecting HRP from the effects of polyaromatic substrates [15] This approach could complement the specific chemical

modification described here, resulting in the production of a HRP derivative with improved phenol removal capabilities at ambient and elevated temperatures.

HRP can be immobilised on surfaces such as paper or plastic or on beads or like surfaces. Materials that could be used as the solid phase encompass the whole range of polymers. Some of the most prevalent are polymers of ethylenically-unsaturated monomers (e.g. styrene, acrylic acid derivatives, ethylene, propylene), polysaccharides (e.g. cellulose, dextran, agarose), polypeptides (e.g. gelatin/collagen, cross-linked proteins such as albumin), nylon and glass. Composites on the foregoing have also been used alone, and in conjunction with inorganic materials other than silica such as crystalline calcium phosphate. Preferably the surface is also useful for filtering the precipitated solids from the solution, such as with filter paper. Additional examples include other forms of cellulose, glass fibre filters and other porous solid phases of natural or synthetic origin.

The choice of immobilisation technique is crucial, as each strategy presents different problems. Nicell *et al.* attached HRP to cellulose filter disks placed in a filter holder to remove 4-CP from water in a heterogenous reactor design. Filter disks were chosen as the reactor matrix to minimise contact time of the enzyme with the free radicals. The system operated on the basis of pumping 4-CP (containing H_2O_2) through the filter disks. Enzyme activity decreased as the concentration of products deposited on the filter disks increased. Results indicated that mass transfer limitations existed in the system. However, the authors did point out that the system was slightly more efficient than utilising a solubilised enzyme system as the enzyme, in its free state, had a finite catalytic lifetime and the rate of enzyme addition to the system was crucial to the system's efficiency.

The entrapment of HRP in alginate beads improved colour removal from phenolic industrial effluents [17]. However, the beads were deemed unsuitable for continuous use as enzymes were rapidly released into solution. HRP has been immobilised on a different reactor matrix, CNBr-Sepharose 4B, for decolourising kraft effluent. Mass transfer limitations and leaching of peroxidase activity to the reaction medium were not apparent [35]. The ability of horseradish peroxidase attached in three different reactor matrices: cellulose filter paper, nylon balls and nylon tubing, to remove 4-CP from aqueous solution was evaluated [18]. Results indicated that over 80 % removal efficiency could be obtained as long as HRP activity was not limiting in the reactor; however, enzyme inactivation by reaction intermediates was observed.

Mass transfer appeared to be a problem when employing immobilised HRP or any other source of peroxidase. Findlay described a novel solid support for practical immobilisation which could be used for either enzymes or cells [19]. Biobone is composed of clean granular chicken bone. The material is extremely porous and is made up of crystals of the mineral hydroxyapatite (calcium phosphate) embedded in a protein matrix of connective tissue. Oxidised phenol is collected on the support and removal efficiencies of over 99.99% were reported. Due to the porosity of the matrix, mass transfer limitations were significantly reduced and the system demonstrated the ability to process large volumes of phenol waste.

3.4.6. Effect of reaction time on 4-CP removal

In any chemical reactor, the conversion achieved is dependent on the reaction time. Experiments were carried out to estimate the time scale of the polymerisation reaction of 1mM 4-CP. Results for the substrates' conversion as a function of time by native and HRP derivatives at 37 and 70^{0}C are shown in Figure 3.7.

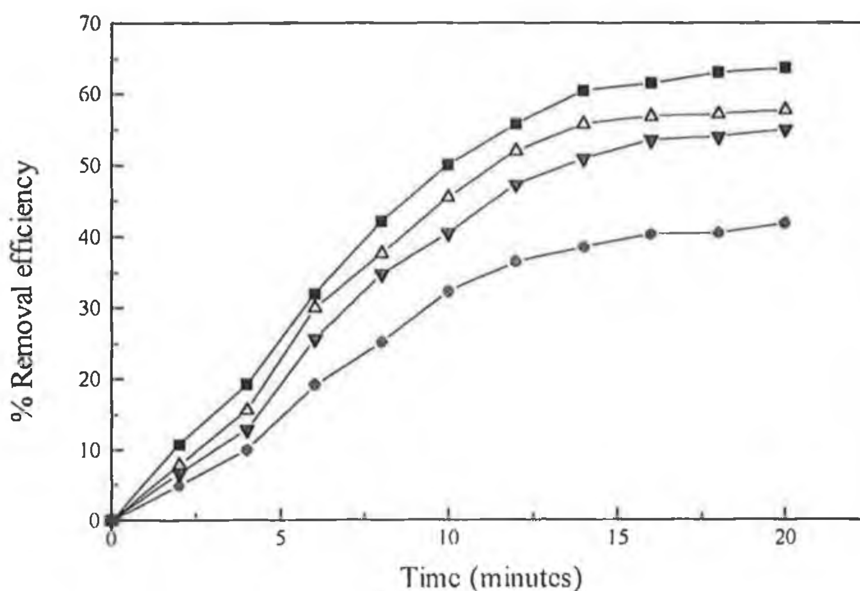
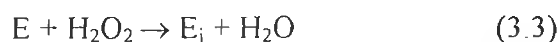


Figure 3.7: % Removal efficiency of 4-CP as a function of reaction time and HRP preparation in a batch reactor at 37 and 70^{0}C. ● Native 70^{0}C; ▼ AA-NHS 70^{0}C; ▲ EG-NHS 70^{0}C; ■ Native 37^{0}C.

Maximum removal was achieved within 10 minutes which indicated a relatively fast reaction rate. This was followed by a very slow removal process (a removal efficiency of >98% after 24 hours). This slowdown in reaction rate could be attributed to the simultaneous decrease in the concentration of all the reacting species (phenol, HRP and peroxide). Similar behaviour was observed for parent phenol. Both derivatives appeared to be more efficient in removing 4-CP at 70^{0}C than native HRP. After 20

minutes, modified HRPs catalysed the removal of approximately 55% of 4-CP in solution whereas native HRP precipitated just over 40% in the same time at 70^0C. Removal efficiencies of succinimide-treated HRP fractions were less than 10% lower than that of native peroxidase at 37^0C, with acetylated HRP possessing marginally better ability over the bifunctional form (EG-NHS HRP).

As previously stated, the slowdown in reaction rate after 15 minutes can be attributed to a decrease in the concentration of all the reacting species. Carmichael *et al.* have shown that multiple additions of cosubstrate (peroxide) resulted in stepwise substrate oxidation [36], an indication of the relative stability of their peroxidase (chloroperoxidase) over the course of the experiment. Naturally this observation is totally dependent on the concentration of individual peroxide spikes. On the other hand, the presence of excess peroxide would result in Compound II (E_{ii}) formation, which can be oxidised to Compound III (E_{iii}); see Reaction 3.6. Compound III is peroxidatically inactive but not terminally inactivated, as it spontaneously decomposes to native peroxidase (Reaction 3.7) [37]. Thus, the slow removal phase may in fact be due to Compound III formation, as any accumulation of HRP in this state results in a loss in catalytic efficiency [38]. The one-electron oxidation of aromatic substrates (AH_2) catalysed by peroxidase is well understood and is usually depicted by the following mechanism where E is the native enzyme and E_i refers to Compound I.



$$E + H_2O_2 \rightarrow E_i + H_2O \qquad (3.3)$$

$$E_i + AH_2 \rightarrow E_{ii} + {}^{\bullet}AH \qquad (3.4)$$

$$E_{ii} + AH_2 \rightarrow E + {}^{\bullet}AH + H_2O \qquad (3.5)$$

$$E_{ii} + H_2O_2 \rightarrow E_{iii} + H_2O \qquad (3.6)$$

$$E_{iii} \rightarrow E + O_2^{-} \qquad (3.7)$$

Nicell *et al.* have postulated that the enzyme is distributed between the Compounds I and II and native enzyme forms immediately following the start of the reaction [10]. Model predictions indicated that the maximum levels of Compounds I and II concentrations are achieved within the first 2 milliseconds under the given experimental conditions (4-CP - 1mM, H_2O_2 - 1mM, HRP - 0.64U ml^{-1}, at 25^0C and pH 7.0). It was assumed that the amount of enzyme inactivated at any time is directly proportional to the quantity of aromatic substrate removed from solution.

3.4.7. Effect of hydrogen peroxide concentration on polymerisation

Experiments have demonstrated that the removal of phenolics by HRP is not a function of H_2O_2 except when its concentration is limiting. It is important to limit the addition of peroxide to the reaction as an excess would inhibit the enzyme's catalytic ability [37]. The oxidative capabilities of native and acetylated HRPs on parent phenol over a temperature range (50-80^0C) at different peroxide concentrations (0.5 and 1.0mM) is shown in Figure 3.8. An initial 1mM peroxide spike in the batch reactor resulted in appreciably greater removal rates for the derivative enzyme at ambient and high temperatures. The removal of 4-CP as a function of H_2O_2 concentration in the presence of excess enzyme at 70^0C was shown to be linear (Figure 3.9) and would suggest a reaction stoichiometry of one. Phenol removal under the same conditions appeared not to exhibit linearity, nevertheless, a one-to-one phenol-peroxide reaction stoichiometry was chosen for further experiments. After the initial levelling out period (see Figure 3.8), one additional spike of 1mM peroxide to the reactor resulted in a further 12% reduction in the level of residual 4-CP over a 30 minute period. Further additional spikes of HRP did not result in any further oxidation of phenolics.

Klibanov *et al.* noted that 2 mol of peroxide were necessary to polymerise 1 mol of phenol when peroxide was added at the beginning of the reaction [9]. The generation of two free radicals per molecule of peroxide has also been suggested [38]. Removal of such compounds from water at low peroxide concentrations (<0.3mM) results in the formation of soluble products which become larger polymers (possessing reduced solubility) in the presence of higher peroxide concentrations. These larger polymers readily precipitate from aqueous solution [12].

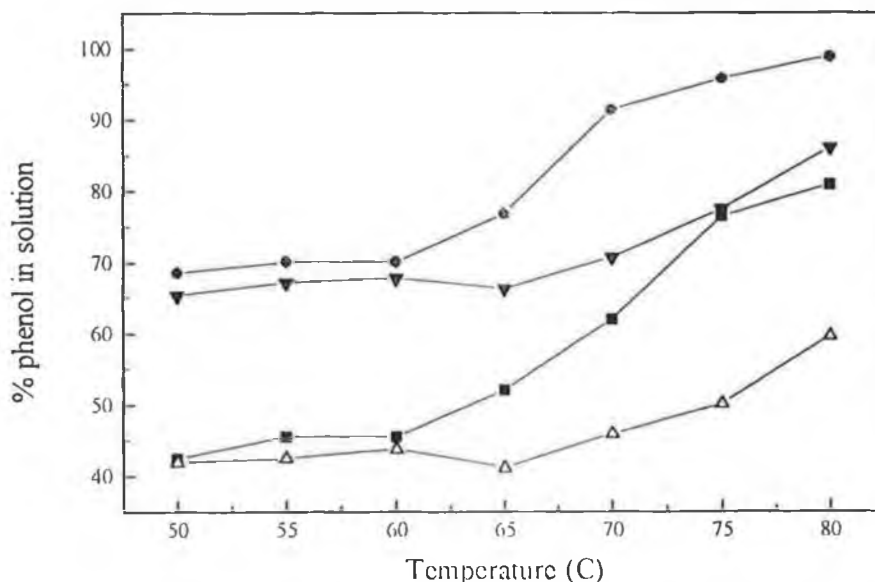


Figure 3.8: Dependence of phenol removal on temperature and concentration of hydrogen peroxide. ▲ AA-NHS HRP (1.0mM H_2O_2); ▼ AA-NHS HRP (0.5mM H_2O_2); ■ Native (1.0mM H_2O_2); ● Native (0.5mM H_2O_2)

Based on the foregoing, peroxide concentrations were generally kept around 1.0mM in all experiments. It was concluded that initial concentrations greater than 1.0mM in the batch reactor did not improve the system's efficiency. Peroxide concentrations greater

than 1.0mM could possibly contribute to the suicide inactivation of the enzyme [37]. It is known that in the absence of a reductant substrate and with excess peroxide, HRP displays the kinetic behaviour of a suicide inactivation, peroxide being the suicide substrate. Greater HRP inactivation can occur as the initial level of peroxide in the system is increased; enzyme inactivation or total loss of activity occurs with an apparently bi-exponential time course. Rather than being inactivated by the cosubstrate, the authors concluded that HRP (due to reduction of Compound I by peroxide) acts as a catalase activity in the absence of any reductant source

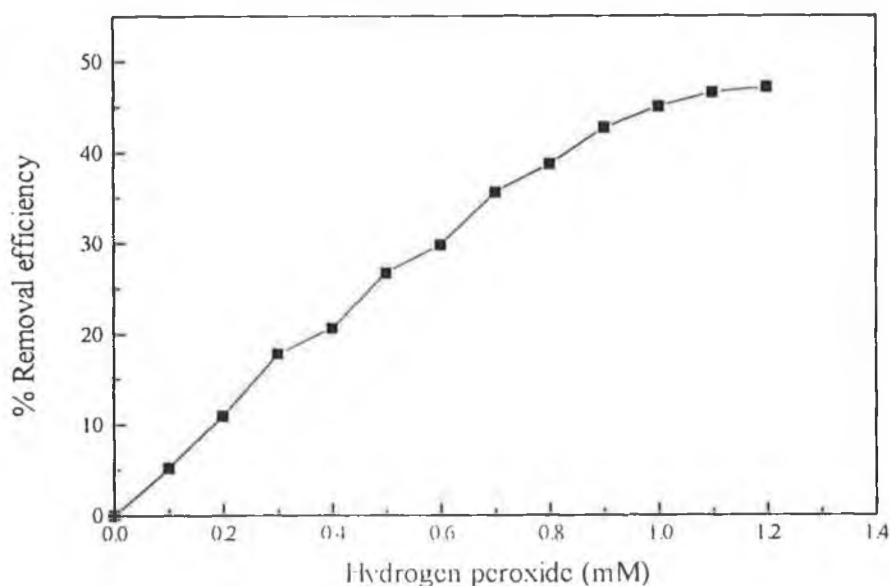


Figure 3.9: 4-CP removal at 70^0C by native HRP as a function of hydrogen peroxide HRP concentration - 1.2U ml^{-1}.

The type of peroxide used in oxidative-type reactions is generally hydrogen peroxide, but other peroxides are useful Other potentially useful peroxides include methyl peroxide and ethyl peroxide The preferred oxidising agent (hydrogen peroxide), can

be dissolved in water for addition to the contaminated water. Pokora *et al.* have recommended the use of a ramped addition system, where higher amounts of peroxide are used at the beginning of the reaction when peroxide is consumed rapidly and scaled down amounts of peroxide are used in the later stages [39]. For reaction on soils and sludges, higher applications of peroxide (as well as of peroxidase) may be required.

3.4.8. Effect of HRP concentration on 4-CP removal

4-CP removal at different concentration levels of HRP preparations is illustrated in Figure 3.10. The amount of enzyme required for aromatic precipitation will depend primarily on its activity The enzyme is not consumed during the course of the reaction, but it does gradually lose activity During this experiment, all reactions were halted after a 20 minute period. Increasing the concentration of the enzyme produced better removal of 4-CP. In the case of enzyme doses up to 0 8U ml^{-1}, the amount of enzyme supplied to the reaction was limiting, as indicated by the significant residual concentration of 4-CP Near-constant removal efficiencies were observed over the range 1.2-4.0U ml^{-1} These initial HRP spike concentrations were therefore well in excess of the amount required for treatment, i.e., a proportion of each HRP fraction remained active following the oxidation of the substrate. Treatments of longer duration (24 hours) required lower initial spike concentrations than those required for 3 hour treatments. Thus, one can increase the time of treatment to offset the reduction in removal efficiency at low enzyme concentrations [25].

The number of reactions catalysed by individual peroxidase molecules can be substantially increased by keeping an initially low concentration of enzyme in the system, i.e. < 1U ml^{-1} [12, 25]. Greater HRP concentrations gave similar removal

efficiencies In this case, each peroxidase molecule catalyses fewer reactions and this represents a decrease in catalytic efficiency. At higher enzyme concentrations (>1.0U ml^{-1}), phenoxy radicals generated may find an enzyme's active site more readily, but as the reaction proceeds and the concentration of the aromatic decreases, free radical polymerisation becomes much more difficult [38].

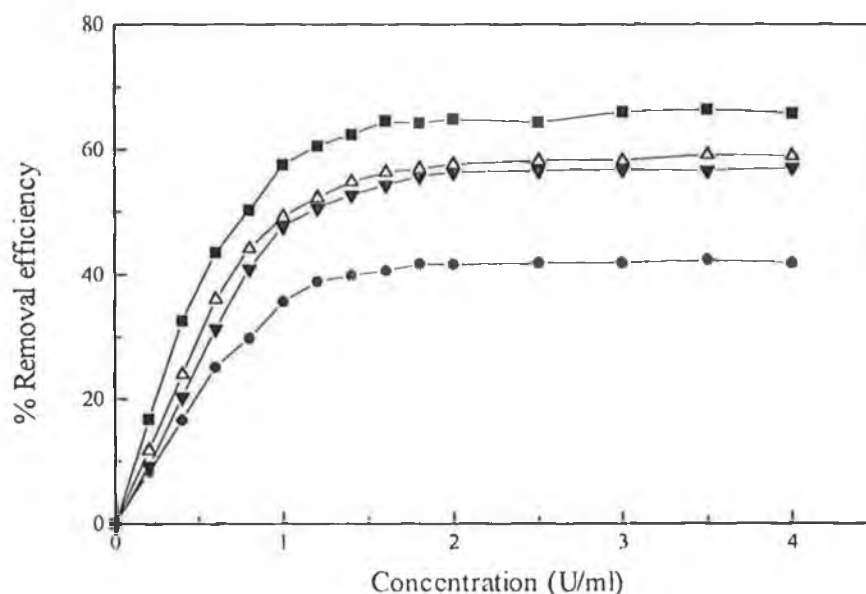


Figure 3.10: 4-Chlorophenol as a function of HRP concentration.
● Native HRP at 70^0C; ▼ AA-NHS HRP at 70^0C; ▲ EG-NHS HRP at 70^0C; ■ Native HRP at 37^0C.

Oxidative enzymes such as HRP are applicable to the removal of a variety of aromatic substrates from industrial wastewater and are effective over a wide concentration range [6, 9]. Peroxidase inactivation during the removal process at high temperatures is presumably due to the unfolding of the protein conformation [40]. It has been demonstrated that the amount of peroxidase required to remove phenolics at 70^0C was

reduced by modification of HRP with ε-amino-specific succinimides. These reagents (e.g. AA-NHS) act by neutralising the amino group's positive charge during the course of the reaction [41]. This charge neutralisation possibly accounts for HRP's enhanced thermostability, which in turn leads to a greater rate of phenol removal at high temperatures. Monofunctional protein modifiers such as AA-NHS esters are unable to form molecular crosslinks. Modification of HRP with bifunctional-type succinimides such as EG-NHS has also been shown to improve thermostability at elevated temperatures [23]; however, their overall removal capabilities were shown to be marginally lower than that of acetylated HRP (see Section 3.4.10). Bifunctional, as opposed to monofunctional succinimides, can form protein crosslinks [37, 41]. Evidence supporting the existence of such links in the EG-NHS form of HRP has been put forward (see Section 2.4.11). Net fluorimetric emissions from modified HRPs were smaller in magnitude than that of the native enzyme at both room 25^0C and 65^0C, indicating that the conformational ability of the derivative was in some way restricted.

The ethylene glycol compound spans a molecular distance of 14Å. It is possible that the presence of such intramolecular crosslinks interferes with the one-electron oxidation of aromatic substrates [12], thus slightly decreasing removal capabilities. It is feasible that crosslinked HRP is less able to contort in binding some substrates, or more likely, both reagent types (EG- and AA-NHS) modify different proportions of NH_2 groups (AA-NHS modifies 3 lysines whereas EG-NHS modified up to 5 residues) [23, 42].

As shown previously, the removal of aromatics from aqueous solution is dependent on the initial concentrations of both HRP and peroxide up to a certain point. However, the amount of peroxidase added to the body of water was not generally

dependent on the concentration of the aromatic hydroxy compound. It was apparent that the more critical variable appeared to be the amount of substrate employed, for the oxidase enzyme used to generate H_2O_2, since the amount of peroxide which can ultimately be generated will be limited by the amount of substrate provided. Thus, the greater the concentration of aromatic in the solution to be treated, the greater the amount of oxidase substrate will preferably be employed. The order of addition of these reagents to the reaction mixture is not critical to the overall efficiency of the reaction. The amount of enzyme required for total water purification could be accomplished by reducing the contact time between the enzyme and aromatic molecules. Shorter contact time prolongs the operating lifetime of the enzyme and, as a result, it may be used to treat a much greater volume of solution than if it had been simply been included in a solution. Strategies of this kind typically involve immobilising the enzyme (Chapter 1) and preferably incorporating the matrix-enzyme complex into a continuous flow reactor [38] so that its contact time with the aromatic and peroxide mixture can be controlled by the flow rate. In this way, the solution can be repeatedly contacted by the enzyme through recycling or by passage through a series of reactor elements on which the enzyme is immobilised [18]. Substantial amounts of the oxidised phenol can accumulate without drastically decreasing enzyme activity [43].

A number of other enzymes apart from peroxidase can be utilised in this type of oxidation reaction. These include haloperoxidases, lactoperoxidase, ligninase (manganese-dependent or -independent), tyrosinase (polyphenol oxidase) and cytochromes as well as heme proteins such as hemoglobin. Some of these enzymes use oxygen as a substrate (e.g. tyrosinase) while others use peroxide as an oxidative substrate to activate the enzyme. All are regarded as "oxidative" or "oxidatic"

enzymes. Equivalent results can be achieved where oxygen is dissolved in the aqueous solution for oxidative enzymes The relative effectiveness of different enzymes possessing phenol-removal capabilities have been assessed [11]

3.4.9. Simultaneous biodegradation of phenol and 4-chlorophenol in batch reactors

The disappearances of phenol and 4-CP catalysed by native and acetylated HRPs at 70^{0}C were monitored. Modified HRP displayed a greater ability to oxidise both aromatics, either individually or when combined. When phenol was incubated with the native enzyme (1U ml^{-1}) at pH 8.0, minimal substrate depletion was noted However, when incubated in the presence of 1mM 4-CP, phenol removal was significantly improved. 4-CP was readily precipitated from solution when exposed on its own to the HRP system. However, when it was exposed to the native enzyme at 70^{0}C in the presence of 1mM phenol, its removal was notably less than that observed when it was the sole substrate (Figure 3.11). Co-polymerisation of both phenolics at the elevated temperature (Figure 3.12) was enhanced when the native enzyme was replaced with acetylated HRP (concentration of 1U ml^{-1}) These results implied that 4-chlorophenol is a better substrate than parent phenol and that acetylated HRP oxidises phenolic mixtures more efficiently than the native enzyme at elevated temperatures. Also, the transformation of phenol was enhanced by the presence of 4-CP and the removal of the latter from aqueous solution was inhibited by the presence of phenol. It has been reported that easily removed pollutants, i.e., those with higher removal efficiencies, aid in the oxidation of more difficult to remove compounds [6]. Free radicals generated from the former-type compounds react with those of the other type, resulting in the

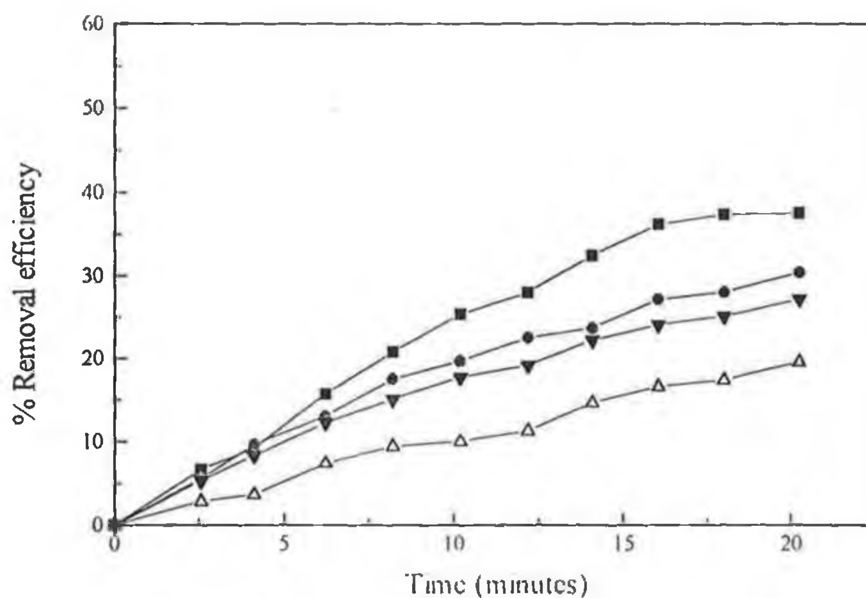


Figure 3.11: Removal of phenol and 4-CP at 70°C by native HRP.
■ 4-CP; ● phenol; ▼ 4-CP with 1mM phenol; ▲ phenol with 1mM 4-CP.

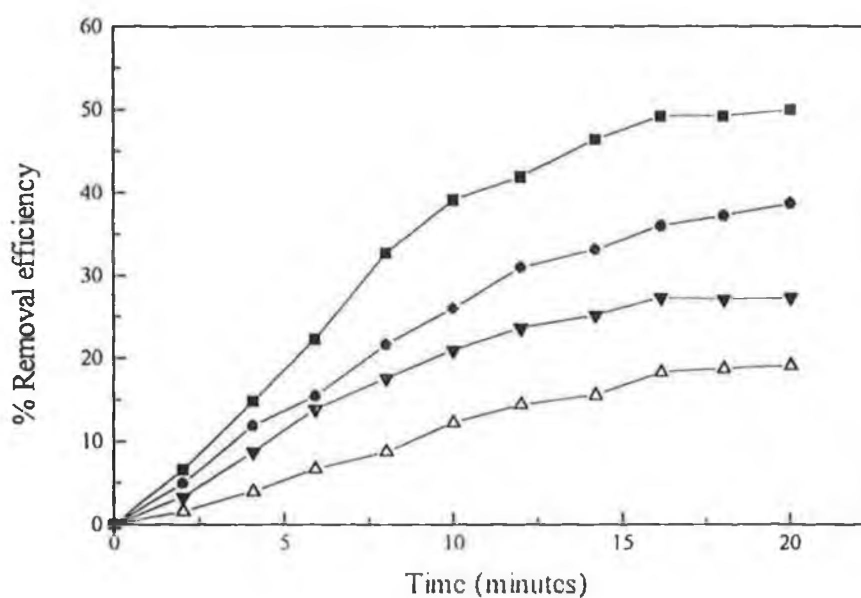


Figure 3.12: Removal of phenol and 4-CP by acetylated HRP at 70°C (as above)

production of high molecular weight insoluble mixed polymers that readily precipitate from solution. This same mechanism has been shown to work with non-phenol and non-amine type compounds [44] Naphthalene and azobenzene (both hazardous pollutants that do not react with HRP) were precipitated in the presence of 2,3-dimethoxyphenol or 8-hydroxyquinoline and the HRP system. Presumably, free radicals enzymatically produced from the latter two compounds attacked and precipitated the more difficult-to-remove compounds. In this case, it is quite possible that both phenol and 4-CP are equally reactive towards HRP, but that the oxidation products of phenol may have a lower combined molecular weight and therefore be fairly soluble in water; 4-CP reaction products may not possess the same degree of water solubility.

The discovery of enhanced enzymatic removal of poorly removed or non-removable compounds in mixtures of pollutants has important application [1]. Real industrial wastewaters contain many different pollutants. Here, even if only a few of them can be easily precipitated by HRP, the removal of others by the HRP system will be facilitated [11]. Nevertheless, the fact that 4-CP has been shown to be more readily oxidised (Figures 3.11 and 3.12) has in its own right important application. Due to the wide use of chlorine as a bleaching agent for chemically produced wood pulps in the pulp and paper industry, dilute pulp mill bleaching effluents contain undesirable levels of adsorbable organic halogens in the form of chlorophenols, chloroaliphatics, chlorocatechols, polymerised chloroaromatics etc. Chlorophenols are major intermediates of phenoxyalkanote herbicides and other pesticides which retain their toxic properties for an indefinite period since they easily form soil bound residues [24]. The effect of chlorinated compounds such as pentachlorophenol (PCP),

polychlorinated biphenyls (PCBs), chlorinated benzene etc released into the environment is an immediate concern for the entire population Increasingly stringent regulations are necessary if the levels of such compounds in the environment are to be reduced

Strategies towards directly enhancing an enzyme's ability to remove such compounds can only improve a system's performance up to a certain point The choice of a suitable reactor could further decrease the cost of the process Batch type reactors suffer from substrate saturation at the start of the reaction in the presence of excess peroxide [18]. In such systems, the enzyme is susceptible to inactivation as reaction intermediates can interfere with biocatalysis, although immobilisation techniques could alleviate this problem [19]. Nicell and colleagues reported that treating wastewater in a continuous stirred tank reactor (CSTR) gave greater removal than in batch systems. A high degree of aromatic conversion can be achieved in batch reactors using long retention times but at the price of high costs and difficult operation at a large scale [12, 38]. A continuous flow system was thus regarded as the only economically feasible option for treating contaminated waters, as reactant and enzyme concentration are lowered immediately upon entering the reactor; therefore inactivation of HRP is automatically reduced by the low stready state concentration of HRP in the reacting mixture. In addition, the concentration of peroxide can be maintained at a low level, thus reducing the possibility of Compound III formation [37] Catalytic turnovers [25] achieved in the continuous set-up were higher than those observed in batch reactors when adequate reaction time was allowed

3.4.10 Removal of a range of phenolics

Clearance tests in batch reactors were performed for a variety of phenolic substrates including parent phenol, chlorinated phenols and alkylphenols (Table 3.2). Data shows the concentration (mM) of each phenolic removed after a 20 minute exposure, by which time a levelling off period was visible in the HRP system. The reaction conditions used in this survey were not optimised for near or complete removal of phenolics but were standardised to determine the relative susceptibilities of these compounds to oxidation by native and derivative peroxidases. Incubation temperatures were 37 and 70^{0}C. In some cases, removal efficiencies were very high (4-chlorophenol and 2-naphthol) whereas 3-chlorophenol was less prone to enzyme-catalysed precipitation. Preliminary results indicated that complete removal of such substrates was feasible with prolonged exposure to the HRP system. For comparative purposes, the reaction time was limited to 20 minutes. The pH for optimum removal of all aromatic substrates was in the range 7.0-9.0 (which is close to the typical pH of coal conversion [9]) with the exception of 2-naphthol (pH 5.5).

Acetylated HRP appeared to possess an overall greater ability to oxidise phenolics than EG-NHS HRP at 70^{0}C. Certain compounds showed significant differences in removal efficiences; however, some compounds were found to be more difficult to precipitate from aqueous solutions than others, e.g. 3-CP. Of the eight aromatics studied, 4-CP was most readily removed from water. There are at least two explanations for the performance of 3-CP: the compound has a low reactivity towards the peroxidase or alternatively, 3-CP may be sufficiently reactive towards the enzyme but the products of its enzymatic oxidation may have a low molecular weight and be soluble in water.

Table 3.2

Concentration (mM) of phenol removed after 20 minutes by the HRP system.

Phenolic	pH	Nat. (37°C)	Nat. (70°C)	EG. (70°C)	AA (70°C)
Phenol	9	0.50	0.32	0.37	0.39
2-CP	8	0.63	0.41	0.57	0.55
3-CP	8	0.31	0.08	0.27	0.27
4-CP	8	0.64	0.42	0.58	0.54
2-naphthol	5.5	0.55	0.30	0.44	0.45
Guaiacol	6	0.52	0.31	0.43	0.47
2-cresol	7	0.57	0.34	0.47	0.47
4-cresol	7	0.56	0.28	0.44	0.45

Addition of an easily removable compound (the oxidation products of which are obviously water insoluble) such as 4-CP might result in the formation of high molecular weight mixed polymers which are nearly insoluble in aqueous media. This approach could indeed facilitate the removal of such difficult aromatic hydroxy compounds by HRP in water. Certain phenols cannot be enzymatically precipitated at all (e.g. nitrophenols); however, co-polymerisation would seem to be the best approach.

Aromatics with electron-donating substituents (methyl groups) at the meta-position favour removal over those with substituents at the ortho- or para- positions. The opposite applies for electron withdrawing groups (e.g. Cl) [6]. Results have shown this to be true as 3-CP was less susceptible to enzymatic oxidation than 2- or 4-CP. Ingols *et al.* showed that resistance to biodegradation increased with increasing

substitution of Cl atoms on the aromatic ring and with their position. For example, phenol has been shown to be more readily oxidised than pentachlorophenol [45]. In the same way, different sources of peroxidase enzymes were found to influence particular oxidative reactions differently. Manganese peroxidase (MnP) was more active towards pentachlorophenol than phenol, the opposite applied for ligin peroxidase (LiP) [46]. Some synergism between the two peroxidases was observed in the degradation of various chlorinated phenolic compounds.

3.4.11. Conclusion

It has been demonstrated that modification of HRP with amino specific bifunctional and monofunctional chemical reagents can substantially increase the rate of phenolic removal from aqueous solutions at high temperatures. Complete recovery of enzyme activity was apparent after modification. These stabilisations persisted on storage at low temperatures. It is possible that the presence of intramolecular crosslinks in the ethylene glycol derivative of HRP in some way hinders the enzyme's ability to catalyse the oxidation of aromatic hydroxy compounds. The acetylating agent (AA-NHS) cannot form such links, stabilisation in this case is possibly due to the charge neutralisation (3 lysines modified). It is therefore plausible that the absence of such crosslinks resulted in the uninterrupted movement of aromatic molecules to and from the enzyme's active centre, even though EG-NHS is capable of forming a considerably sized link of 14Å length. Nevertheless, this relatively straightforward procedure makes the HRP-catalysed removal of aromatics more feasible in view of the costs involved.

There is a possibility of retarding enzyme inactivation by controlling the rate of hydrogen peroxide addition to the reaction mixture. Further improvement could be achieved by using an immobilisation technique or through modification of the enzyme's carbohydrate moiety (which has been left intact after succinimide modification).

Previous authors have shown that the use of additives such as polyethylene glycol and gelatin can increase HRP's efficiency in removing aromatics. This strategy could be used in conjunction with chemical modification, thus offering a form of peroxidase with enhanced oxidative capabilities at ambient and high tempertures. Incorporation of such a derivative in a continuous flow reactor system whereby the immobilised activity could deliver substantial improvements in water detoxification.

Proposals such as these could possibly result in the production of an enzyme with tolerance comparable to that of a bacterial or fungal cell. The genes of this potential HRP derivative could then be cloned into efficient enzyme-producing microorganisms, as recombinant peroxidases have been previously shown to improve phenol removal [47], resulting in the formation of harmless end products such as CO_2, H_2O and native peroxidase.

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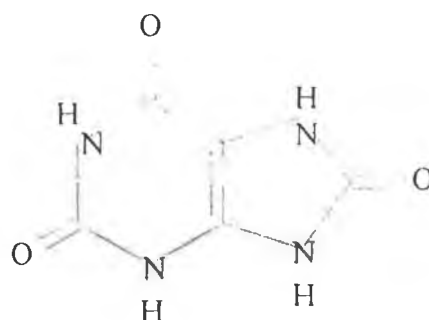
CHAPTER 4

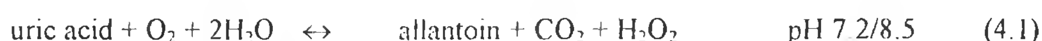
THE USE OF HORSERADISH PEROXIDASE IN A POLYMER-BASED SENSOR FOR DETECTING URIC ACID

4.1 INTRODUCTION

Uric acid (2,6,8-trihydroxypurine), a primary end product of purine metabolism, is a constituent of many body fluids It consists of fused pyrimidine and imidazole rings. With the exception of urea, it represent the most significant fraction of non-protein nitrogen in uricotelic organisms such as man. As uric acid (urate) is the chief end-product of purine metabolism, its measurement remains the most important means of assessing a range of disorders associated with altered purine metabolism, most notably gout and hyperuricaemia [1] Other medical conditions, such as leukaemia and pneumonia, have been associated with enhanced urate levels. Additionally, the activity of chemotherapeutic drugs can be assessed by monitoring the uric acid, as increased nucleoprotein degradation results in elevated purine excretion. Concurrent with its



clinical significance, an entire spectrum of methodologies has evolved for its determination. As early as 1894, Offer reported on the production of a blue colour (tungsten blue) by uric acid in alkaline solution with phosphotungstic acid [2, 3]. Numerous modifications on this colorimetric procedure have ensued. The poisonous nature of the oxidising agents involved and susceptibility to other reducing agents has restricted the analytical utility of colorimetric methods [3]. Bulger and Johns introduced uricase to enhance the selectivity of uric acid determinations [4]. Uricase specifically catalyses the scission of the purine ring (see above).


uric acid + O_2 + $2H_2O$ $\leftrightarrow$ allantoin + CO_2 + H_2O_2 pH 7.2/8.5 (4.1)

uric acid + O_2 + $2H_2O$ $\leftrightarrow$ urea + allantoin pH 7.2 (4.2)

uric acid + O_2 + $2H_2O$ $\leftrightarrow$ urea + allantoin + alloxanic acid pH 9.0 (4.3)

It is known that the chief products of urate oxidation in acidic media are alloxan and urea. Allantoin and its enzymatically reduced product, allantoic acid (both known as ureides), account for 70-80% of the organic nitrogen in the xylem of nitrogen-fixing soybean plants and other species of tropical grain legumes [5]. However, many variations on the oxidation of uric acid by uricase have evolved, generally following the same principles.

Uric acid has an absorption peak in the region 290-293nm [3]. Absorption in this range is due to the chromophore existing at the C(4)=C(5) bond. As this is the site of uricase activity, no absorption is observed for its oxidation products. The decrease in absorbance is directly proportional to substrate concentration [6]. Practical considerations such as deproteinisation of the sample and the use of expensive equipment have limited spectroscopic uricase methods. Hydrogen peroxide liberated from the oxidative decarboxylation of urate by uricase (Equation 1) has been used to indirectly quantify uric acid [7]. This colorimetric method, by Lorentz and Berndt, decribes the oxidation of *o*-dianisidine by peroxidase in the presence of H_2O_2 to a brown quinonediimine (3,3-dimethoxy-4,4-diimino-diphenoquinone). A linear response in colour development up to 20μg was observed, corresponding to 8mg of substrate per 100ml of serum.

Electrochemical sensors have been previously shown to be particularly suited to the determination of uric acid [8-10]. The first amperometric method for its quantitative determination in biological fluids was reported by Nanjo and Guilbault

[11]. Using a platinum electrode, which was covered with a thin layer of immobilised uricase, the disappearance of dissolved oxygen (no measurable amounts of H_2O_2 were detected) was used as a means of measuring uric acid present (applied potential: -0.6V vs. Ag/AgCl). A suggested problem was that H_2O_2 complexation to allantoin occurred, possibly due to the choice of background electrolyte employed. Keedy and Vadgama, also using a platinum working electrode, were able to measure uric acid via peroxide liberation [12].

Uric acid has also been shown to readily adsorb onto carbon paste electrode surfaces [13-14], which has led to the development of a controlled adsorption process for its selective determination in flowing streams [15]. Carbon, in the form of graphite, is a relatively inexpensive and versatile material and is particularly suitable for the fabrication of electrodes. Although uric acid is electroactive at such surfaces, its oxidation requires undesirably high overvoltages (typically greater than +0.4V) [16]. Preanodisation of carbon paste electrodes in alkaline media at 1.4V vs. SCE was reported to enhance sensitivity [14]. Such electrochemical pretreatment strategies are unique to carbon-based electrodes. Such improvements are thought to be due to the generation of hydrophilic electron transfer mediating groups. Oxidation of the electrode material also results in a lowering of the overpotential and increased wettability [14]. Coulometry, using a porous carbon felt electrode, has been applied to urate determination in human urine. The analyte of interest was electrolysed with nearly 100% current efficiency at the working surface [17].

The disappearance of O_2 [11], H_2O_2 [12] or CO_2 production [18] have been exploited for urate detecting. In the first situation, dissolved O_2 may also be consumed by compounds such as ascorbic acid and thiol-containing substances [19]. Moreover,

ascorbate and thiols may also react with enzymatically liberated H_2O_2. Various systems have exploited the anodic electroactivity of H_2O_2 in urate sensing [20-22], regardless of the known disadvantages. The elimination of interferences was achieved by Kulys *et al.* when HRP served as a catalyst for the reaction between H_2O_2 and hexacyanoferrate(II) followed by reduction of the resulting hexacyanoferrate(III) at 0V vs. Ag/AgCl [23]. Tatsuma *et al.* noted that the anodic current (potential: 0.5V) using a bienzyme electrode was due primarily from urate oxidation and partially to H_2O_2 oxidation. Its detection in peroxidase-based systems can be affected as oxidised urate can donate electrons to HRP, thus reducing its capacity to detect biocatalytically generated peroxide [24].

The feasibility of employing HRP for amperometric detection of organic peroxides is well known [25]. In 1979, direct electron transfer between carbon black and HRP's active site was reported [26]. Gorton has reviewed the increasing number of reports on mediatorless electrodes where charge transfer occurs between the electrode and enzyme only if the enzyme is in intimate contact with the conducting surface [27]. A mediatorless bienzyme sensor for glucose has been reported even though the nature of electron transfer was unclear [28], although Wollenberger *et al.* have suggested the role of surface functionalities in reagentless electron mediation [29].

In this chapter, the development and application of a novel reagentless bienzyme carbon paste electrode for the indirect amperometric determination of uric acid is described via the biocatalytic production of H_2O_2. The co-immobilisation of HRP and uricase in carbon paste in the absence of an added electron transfer mediator, coupled with the electropolymerisation of *o*-aminophenol at the working surface of the

electrode acting as a conducting polymer, has proven to be an interesting alternative to conventional methods for constructing biosensors. Modified surfaces are of interest from an analytical viewpoint in that they may be used to enhance analyte permeability, while at the same time reducing interference effects. The sensor was also examined for its applicability to uric acid quantification in human serum.

4.2 EXPERIMENTAL

4.2.1 Materials

Horseradish peroxidase (HRP, E.C 1.11.1.7, type VI A), uric acid, uricase (uric acid oxidase, E.C.1.7.3.3), allopurinol, ascorbic acid, bilirubin, cellulose membrane dialysis tubing (12000 Da cutoff) and EDTA were purchased from Sigma Chemicals.

Hydrogen peroxide was obtained from Aldrich Chemicals.

o-Aminophenol was purchased from Fluka Chemika.

Spectroscopic grade graphite powder was purchased from Ultra Carbon, Dicoex, Bilbao, Spain, and paraffin oil from Uvasol, Merck, Bilbao, Spain.

All other reagents (e.g. sulphuric acid, sodium hydroxide) were of analytical grade.

Serum samples were obtained from Bio-Quim Laboratories, Oviedo, Asturias, Spain.

4.2.2. Equipment

Cyclic voltammetry and amperometry were performed using a Metrohm E612 VA Scanner in conjunction with an E641 VA detector.

A Linseis LY1600 *x-y* plotter was used to record all cyclic voltammograms.

A Linseis L6012B recorder was used for amperometric measurements.

Static measurements were carried out in a 20ml Metrohm glass cell, incorporating a conventional three electrode system.

The working solution in the glass cell was magnetically agitated at 500rpm. The rate of agitation was measured using an electronic speed meter (Heidolph 2001).

A piston-type carbon paste electrode with a Teflon body and stainless steel contact acted as the working electrode. The active surface was a disk with geometric proportions of 7.1mm^2.

A silver/silver chloride/saturated potassium chloride electrode acted as the reference electrode. A platinum wire served as the auxillary (counter) electrode.

The Flow Injection Analysis (FIA) consisted of a twelve cylinder Spetec Perimax 12 peristaltic pump and a six-port rotary valve (Rheodyne 7125) as carrier propulsion and injection systems, respectively.

Analysis was carried out in a home made thin-layer flow cell (Kissinger design) [30], equipped with a working electrode of geometric proportions as described previously.

A downstream compartment connected to the thin layer cell outlet contained the reference electrode incorporating a low resistance liquid junction and a stainless steel waste tube acting as a counter electrode.

The kinetic parameters K_m and V_{max} were calculated using the Enzfitter programme (Biosoft, Cambridge, U.K.).

4.3. METHODS

4.3.1. Carbon paste preparation

Carbon paste was prepared by thoroughly mixing 1.8ml of paraffin oil with 5g of spectroscopic grade graphite powder using a pestle and mortar for approximately 30 minutes, at which stage the mixture was uniformly wetted and homogeneous. Gritty graphite types were avoided as they can yield irregular electrode surfaces, resulting in lower peak currents. The mulling agent should be of low volatility, possess very low solubility in the medium of interest and contain no electroactive impurities.

4.3.2. Electrode preparation

Uricase, HRP and ferrocene (used in preliminary trials) were added to unmodified paste and mixed thoroughly for 30 minutes. To pack the electrode, a small but excess amount of enzyme-modified carbon paste was pressed into the electrode well (depth of approximately 2mm). The electrode was then inverted and the modified paste pressed onto a flat sheet of white paper. The surface of the electrode was then smoothed by moving the electrode in a circular motion. It was important not to apply too much pressure as the graphite-oil mixture can be separated, resulting in high resistance contact between the paste and metal contact. Any excess paste remaining on the teflon body was wiped away with a lens tissue, taking care not to damage the carbon paste surface. Electrodes (with no enzymes incorporated) could be renewed by removing a small layer of carbon paste from the surface and re-packing as before. If a completely new electrode was required, the electrode well was cleaned out in an ultrasonic bath, dried and re-packed.

4.3.3. Polymer film preparation

Electropolymerisation of the monomer (*o*-aminophenol) at the working surface area of the electrode was carried out in 0.1M acetic acid, pH 5.0, in the potential range 0-0.7V for 10 minutes (or a defined number of individual polymer layers) at a scan rate of 50mV s^{-1}. The acetic acid medium was purged with helium to remove molecular oxygen present. The quality of the electro-deposited layer was verified by carrying out a cyclic voltammogram scan in the afore mentioned potential range. The electrode was then rinsed for a short period of time in 0.1M phosphate buffer (pH dependent on the working background electrolyte).

In both static and flow injection systems, the assembled cell was equilibrated with the supporting or background electrolyte (which was not purged with helium as molecular oxygen acts as a natural cofactor to the uricase reaction) while applying the working potential. Amperometric signals were recorded after the transient signal decayed.

4.4 RESULTS AND DISCUSSION

4.4.1. Cyclic voltammetry studies on poly(*o*-aminophenol)

Cyclic voltammetry (CV) was performed on bare and polymer-modified electrodes to examine the permselective and electrocatalytic properties of the electropolymerised polymer membrane. Working electrodes were composed of 100% carbon paste (no enzyme present). Figures 4.1 and 4.2 depict the electro-oxidation of uric acid to allantoin at bare and modified electrodes, respectively.

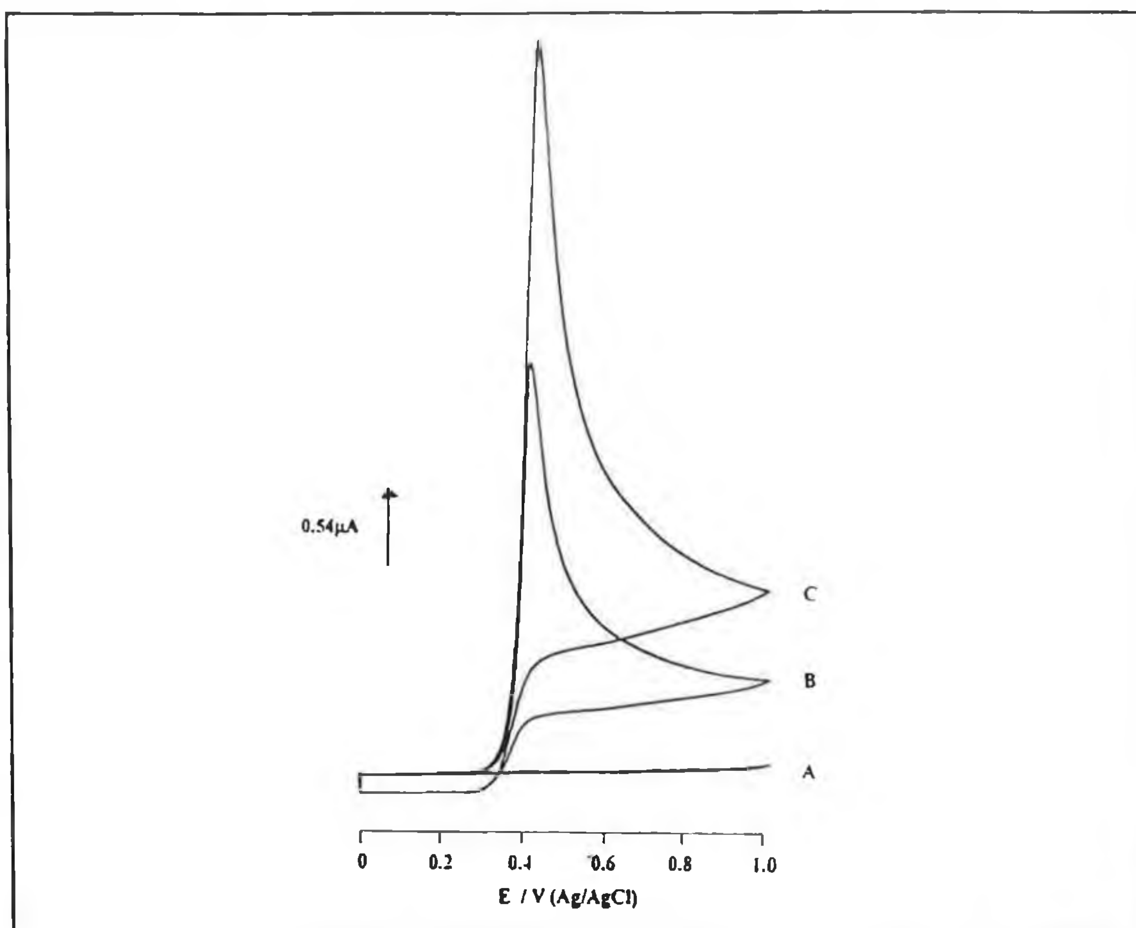


Figure 4.1: Cyclic voltammetric behaviour of uric acid at bare carbon paste electrode (CPE). A- phosphate buffer pH 6.5; B- 0.5mM uric acid; C- 1.0mM uric acid. Sensitivity- 5µA, Scan range- 0→ +1.0V, Scan rate- 50mVs^{-1}.

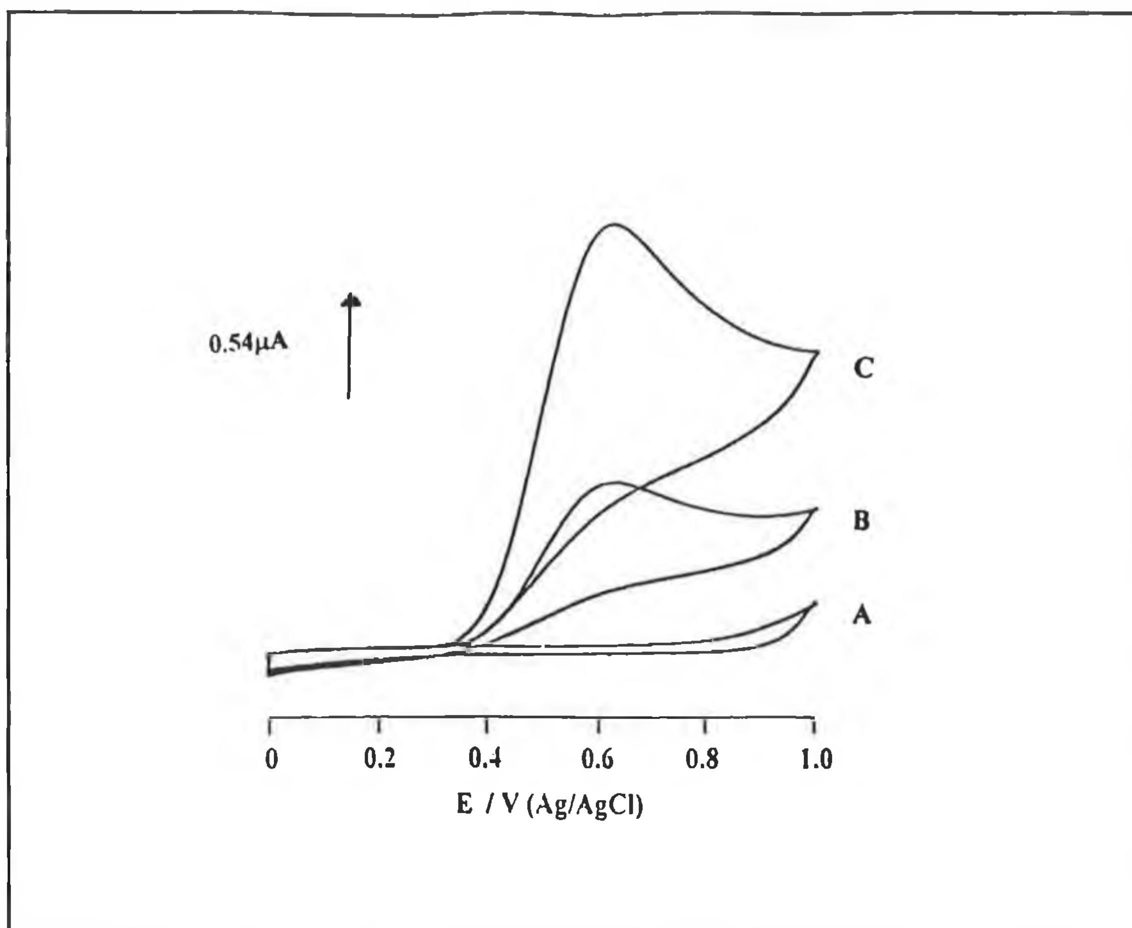


Figure 4.2: Cyclic voltammetric behaviour of uric acid at a poly(*o*-aminophenol)-modified carbon paste electrode (CPE). A- phosphate buffer pH 6.5; B- 0.5mM uric acid; C- 1.0mM uric acid. Sensitivity- 5μA; Scan range- 0→+1.0V; Scan rate- 50mVs^{-1}.

Oxidation type reactions of reduced biomolecules such as uric acid involve anodic (oxidation) processes, the magnitude of this current being proportional to the concentration of analyte present. This process occurred using both electrodes. At an almost identical applied potential (0.4V), the anodic current produced at the poly(*o*-aminophenol) modified electrode was approximately half of that observed at the bare carbon paste electrode. This was expected as membrane-covered electrodes generally yield currents of restricted magnitude due to the imposition of additional diffusion barriers. The ratio between the current at the polymer-coated electrode and that of the

bare one is a measure of coating permeability. Under such conditions, the cyclic voltammograms of uric acid did not exhibit any cathodic peaks (reduction current), therefore the oxidation process was considered to be irreversible. It was noted that as the cyclic scan rate was increased, the oxidation potential value (E_p) shifted in the anodic direction. Similar findings were reported by Gilmartin *et al.* [31]. Cai and colleagues pointed out that by applying a preanodisation potential (1.4V) for 40 seconds, the overall performance of a carbon paste electrode (CPE) for detecting uric acid could be substantially improved [14]. Changes include enhanced sensitivity, improvement in the shape of the signal and a shift of the oxidation potential in the cathodic direction. When using a glassy carbon electrode instead of a CPE, the same effects could be observed although peak currents at CPEs were notably less due to slower rates of charge transfer due to layers of non-conducting pasting liquid at the electrode surface [32]. This problem could be overcome by preanodisation [33].

As previously stated, cyclic voltammetry of uric acid did not produce any reduction peaks. Goyal *et al.* reported on a single oxidation peak and on the reverse negative sweep, two reduction peaks. One of these peaks was shown to form a quasi-reversible couple with the lone oxidation peak [34]. The authors postulated that both cathodic peaks represented urea and 6-thioalloxan. The greater susceptibility of uric acid to electrochemical oxidation compared with 6-thiouric acid (purine) was believed to be due to the mercapto group, which can easily undergo oxidation.

It was deduced that direct electron transfer occurred between the uric acid and the electrode. The difference in the shape of the oxidation waves suggested that the polymer-coated electrode did in fact suffer from mass transfer limitations. Such polymer films have advantages over more conventional films [35].

4.4.2. Mediator studies

Direct electron transfer is rarely encountered with high molecular weight enzymes whose active centres lie deeply buried in the polypeptide structure. Nevertheless, a knowledge of the electron transfer mechanism is fundamental in the development of amperometric enzyme electrodes. One must distinguish between oxidoreductases with prosthetic groups bound tightly to the protein matrix (flavoproteins) and oxidoreductases without an integrated cofactor (NAD^+-dependent dehydrogenases) whose substrate can be used as a redox mediator. Steric properties of some oxidoreductases decrease the accessibility of the active site. Hence, mechanisms involving natural or artificial electroactive compounds, which act as "electron shuttles" or mediators, must provide redox coupling between the electrode and the redox centre in the biological component [36].

A study was therefore undertaken to examine the necessity for an electron mediator [27]. CPEs incorporating 5% w/w ferrocene (and 85% paste, 7% uricase and 3% HRP) exhibited slow responses and less than desirable sensitivity in response to uric acid (Figure 4.3). Peaks in current were followed by slow, gradual decreases to the existing baseline level. This was thought to be due to the diffusion of ferricinium ions to the bulk solution. Coating the electrode's working surface area with a cation-exchange material such as Nafion could prevent such a diffusional effect [37]. Non-ferrocene containing CPEs did not exhibit this behaviour.

It would appear that the nature of the electrode's response is independent of mediator-type compounds and that direct electron transfer possibly exists between the the enzyme(s) and the electrode (carbon paste). More direct evidence of this hypothesis is given later. However, in light of the above result, it is possible that HRP's

heme group is exposed and orientated in the direction of the electrode, thus facilitating the direct electron transfer process Electron transfer must run over a distance of at least 1.2nm, the minimum distance between the electrode and the plane of closest approach to the heme edge. These findings do prove that a well orientated and positioned enzyme increases the probability of rapid electron transfer, a prerequisite for umediated biosensing. Although direct electron transfer based on small redox proteins and modified surfaces has not been reported to any great extent, the understanding of possible electron transfer mechanisms is important for systems incorporating proteins with catalytic activity.

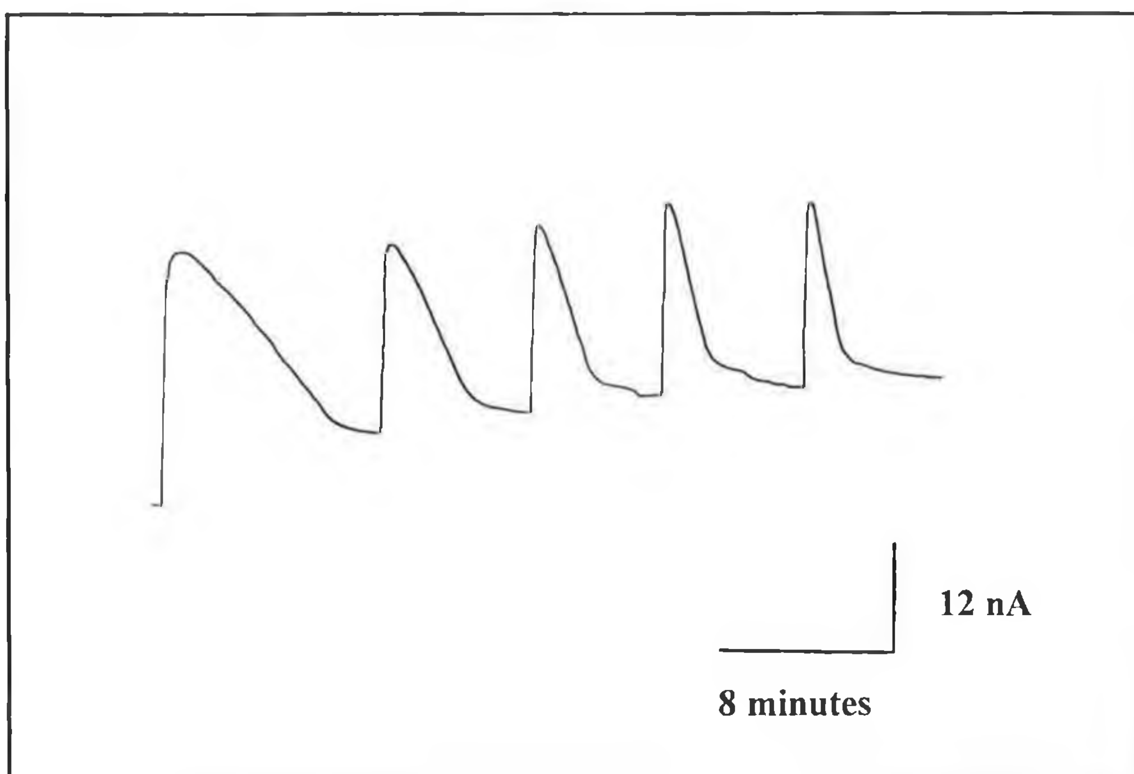


Figure 4.3: Typical amperometric responses obtained when ferrocene was included in the carbon paste. Electrode composition: 85% carbon paste, 5% ferrocene; 7% uricase and 3% HRP. Responses to 1×10^{-4}M injections of substrate.

4.4.3 Effect of enzyme loading on sensor response

The variation of enzyme loading is a means for determining the minimum amount of enzyme required for maximum sensitivity. This reveals the magnitude of the enzyme reserve of diffusion-controlled sensors.

An investigation was performed to elucidate the optimum ratio of HRP and uricase required in the paste mixture for immobilisation. The percentage of immobilised enzyme in the paste in all preliminary experiments did not exceed 15% w/w. Greater percentages did not improve the response characteristics of the electrode. Different paste mixtures were prepared with either HRP or uricase maintained at a fixed level while the amount of the other was varied. Figure 4.4 illustrates the responses to 3×10^{-4}M uric acid for various paste compositions. As the proportion of uricase in the paste was increased, sensitivity also increased. Response times were also noted to decrease with increasing amounts of uricase up to a certain point, after which they were stable. Carbon paste incorporating 3% w/w HRP gave rise to the greatest sensitivity; higher percentages gave no further improvement. The relationship that exists between the sensitivity of an electrode and the immobilised biocomponent depends primarily on the affinity of the oxidase (uricase) for its specific substrate (uric acid). Generally, in conventional enzymology where coupled enzymatic reactions are used in solution for determining the concentration of a target analyte, it is recommended that a higher auxilliary enzyme activity be maintained compared with that of the detecting one, thus preventing the response of the first enzyme being limited [38]. It is neccessary for the auxilliary enzyme to oxidise as much of the intermediate metabolite in the microenvironment as possible into substances that can be detected by an electrochemical transducer. In addition, it is unnecessary to load higher activity into

the paste if no improvement in sensitivity is achieved. Thus a paste mixture of 7% w/w uricase and 3% w/w HRP was subsequently used. The co-immobilisation of enzymes

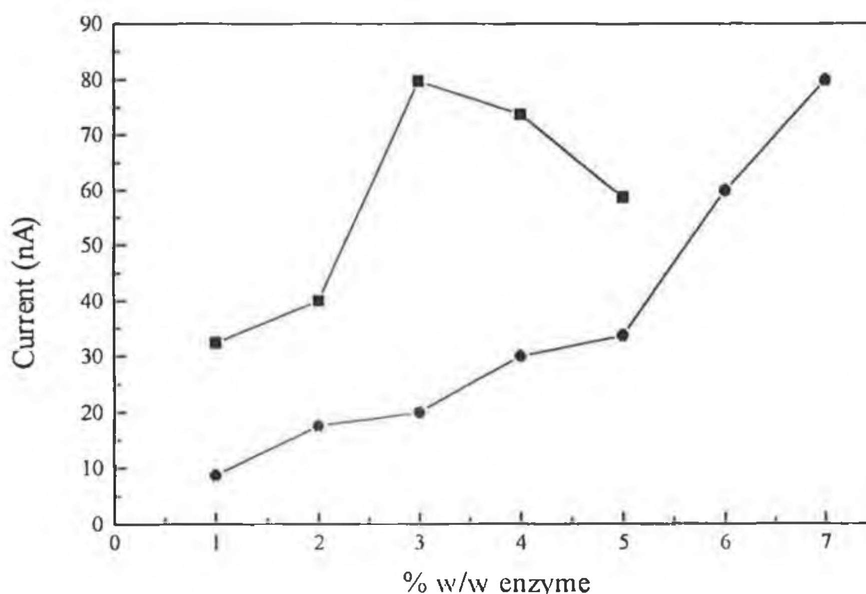


Figure 4.4: Effect of enzyme loading on sensor performance.
■ uricase fixed at 7% w/w; ● HRP fixed at 3% w/w; Measurements were performed in 0.1M phosphate buffer, pH 6.5, on addition of 3 $\times 10^{-4}$M substrate.

on a support matrix poses a number of problems. Commercially available enzymes generally have different specific activities that are difficult to modify and the yield of immobilisation (depending on several parameters such as the nature of the support, the enzyme composition and the immobilisation procedure) could be completely different for the two enzymes involved. Also, as immobilisation of two biocatalysts is carried out in one step, a competition may occur between the enzymes for the available binding sites of the activated matrix.

The enzyme loading, to a major extent, determines the overall stability of a biosensor. An enzyme reserve can be built up by employing more enzyme activity in front of the electrochemical probe than is minimally required to achieve diffusion control. As long as this reserve lasts, the sensitivity will remain essentially constant. This is relevant only to sensors for substrate determinations. If inhibitors of biosensing catalytic reactions are to be measured, kinetic control is desirable This permits the enzyme loading to be varied only with in a relatively narrow range. It is practical to use a sensor intermittently for analysis, rather than prolonged continuous use. Exceptions to this rule are biosensors intended for *in situ* applications where an extended usage period is imperative. The Fuji Electric (Japan) UA-300 analyser uses a uricase membrane fixed to a hydrogen peroxide-selective layer, as little as 20μl of blood serum is required and a sample throughput of 50-60 per hour with a relative precision of 3.0% [39].

The method for preparing the graphite/paraffin oil mixture was similar to that of Cai *et al.* [14]. The procedure for incorporating HRP and uricase into the carbon paste was based on that of Wollenberger *et al.* [29] and Dominguéz-Sanchez *et al.* [40]. The carbohydrate side chains of HRP (18% of the enzyme) allow the enzyme to be immobilised onto an insoluble matrix such as carbon paste [41]. It may be true that higher responses are obtained in response to substrate additions when enzymes are added to graphite prior to adding paraffin oil [27]. As it is well known, dry graphites have very high capacitive current (one of the original reasons for producing paste electrodes was to lower the residual current) [42]. Obviously the magnitude of a carbon paste electrode's current drops with an increasing quantity of the pasting liquid in the graphite, however, this is often accompanied by a decrease in sensitivity of the electrode. CPEs incorporating paraffin oil as the mulling agent (as opposed to such

materials as silicone oil, bromonaphthalene and nujol [mineral oil]), have the widest useful potential range [43].

Most CPEs exhibit background current of 200nA or even lower over a wide potential range [44]. Methods proposed to decrease such currents are based on removing adsorbed electroactive species, in particular oxygen. Prior to paste preparation, graphite can be pretreated by exposure to high temperatures with subsequent impregnation of the pores by cerasin wax, by nitrogen saturation or hydrogen reduction of preheated carbon [45]. CPEs suffer substantially from the presence of oxygen entrapped in the paste, brought in by the carbon molecules but also by the preparation of the paste [42]. In voltammetric measurements, oxygen seriously interferes with detection when the applied potential is in the negative range. Parasitic oxygen signals can be partially suppressed by using highly lipophilic pasting liquid such as tricresyl phosphate [46]. The heterogeneity of carbon paste is disadvantageous with respect to applications of CPEs in nonaqueous media, because the electrode material obviously disintegrates [42]. Admixing a surfactant to the paste may lead to a greater level of stability in the paste in some solvent systems. Silicone oil-based carbon paste materials can be exposed to media such methanol, acetonitrile, dimethylformamide or dimethylsulphoxide (50% mixtures with water) [47]. The first reported CPEs incorporating an enzyme appeared in 1988, where glucose oxidase (GOD) was directly blended into the organic phase consisting of graphite powder and silicon oil [48]. Since then, immobilisation of an enzyme into an organic phase composite electrode (carbon paste, carbon cement, carbon epoxy resins) has become an increasingly popular way for the construction of enzyme electrodes. Peroxidase-modified CPEs have been used

for the determination of H_2O_2 and other organic peroxides, with both soluble and immobilised electron donors acting as mediators [25, 27, 40].

4.4.4. Amperometric studies on poly(*o*-aminophenol)

The permeability characteristics of poly(*o*-aminophenol) acting as a conducting polymer were compared to those of a cellulose dialysis membrane (12000Da cutoff) by amperometry. An optimised enzyme loading was used for electrode designs. The polymer modified electrode (bienzyme reaction) displayed a higher maximum enzyme reaction rate (V_{max} of 3.17×10^{-1}min^{-1}) compared to that of the cellulose membrane encapsulated electrode (V_{max}-1.91×10^{-1}min^{-1}). The K_m of the latter electrode was 1.99×10^{-3}mol which was slightly higher than that of the polymer coated electrode (K_m-1.92×10^{-3}mol). The higher K_m value would suggest mass transfer limitations between the enzyme layer and the analyte solution (Figure 4.5). The parameter which describes this behaviour is referred to in chemical engineering literature as the Damkoehler number [49];

$$D_a = \frac{V_{max}}{[K_m]\frac{D}{\delta}}$$

where V_{max} is the maximum rate of the homogenous reaction rate and K_m is the Michaelis constant (assuming that the enzyme(s) obey(s) Michaelis-Menten kinetics). For a D_a value of <0.1, the reaction is catalysis-controlled, while for values greater than 10, the reaction is controlled by mass transfer. As stated above, mass transfer limitations cause the K_m value to increase; however, increased oxygen concentrations also cause this effect.

If an enzyme electrode was actually operated under kinetically controlled conditions, the current-concentration relationship would be non-linear and a useful range of less than one magnitude would result However, such sensors are operated with a membrane between the enzyme and solution. This provides a barrier and a response proportional to the diffusional flux which is not limited by enzyme kinetics.

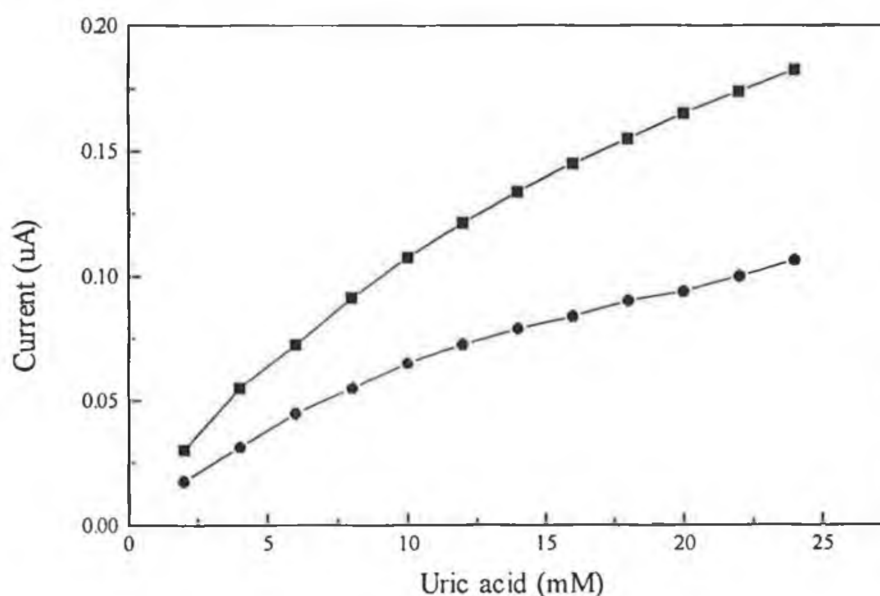


Figure 4.5: Dependence of response on the type of membrane encapsulation. ■ Poly(*o*-aminophenol) CPE; ● Cellulose membrane CPE; Applied potential - +0.05V.

This can be a factor when the enzyme's activity becomes too low. This is the reason why an amperometric electrode's response remains constant for an extended period. The response of a biosensor is independent of enzyme activity as long as the bio-activity is high enough [50]. However, the enzyme decays gradually and reaches a point where the response becomes kinetically controlled. At this point, the sensor response is no longer constant.

Hydrolysis of membrane films has been reported to increase porosity [51]. A similar idea was investigated for poly(*o*-aminophenol). On storage at 25^0C in 0.1M phosphate buffer, pH 7.5, amperometric responses to standard additions of urate increased by approximately 150% after 2 hours. The enzymatic production of peroxide due to uricase biocatalysis may have altered the selectivity characteristics of the polymer in some way, causing an increase in film permeability with time. Such increases in signal size remained for 24 hours followed by a return to initial signal intensities; this phenomenon was probably due to a variety of causes.

The effect of increasing the concentration of monomer during the electropolymerisation process was examined. In quiescent solution, an increasing concentration resulted in more intense signals to injections of 5 $\times10^{-3}$M uric acid standards (Figure 4.6).

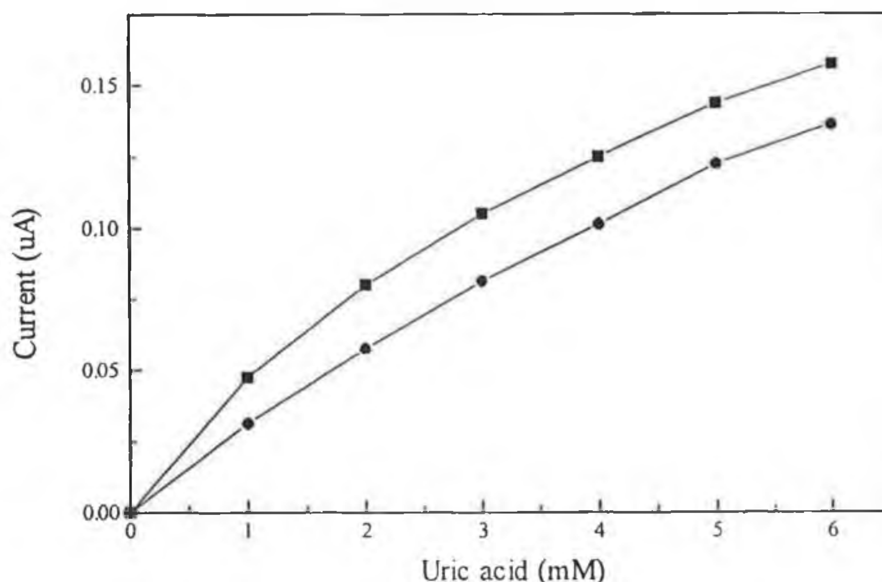


Figure 4.6: Effect of monomer concentration on biosensor response.
■ 5 $\times10^{-3}$M *o*-aminophenol; ● 1 $\times10^{-4}$M *o*-aminophenol; Applied potential - +0.05V.

This effect was also observed when the sensor was incorporated into a flowing stream set-up. Faster response times were recorded (24 seconds for $t_{95\%}$ of signal) for a polymer built from 5 $\times 10^{-4}$M *o*-aminophenol as opposed to 39 seconds for a 10-fold increase in the concentration of monomer (flow rate: 1ml min^{-1}). Operation at a higher flow rate of 2 ml min^{-1} resulted in a 42% reduction in response times. These results suggested that a rapid replenishment of the analyte solution from the surface of the electrode occurred, i.e. lack of analyte trapping. Another origin of the difference in response between monomer concentrations is that the faster the sample plug passes the electrode, the smaller the fraction of consumed sample in the enzyme layer at the electrode surface (kinetic limitations). The parameters affecting FIA of uric acid are discussed later. However, fast response times are an important prerequisite in flowing stream set-ups; typically the quicker the response, the smaller the signal.

In electrochemical polymerisation, the material formed will be in the oxidised form. Reduction of the polymer can be carried out either electrochemically (with the potential kept at the reduction potential of the polymer) or chemically (whereby the film is washed with a reducing agent such as ammonia). It has been demonstrated that the use of polymer coating to produce modified surfaces provides certain advantages during analysis [52]. Perhaps the most significant of these is that multilayered, dynamic, polymer coatings provide a three dimensional reaction zone at the electrode surface. This gives rise to an increase in the flux of reactions that can occur, which in turn increases sensitivity. Historically, the first polymerisation processes carried out by electrochemical means were indirect as they involved production of an initiator at the electrode and the remainder of the process occurred in solution. A description of such

processes and other electropolymerisation schemes was the subject of a recent review by Beck [53].

The polymer film was formed at the CPE surface by electrodeposition (Figures 4.7A and 4.7B) Polymerisation of *o*-aminophenol continues until the surface is completely covered, which is signalled by the current decreasing to a minimum as the monomer cannot penetrate this film [54] This polymer is required only for retaining the immobilised enzyme deposition, thus it was not mandatory to assure complete coverage of the elecrode surface [52]. Nyugen *et al.* pointed out that if less than a

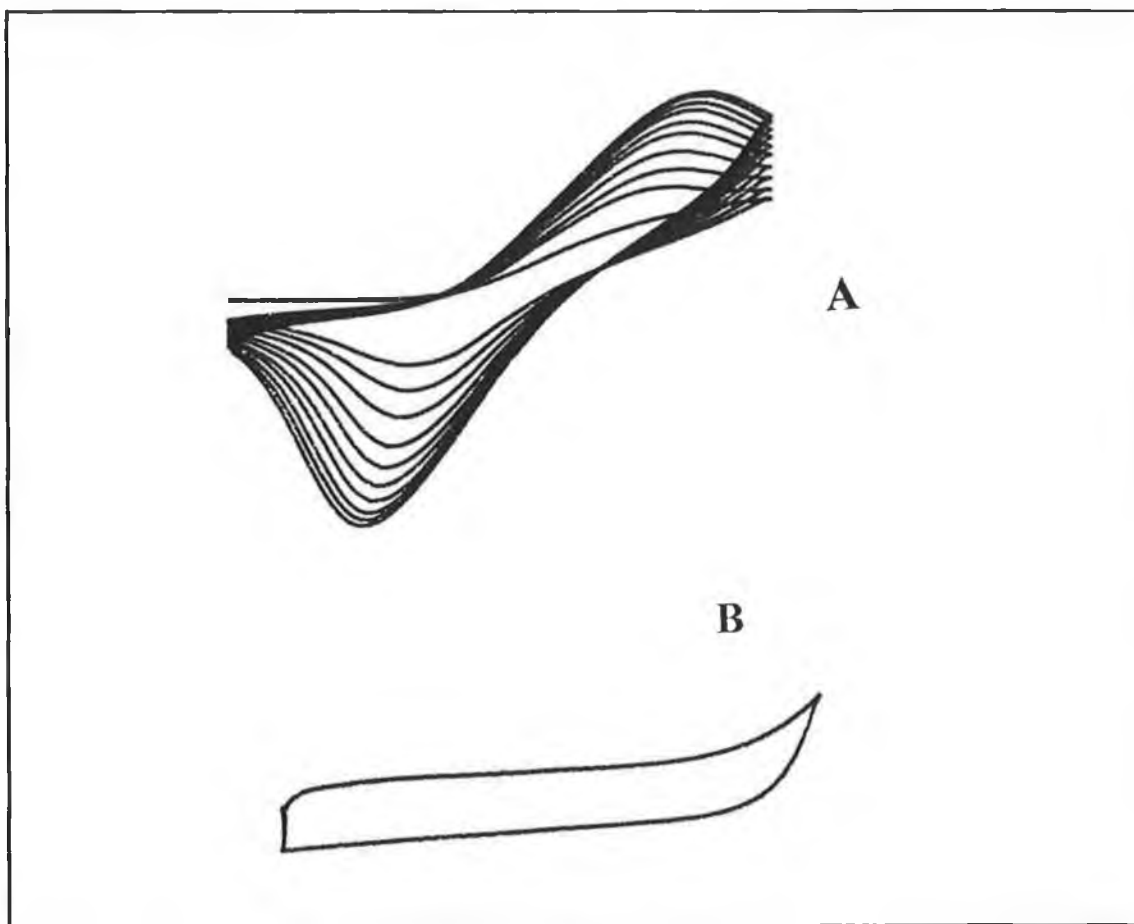


Figure 4.7: **(A)** Polymerisation of 5 $\times 10^{-3}$M *o*-aminophenol 0.1M acetic acid, pH 5.5, at the working surface of the CPE. **(B)** Single cyclic scan verifying successful polymerisation (carried out in background electrolyte. Scan range - 0$\rightarrow$ +0.7V, Scan rate 50mVs^{-1}, Sensitivity 0.5μA.

monolayer of polymer was formed over the working surface, the immobilised enzyme(s) could still be retained and, more importantly, the electrode's recovery and response was improved [55]. This compares favourably with the findings above.Growth in the biosensor field is expected to include substantial development in biosensors that incorporate polymer films in their design. Frequently, incorporation of a polymer film renders a biosensor feasible for an application for which it would be otherwise unsuitable. Such a film may prevent interferences and electrode fouling that would otherwise preclude its use (interferences are discussed later). The low cost of manufacture of such electrode materials should result in a range of disposable devices for routine use in analytical laboratories in the near future.

4.4.5. Hydrodynamic studies

Even though amperometric signals obtained were characteristic of membrane diffusion processes, it was important to ensure that these currents were due to enzymatically generated H_2O_2, and not to direct electron transfer of urate electrons at the working surface of the CPE. Voltammograms were constructed for four electrode designs over the range -0.1-0.3V (Figure 4.8). The four electrode types were:

1. 100% w/w carbon paste;
2. 93% w/w carbon paste and 7% w/w uricase;
3. 97% w/w carbon paste and 3% w/w HRP;
4. 90% w/w carbon paste, 7% w/w uricase and 3% w/w HRP.

Polymer (PAP) concentration was 5 $\times 10^{-3}$M. Stepwise potential increases (50mV) were applied and the resulting steady state currents (in response to 4 $\times 10^{-4}$M urate)

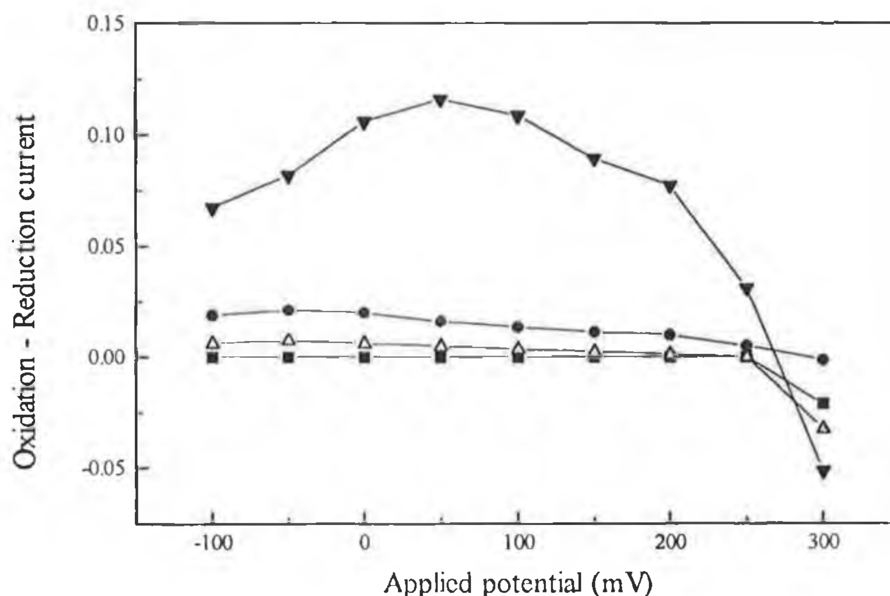


Figure 4.8: Hydrodynamic voltammograms of 4 $\times 10^{-4}$M uric acid recorded in 0.1M phosphate buffer, pH 6.5, for different electrode configurations. All electrodes were modified with 5 $\times 10^{-3}$M poly(*o*-aminophenol). ■ 100% w/w carbon paste; ▲ 7% w/w uricase; ● 3% w/w HRP; ▼ 7% uricase and 3% w/w HRP.

were recorded and plotted versus applied potential (mV). At potentials more negative than -0.2V, a rapid loss of activity occurred, possibly due to the irreversible reduction of HRP [56]. Reduction processes were apparent for all electrodes with the exception of the unmodified CPE. Moreover, the respective electrodes containing uricase and HRP only, exhibited relatively weak cathodic currents in the presence of uric acid. Reduction current was significantly larger in the case of the bienzyme electrode (7% uricase and 3% HRP). The optimum working potential was found to be 50mV. All voltammograms showed a sharp anodic rise in the range 0.30-0.65V (Figure 4.9).

Direct oxidation of uric acid occurred at potentials greater than 0.25V, peaking at approximately 0.5V [22] (for a 100% w/w CPE, no enzyme present).

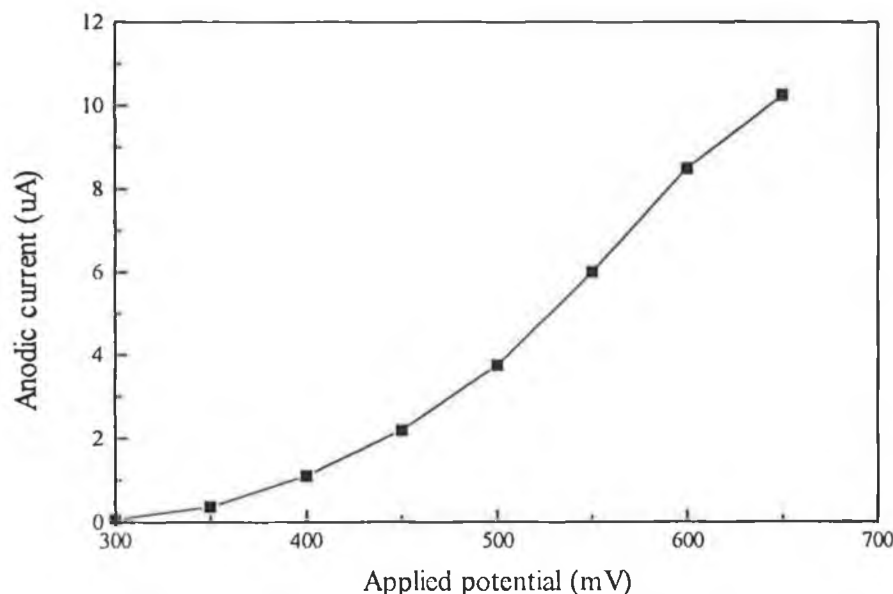


Figure 4.9: Hydrodynamic voltammogram of optimum bienzyme electrode design. Responses to 4 $\times 10^{-4}$M uric acid were recorded in 0.1M phosphate buffer, pH 6.5.

The magnitude of this oxidation current was greater when uricase was added to the carbon paste. In the aforementioned potential range, a great deal of noise due to background currents and potential interference from other electroactive compounds exists. It was previously attempted to elucidate the observed reduction current (at 0.05V) by cyclic voltammetry; however, no cathodic return was visible therefore, under the given experimental conditions, the oxidation of the substrate was considered as being irreversible. These findings were similar to that of Jawad *et al.* [57]. From hydrodynamic studies, it appeared that the magnitude of the urate oxidation wave

masked the reduction process, i.e. CV was not sensitive enough to detect the reduction current (in the range -0.1-0.25V). Figure 4.9 depicts the anodic response of the bienzyme CPE in the potential range 0.3-0.65V. The oxidation of 4 $\times 10^{-4}$M uric acid increased substantially as the potential was increased. It was calculated that the reduction current at 0.05V was approximately 1% of the corresponding oxidation process at 0.65V. The implication of these findings is that the response of the sensor was due to the biocatalytic production of H_2O_2 and not to direct transfer of uric acid to the carbon paste. These results, however, do not show whether direct electron transfer between HRP and the carbon paste has occurred.

4.4.6. Kinetic studies

As no artificial mediator (soluble or insoluble) was added to the sensor configuration, the existence of a naturally occuring mediator for the uricase-peroxidase reaction was investigated. The nature of the electrode has by now been shown not to be due to direct urate transfer to the electrode (implying a bienzyme reaction). At this point, poly(*o*-aminophenol) and uric acid were considered as potential cofactors to the reaction as depicted below.

$$\text{uric acid} + O_2 + 2H_2O \xrightarrow{uricase} \text{allantoin} + CO_2 + H_2O_2 \qquad (4.4)$$

$$H_2O_2 + Med_{red} \xrightarrow{HRP} H_2O + Med_{ox} \qquad (4.5)$$

HRP catalyses the reduction of H_2O_2 in the presence of an electron transfer mediator according to reaction (4.5). The electrochemical reduction of the oxidised mediator generated by the enzymatic reaction provides the amperometric signal for the

measurement of peroxide It is well known that HRP can utilise a wide range of cofactors [27]. Oxidoreductases such as HRP, possess a redox active site [41] capable of undergoing oxidation by the peroxide and reduction by the mediator.

A CPE modified with 3% w/w HRP only was used in all kinetic trials. Various configurations of the HRP electrode were then prepared:

1. A HRP CPE with 5 $\times 10^{-3}$M poly(*o*-aminophenol) electrodeposited at the surface;
2. An electrode similar above except that 5 $\times 10^{-3}$M uric acid was included in the background electrolyte solution (voltammetric cell);
3. A HRP CPE with a cellulose acetate membrane (12000Da cutoff) encapsulating the working surface area instead of the polymer present; also 5 $\times 10^{-3}$M *o*-aminophenol in the bulk solution.

The poly(*o*-aminophenol) modified electrode (number 1) was compared to the cellulose membrane configuration (number 3) employing uric acid as the test mediator. The latter electrode displayed a significantly higher affinity for H_2O_2 (K_m-2.83 $\times 10^{-3}$M). Electrode number one had a K_m value of 2.45 $\times 10^{-5}$M. The reaction rate also increased in the presence of uric acid With polymer-based electrodes, the affinity of HRP for H_2O_2 was not enhanced in the presence of urate. This suggested that poly(*o*-aminophenol) did not act as mediator to the reaction, even though both *o*- and *p*-aminophenol have been reported as being excellent electron donors to oxidised HRP. This was further illustrated when H_2O_2 was added to 0.1M phosphate buffer containing 5 $\times 10^{-3}$M *o*-aminophenol (electrode number 3). The resulting current was anodic in

nature. A relatively small reduction current was observed from an electrode incorporating uricase and poly(*o*-aminophenol) on injection of uric acid. It was therefore concluded that the electrocatalytic reduction of H_2O_2 by HRP immobilised on carbon paste took place without an artificial or naturally existing mediator; thus the nature of the sensor's response was due to direct electron transfer from the enzyme (HRP) to the carbon paste.

Peroxidases are capable of catalysing the oxygenation of a variety of substrates in the presence of H_2O_2. The enzymatic reaction involves Compound I (HRP-I) which contains Fe(IV) and a porphyrin-radical cation, and Compound II (HRP-II) with one Fe(IV) while a donor (AH) is oxidised (see reaction numbers 4.6-4.9).

$$HRP + H_2O_2 \rightarrow HRP\text{-}I + H_2O \quad (4.6)$$

$$HRP\text{-}I + AH \rightarrow HRP\text{-}II + {}^{\bullet}A \quad (5.7)$$

$$HRP\text{-}II + AH \rightarrow HRP + {}^{\bullet}A \quad (5.8)$$

$$HRP + H_2O_2 + 2H^{+} \rightarrow HRP\text{-}I + 2H_2O \quad (5.9)$$

HRP adsorbed on carbon black has been reported for the electrocatalytic reduction of H_2O_2, i.e., the formation of HRP-I (reaction 4.6) and regeneration of HRP by direct electron transfer from the electrode to the hemin Fe(IV) without a mediator [26]. Catalytic current was found to be proportional to peroxide concentration. The overall reaction involves the production of water while electron transfer occurs, thus a current can be measured (reaction 4.9). An intimate contact between HRP and the conducting surface is required to achieve such direct charge transfer between the enzyme and the electrode. Recent studies have documented the possibility of direct electron transfer to the peroxidase active centre. The proposed mechanism is depicted in Figure 4.10. The

elimination of artificial electron transfer mediation is desirable for practical sensing applications. Jönsson-Pettersson described reagentless bienzyme sensors based on HRP adsorbed onto spectroscopic graphite for detecting H_2O_2 and glucose at a very variable applied potential (-50mV) and with short response times [28].

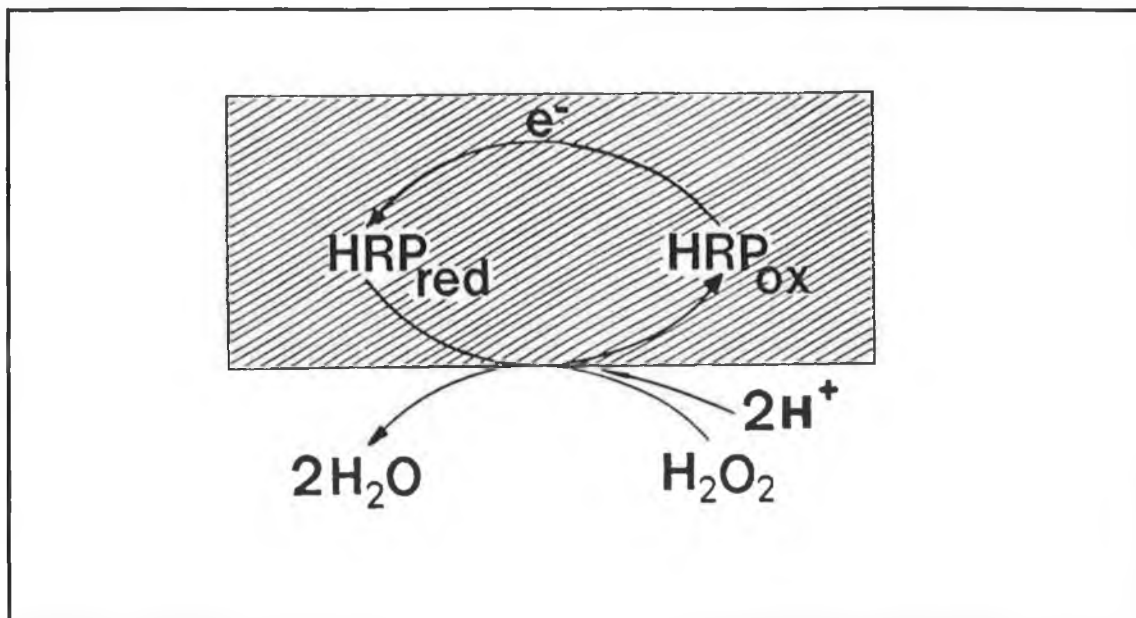


Figure 4.10: Proposed mechanism of direct electron transfer from the electrode to the peroxidase active centre

The mechanism responsible for the electron transfer between HRP and the electrode was unknown. Bogdanovskaya *et al.* have postulated that the protein-bound prosthetic group may be involved in the heterogenous electron transfer, as such signals differ from those obtained from the free prosthetic group and also from that of the apoenzyme [58]. The authors also suggested that the entire adsorbed enzyme is involved in electron transfer, however, Ikeda *et al.* surmised that only a small fraction of adsorbed enzyme molecules have a role in direct electron transfer [59]. This fraction

is thought to be biocatalytically inactive and structurally altered; the greater proportion of enzyme molecules are active and therefore play no role in electron mediation. Detection of H_2O_2 has been described where HRP was entrapped by electropolymerisation of pyrrole [60]. Efficient charge transfer occured between the electrode and HRP without added mediation. When covered with a layer of detecting enzyme (GOD), this modified peroxide electrode served as a glucose sensor which worked well at a low potential.

Wollenberger *et al.* have suggested that electon transfer in reagentless sensors may be aided by surface functionalities on the electrode surface [29]. The feasibility of HRP to facilitate fast electron transfer from different organic peroxides (2-butanone, cumene, hydrogen peroxides etc.) to carbon material when incorporated in the bulk of the electrode is known. Reports also exist on direct transfer between graphite electrodes and peroxidases other than HRP, such as cytochrome c peroxidase [61] and a fungal peroxidase from *Arthromyces ramosus* [62].

Wang *et al.* have described a simple approach to enhance the sensitivity of mediatorless HRP electrodes. Signal amplification is attributed to the accumulation and subsequent detection of the oxidised form of HRP (Figure 4.10) [63]. The biospecific enhancement of the substrate response while facilitating the quantitation of micromolar concentrations of H_2O_2 could also minimise interferences from electroactive species present.

The possible role of surface functionalities in reagentless biosensors has been previously mentioned [29]. Another valid explanation might be that thermal pre-treatment of carbon paste (prior to enzyme immobilisation), which is most efficient for obtaining a catalytic effect, introduces oxygen-containing functionalities on the

electrode surface which are capable of mediating the electron transfer from Compounds I and II (Reactions 4.6 and 4.7) [64]. Thus, it appears that the method for preparing the carbon paste is critical to the efficiency of direct electron transfer.

Examples of reagentless enzyme sensors for glucose, alcohols and amino acids have been reported [64]. The uric acid selective sensor reported here would appear to fit into this class of biosensor. Accordingly, it is an example of a third generation biosensor, which is defined as a device that does not require the addition of any external reagents [41].

4.4.7. pH dependence of biosensor

The response of the biosensor was found to be pH-dependent (Figure 4.11). The range studied was 5.0-9.0. Greater sensitivity was achieved at lower pH values; a best fit approximation suggested an optimum pH of 6.6. However, a "compromise" pH value of 7.5 was chosen in order to mimic the macroenvironment of physiological fluids and to promote the long term stability of the electrode. Noisy baselines were recorded when using background solutions in the pH range 5.0-7.0. As variation in the ionic strength of the electrolyte solution is thought not to have an effect on uricase-HRP sensitivity when immobilised [12], it was not investigated. Nanjo and Guilbault noted, however, that the type of buffer solution employed had an effect on overall sensor response (recall here that the concentration of uric acid was related to the rate of oxygen consumed) [11]. Greater buffer concentrations were directly proportional to decreasing electrode reaction rates. Ammonium sulphate concentration also had an effect on the initial reaction rate, the rate decreasing with a proportional increase in

concentration. This suggested that low ionic media are more favourable for measurements with a uricase *only* electrode.

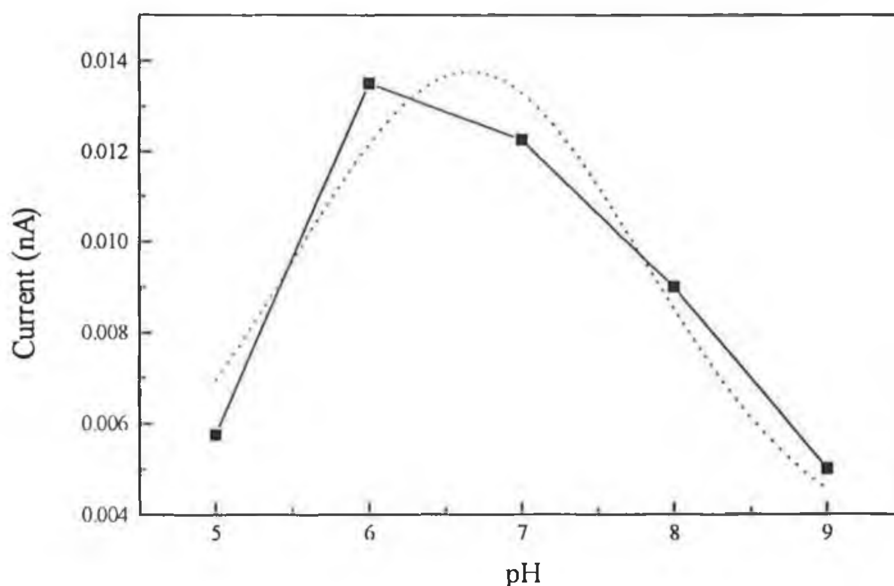


Figure 4.11: Effect of pH on the amperometric response of the PAP-modified carbon paste biosensor. Applied potential - +0.05V. Background electrolye - 0.1M phosphate. ■ Experimental data; ······· Best fit approximation curve.

The pH of a working medium is a factor that affects the response of a biosensor when the enzyme's activity is controlled by ionising groups. It is important to elucidate the best pH range in order to make the electrode more compatible with the matrix of interest. The reported optimum pH of immobilised uricase is in the range 8.5-9.2 [17, 31, 57], where as HRP works well in the range 6.0-8.0 [65]. However, the pH of most physiological fluids lies below this. Keedy *et al.* reported a low pH-dependence for a uricase electrode in solution [12]. It must be remembered that the pH and ionic strength affect not only the immobilised activities, but possibly diffusion of urate to the working surface area of the electrode and also, the rate of direct electron transfer

between HRP and the carbon paste. Thus, it is important to obtain a compromise configuration when designing amperometric biosensors

A large excess of immobilised enzyme in the carbon paste can minimise the effect of pH variations on the measuring process. Therefore, the pH profiles in the linear measuring range (dynamic range) control should, in theory, be substantially less sharp than those of the respective enzymes in solution, i.e. on injecting significantly lower concentrations of uric acid, the response of the sensor is not overly dependent on pH. A strategy towards minimising pH-dependency would be to carry out analysis in the presence of dithiothreitol (DTT), a reducing agent [66]. Increased current responses were reported in increasing DTT concentrations in the sample solution, the amplification factor for a 1×10^{-6}M uric acid solution was increased to 133 when 5×10^{-2}M DTT solution was used. The process involved the regeneration of uric acid by the reduction of the ternary complex consisting of uricase, uric acid and oxygen and by the accompanying formation of the hydroxyl radical from the coordinated oxygen

All amperomeric studies were carried out between 25 and 30^0C. It is known that the rate of reaction can increase with temperature up to a point, above which the effects of thermal inactivation dominate over that of the increase in the collision frequency. Enzyme stabilisation by immobilisation is frequently reflected in an increase in the optimum temperature for substrate conversion. If kinetic and diffusional control are superimposed, the higher collision frequency results in a substantial acceleration of enzyme efficiency with increasing temperature. Therefore, the activation energy determined at lower temperatures is ascribed to the enzyme reaction and that at higher temperatures to diffusion. In addition, the temperature profile is affected by

temperature-dependent conformational changes of the enzyme [65] and decreasing solubility of the cosubstrate

4.4.8. Interference studies

In reactions between the immobilised biomolecule and the sample (which is often a highly complex matrix e.g. serum), undesired binding events or measuring effects may occur. Particulary in biosensors that use coupled enzyme reactions, the substrates of each individual reaction will interfere, therefore, increasing complexity of biosensors results in decreased selectivity Interferences can also occur on the level of the transducer reaction. Nevertheless, an important prerequisite of quantifying an analyte in physiological fluids is a high degree of selectivity

A range of naturally occuring substances in blood were tested for their potential interference effects. Results were compared with a standard uric acid response which acted as a control (5×10^{-4}M) Alternate uric acid injections were made in a voltammetric cell containing 0 1M phosphate buffer (control) and another cell containing a known concentration of the test interferent made up in background solution Ascorbic acid gave approximately 2 and 15% increases in signal for mean (2.8×10^{-4}M) and upper (3.41×10^{-4}M) levels found in serum, respectively. The presence of bilirubin was found to have a detrimental effect on urate amperometric signals. Figure 4.12 illustrates the responses obtained in the presence and absence of the substance. A concentration of 1.7×10^{-4}M bilirubin resulted in a 35% decrease in signal intensity As bilirubin is too big to pass through the polymer layer, it is possible that some form of interaction occurs with uric acid at the working surface, resulting in a diminished current As the bilirubin concentration tested was high and anticipated

physiological levels would be significantly lower, it was hoped that this fact coupled with dilution of serum samples, would minimise this effect. The observed increases in signals in the presence of ascorbic acid may be attributed to changes in the

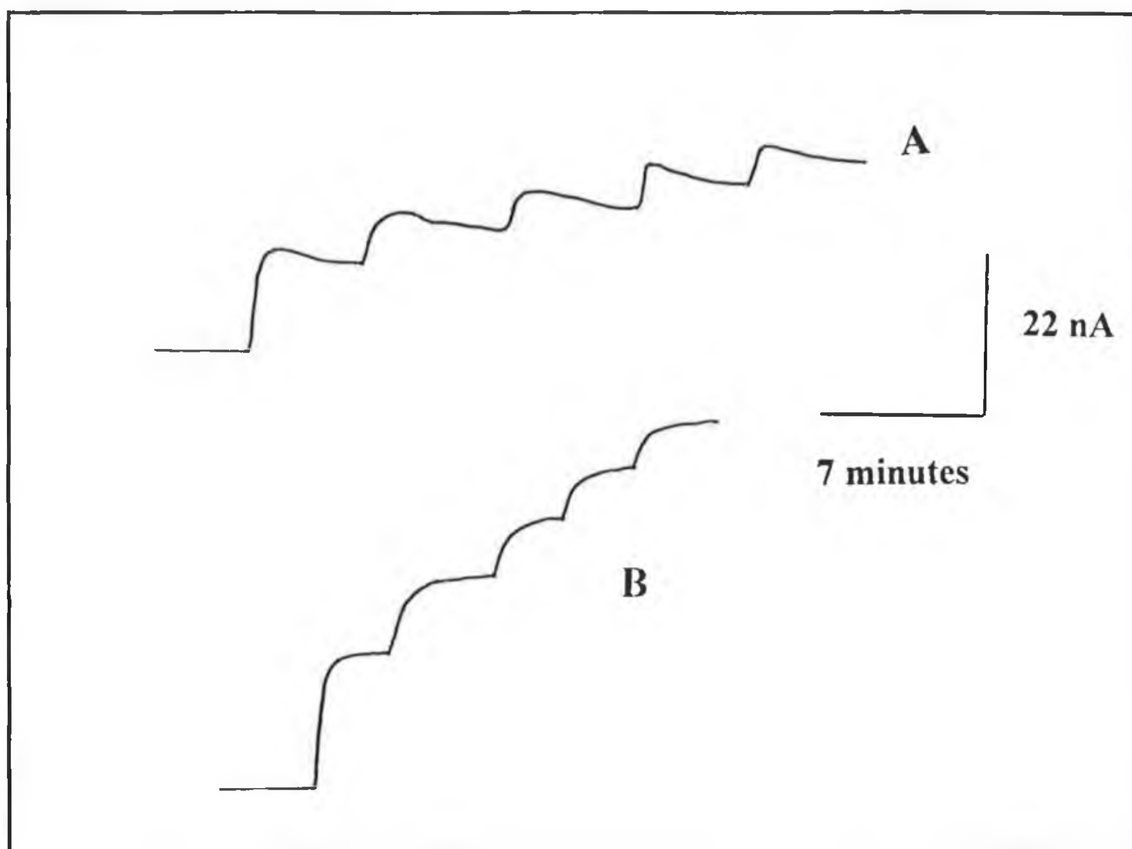


Figure 4.12: Typical amperometric responses in the presence (A) and absence (B) of bilirubin. Multiple injections of 1×10^{-4}M uric acid standards in phosphate buffer. Applied potential - +0.05V

permeability of the polymer layer. Alternatively, the presence of ascorbic acid may in some way interfere with electron transfer between HRP and the paste.

A number of approaches have been employed to prevent interferences. These include using polymeric films to block potential electroactive substances, using a low potential in performing the determination, utilising differential measurement and

removing the interferences by chemical means. Polymeric films prevent interferences from reaching the electrode surface that could otherwise be oxidised. A few examples of the use of such films used in this capacity have been described by Emr and Yacynch[35].

A minor problem that can occur in the analysis of biological samples is electrode fouling. It is defined as "the passivation of the electrode surface by the adsorption of non-electroactive species". High molecular weight compounds such as proteins are a major source of fouling, which can result in a decrease in overall efficiency. Electropolymerised films of nanometer thickness can effectively block interferents and prevent fouling, while minimising problems associated with diffusion. Sasso and co-workers addressed fouling in a biological matrix with an electropolymerised poly(1,2-DAB) film that prevented fouling of the biosensor by proteins in blood serum [66].

The use of an appropriate polymer film to impart biocompatibility is important in preventing interferences from reaching the electrode surface and to preclude fouling. Additionally, for *in vivo* applications, the use of biocompatible polymeric layers can eliminate blood clotting effects as well as immune reaction products.

4.4.9. Calibration characteristics in static systems

Figure 4.13 shows the typical amperometric responses recorded as a function of uric acid concentration. Responses were linear up to a concentration of 1 $\times 10^{-4}$M (Figure 4.14) with a detection limit (L.O.D.) of 3.14 $\times 10^{-6}$M and a correlation coefficient of 0.9996 (n=6). The reproducibility expressed in terms of relative standard deviation was 2.2% (n=4) for a concentration of 2 $\times 10^{-5}$M urate. The sensor had a response time ($t_{98\%}$) of 37 seconds. The signal was stable for 2 days while the electrode was stored in background electrolye at room temperature.

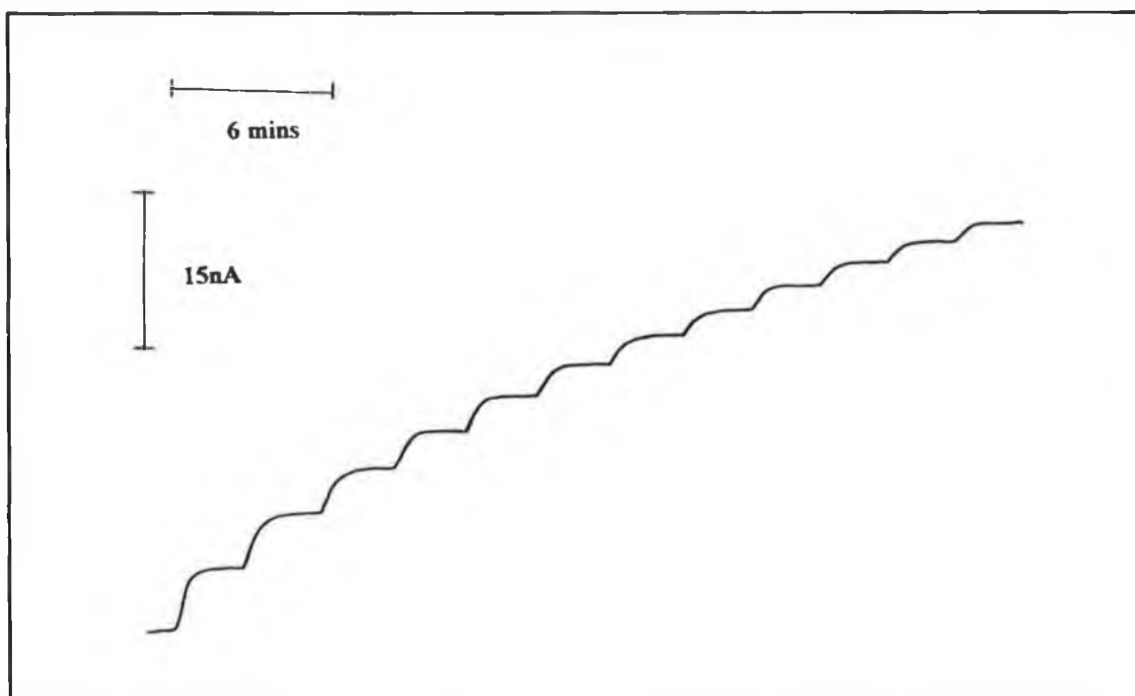


Figure 4.13: Amperometric responses to increasing concentrations of uric acid (10^{-5} to 10^{-4}) at a PAP-modified bienzyme carbon paste electrode at an applied potential of +0.05V. Supporting electrolye was 0.1M phosphate buffer, pH 7.5.

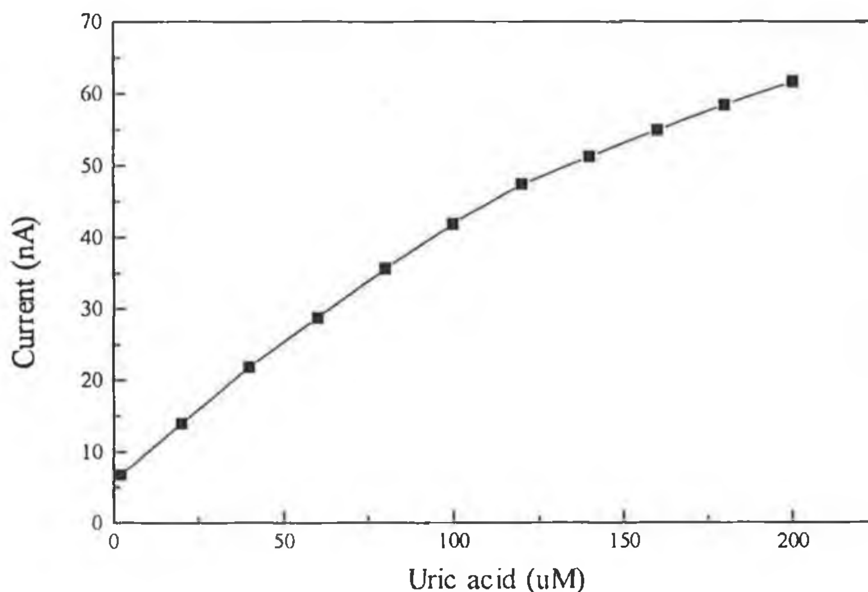


Figure 4.14: Calibration plot for the amperometric determination of uric acid in a static system. Applied potential - +0.05V vs. Ag/AgCl. Supporting electrolyte was 0.1M phosphate buffer, pH 7.5.

The use of polymer films to extend the dynamic range of a biosensor has been readily accomplished by employing a film that blocks a certain percentage of analyte from reaching the working surface. This reduces the response for a given concentration of the analyte, which in effect increases the linear range. The choice of polymer membrane is important as not all films are capable of blocking a portion of a particular analyte.

4.4.10 Application of sensor in a flow injection system

The bienzyme electrode was incorporated as the working electrode in a flow injection (FI) system. Preliminary results suggested that uric acid detection was feasible over a wide range of experimental conditions. The applied working potential was fixed at 0.05V vs. Ag/AgCl. The carrier stream used was 0.1M phosphate buffer, pH 7.5. Carrier solutions were thoroughly degassed with helium prior to operation. Somewhat noisy baslines and high background currents were found with carrier streams in the pH range 5.0-7.0. As with analysis in quiescent solution, peak currents were marginally higher at pH values below 7.0; for a 2×10^{-4}M urate standard, an increase of 33% in signal was noted (using a carrier stream of pH 6.0) relative to injecting the same standard into a carrier stream with a pH of 7.5. Signals in quiescent solution were approximately 19.5% higher.

The effect of varying the flow rate of the carrier stream was examined (Figure 4.15). Three electrodes were tested in the FI system with a % R.S.D. of less than 6.0%. Highest sensitivity for a 5×10^{-5}M injection of uric acid was obtained at a flow rate of 0.85ml min^{-1} with appreciable decreases in responses at higher flow rates. Moreover, operation at high flow rates resulted in distorted baselines. Decreasing the sample loop volume from 500μl to 300μl decreased sensitivity, resulting in a narrowing of the dynamic range and lower detection limits. Use of the smaller loop resulted in slight memory effects. Longer washing times at the expense of sample frequency could alleviate this problem; however, use of the larger loop eliminated this problem. The most suitable monomer concentration for poly(*o*-aminophenol) coating of the electrode surface was 5×10^{-3}M, even though a 10-fold less concentration improved response times and more rapid returns to baseline levels (see Section 4.4.4.).

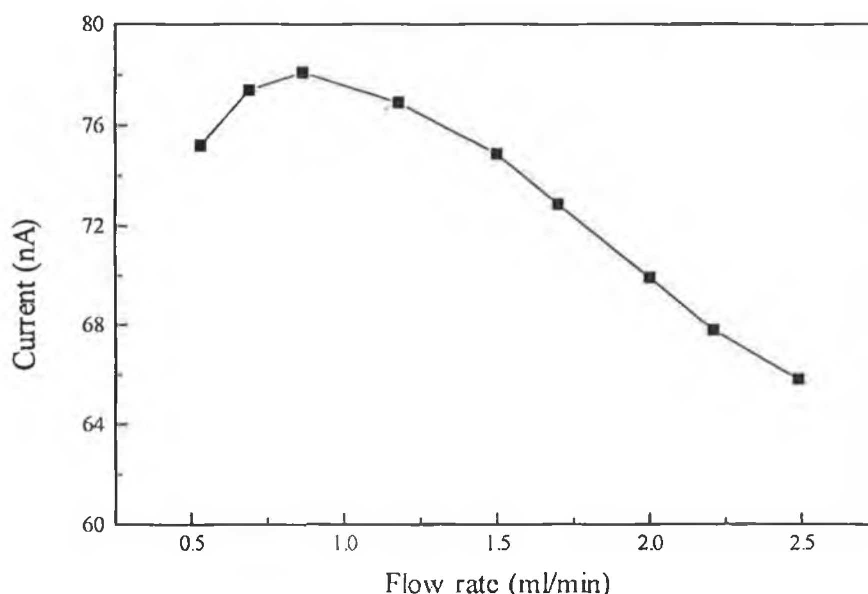


Figure 4.15: Variation of the electrode response with flow rate. Applied potential - +0.05V vs. Ag/AgCl. Uric acid concentration - 5 ×10-5M

The calibration characteristics of the electrode in flowing streams were examined. Figure 4.16 shows the typical signals to uric acid standards. The calibration plot (Figure 4.17) provided a linear response up to 2 $\times 10^{-4}$M with a slope, intercept and correlation coefficient of 2.13 $\times 10^{4}$ nA.L.mol^{-1}, 0.71nA and 0.9996 (n=6), respectively. No linear ranges existed at higher substrate concentrations. The limit of detection was calculated at 6.8 $\times 10^{-6}$M. Increasing the flow rate from 0.85 to 2.0ml min^{-1} reduced the L.O.D. to 8.1 $\times 10^{-6}$M and also the dynamic range. A series of 6 repetitive injections of (1 $\times 10^{-4}$) uric acid yielded a % R.S.D. of 2.0%. The maximum allowable sample frequency was estimated as being 20 per hour. The electrode when incorporated into the FI system, was found to be more stable. This may be partly

explained by the continuous flow of the carrier stream washing unreacted urate from the carbon paste of the working electrode. The influence of allopurinol on urate amper-

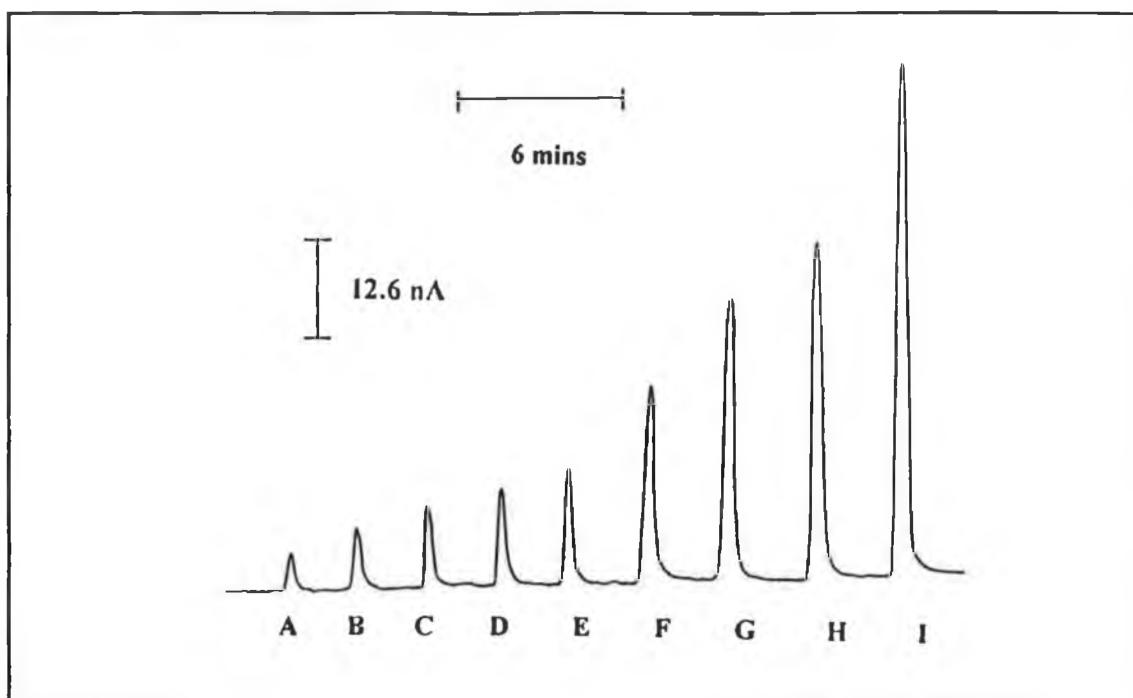


Figure 4.16: Flow injection amperometric responses to uric acid (M) additions. **A** - 2 $\times 10^{-5}$; **B** - 4 $\times 10^{-5}$; **C** - 6 $\times 10^{-5}$; **D** - 8 $\times 10^{-5}$M; **E** - 1 $\times 10^{-4}$; **F** - 2 $\times 10^{-4}$; **G** - 4 $\times 10^{-4}$; **H** - 6 $\times 10^{-4}$; **I** - 8 $\times 10^{-4}$. Carrier stream was 0.1M phosphate buffer, pH 7.5; Flow rate - 1ml min^{-1}; Sample loop volume - 500μl; Applied potential - +0.05V vs. Ag/AgCl.

ometric signals was then studied. Allopurinol, a drug, inhibits xanthine oxidase, in that its oxidised form (oxypurinol) binds tightly to the reduced form of the enzyme The oxidase catalytically converts hypoxanthine to uric acid and peroxide [67]. Allopurinol is used in the treatment of chronic gout and hyperuricaemia (symptoms associated with enhanced urate levels). A high concentration of the oxidase inhibitor (1 $\times 10^{-3}$M) was added to the carrier stream (0.1M phospahte buffer, pH 7.5) No adverse effects such as high background current, basline drift or signal noise were observed Repeated

injections of 1 $\times 10^{-4}$M uric acid were carried out in the presence and absence of the drug. A reduction of 10.5% in response was observed. Two possibilities may account for this: oxidation of allopurinol (thus interfering with the oxidase's catalytic mechanism) and/or blockage or interaction at the working surface. Again, it was hoped that dilution of real samples would alleviate such a problem. Flow injection analysis

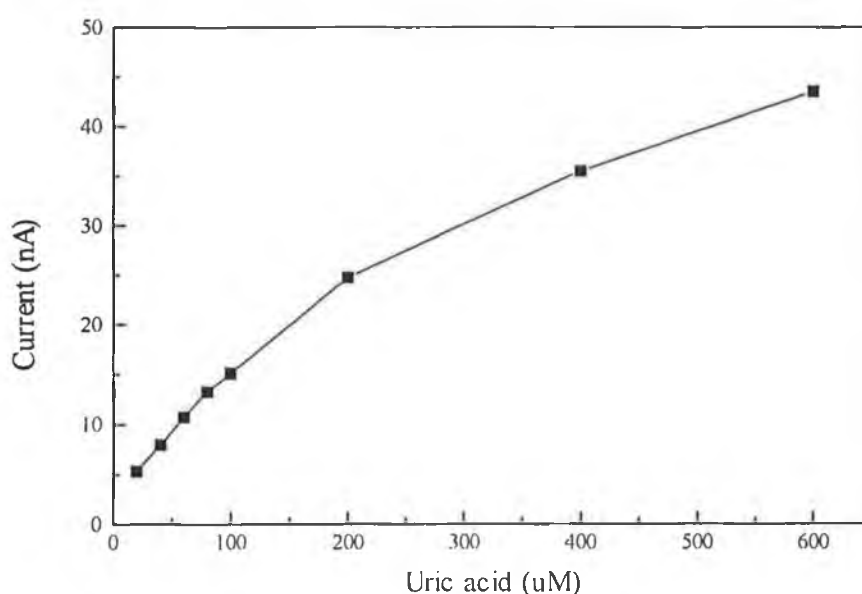


Figure 4.17: Calibration plot for uric acid in a flow injection system. Applied potential - +0.05V; Carbon paste modified with 7% uricase and 3% HRP and coated with 5 $\times 10^{-3}$M *o*-aminophenol.

(FIA) has gained tremendous importance for bioprocess control during the last few years. Although it is not an *in situ* or a real on-line analytical technique, it can be automated and operated at very high analysis cycle frequencies. One of the most important advantages of FIA is the use of very small volumes. The principle of FIA is depicted in Figure 4.18. Defined samples volumes are injected periodically into a buffer carrier stream (C). When an analyte reagent (R) is combined with the buffer stream,

the sample and reagent can react in a reaction coil (RC) before the reaction products are monitored by a detector system (D) Mixing of the sample with the buffer and reagent flow occurs by controlled dispersion The sample is transported through the system by a rapidly flowing buffer stream. It is not necessary for the analytical reaction to reach its equilibrium. When all of the parameters such as

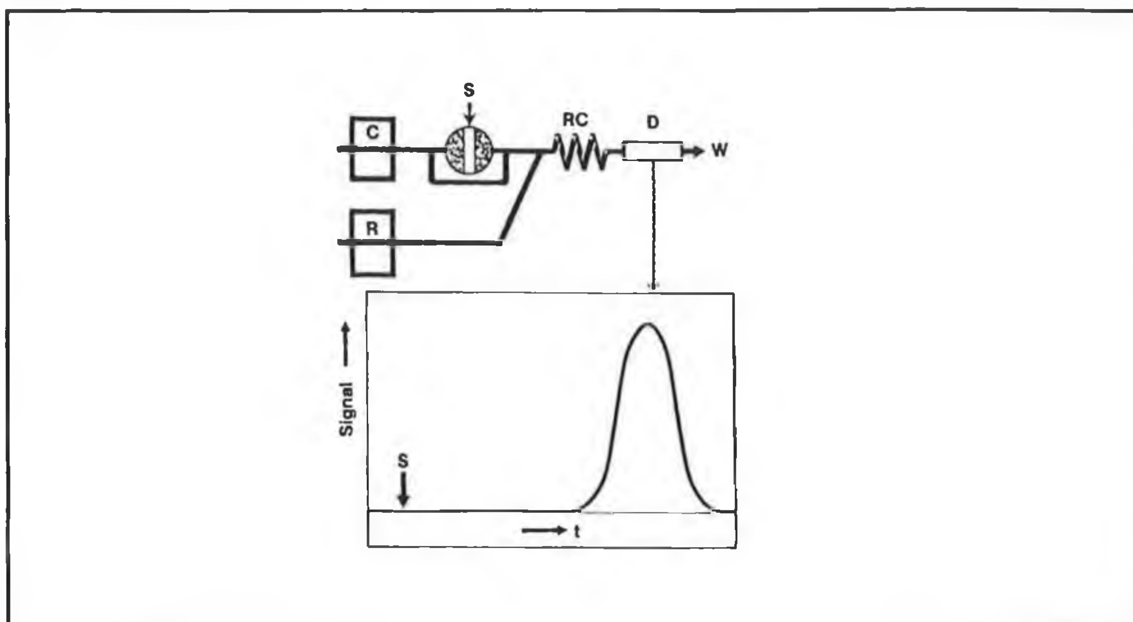


Figure 4.18: Principle of flow injection analysis. (C: carrier flow, R: reagent flow, S: sample, RC: reaction coil, D detector, W: waste

flow rate and reaction time are constant, reliable detection can be made before the reaction equilibrium is attained (dynamic measurement). This results in high frequencies of analysis and short analysis times. Another advantage of FIA is the provision for sample preconditioning before reaction and monitoring. Thus, the reaction can be run under its optimal conditions Sample preconditioning can involve achievement of an optimal reaction matrix and/or an optimal sample dilution [68].

Immobilised enzymes can be used in the detection step. The injected sample passes the immobilised enzyme(s) and the enzymatic reaction is measured by a special

transducer. The system can be recalibrated frequently by injecting calibration solutions instead of a sample. The entire analyte system (enzyme and sensor) is calibrated under real analysis conditions. Deactivated enzyme preparations or sensors can be replaced without any problems.

As previously described in Section 4.4.4, faster response times occured when the polymer concentration at the electrode surface was reduced. Greater concentrations resulted in somewhat delayed returns to the original baseline levels. An explanation for this may be where a sample of the substrate passes the electrode surface, some peroxide could still be produced by the enzyme-substrate complex, i.e. analyte trapping. The dispersion of the flow system at 0.85 and 2.48ml min^{-1} was evaluated from measurements of 5×10^{-5}M uric acid. The dispersion factors [69], given below, of

$$D = \frac{i_{steadystate}}{i_{peak\,max}}$$

the flow system, were 1.0 for a flow rate of 0.85ml min^{-1} and 1.15 for 2.48ml min^{-1}.

4.4.11. Analysis of real samples

The justification for using a bienzyme CPE with a conducting polymer layer relates to the need to overcome electrochemical interference and fouling by sample components such as ascorbic acid, bilirubin and proteins present in physiological samples. Therefore, it was pertinent to evaluate the sensor in analysing real samples.

The biosensor was used for the analysis of uric acid in humam serum and results were compared to a standard UV spectrophotometric method. Samples were diluted 1:10 with the background electrolyte and the pH adjusted to 7.5 (when necessary). Diluted serum samples were then injected directly into the flow system and the concentration of urate present in each sample was estimated using a calibration plot. The data obtained (summarised in Table 4.1) compared favourably with the results determined by UV specrophotometry. Repeated exposure of the electrode to diluted sera had no adverse effect on the size of the amperometric signal. The lower measured urate levels in samples were likely to have been the result of a greater selectivity of the uricase enzyme for its substrate, although an adverse effect of physiological sample components on the electrochemical reaction cannot be ruled out even though membrane fouling was not evident.

To date, analysis of unspiked or real human serum compared as well as, if not better, than other previously reported enzyme-electrode methods for detecting uric acid in a complex physiological matrix. The polymer appeared to have effectively protected the electrode from interferences and fouling. In interference studies, the concentrations of potential interferents tested were much higher than anticipated physiological levels. Moreover, serum samples were simply diluted with background

electrolyte rather than carrying out extraction procedures which are time consuming and restrict the frequency of sampling.

Table 4.1

Determination of uric acid in real serum samples with the poly(*o*-aminophenol) bienzyme carbon paste electrode. Experimental conditions as described in text.

Serum sample	Urate (mg L^{-1}) *	Urate (mg L^{-1}) +	Results (%)
1	5.3	4.9	93
2	4.6	4.1	90
3	5.5	5.2	95
4	6.2	5.6	91
5	3.9	3.7	95

* **Concentration (mg L^{-1}) as determined by UV spectrophotometry at 293nm.**
+ **Concentration (mg L^{-1}) as determined by sensor at +0.05V vs. Ag/AgCl**

Bienzyme electrode flow injection analysis approaches are convenient to use and lend themselves to dealing with large numbers of samples. In particular, the uricase-HRP electrodes offer possibilities for quality control monitoring in the clinical environment.

4.4.12. Conclusion

In this study, a novel reagentless sensor for detecting uric acid has been described based on the co-immobilisation of HRP and uricase in carbon paste. The sensor was incorporated into a flow injection analysis system where the concentration of uric acid in a complex physiological matrix could be determined with great accuracy. It was found possible to amperometrically detect uric acid by immobilising uricase alone; however, such a method required high overpotentials (>0.4V) and would be therefore susceptible to interference from electroactive species present in real samples. It is therefore desirable to construct a sensor that operates efficiently at a favourable potential (<0.25V) and that possesses a short response time.

The response of the biosensor was shown to be due to the biocatalytic production of hydrogen peroxide and not due to direct transfer of urate ions across the polymer film. Peroxide was shown to be electrocatalytically reduced via the relatively facile direct electron transfer from the electrode to the Compound I form of immobilised HRP. HRP is then regenerated by direct transfer from the paste to the hemin Fe(IV) without the necessity for an artificial mediator or cofactor. The precise nature of this mechanism is still somewhat unclear; however, oxygen-containing functionalities on the electrode surface which were introduced during paste preparation may offer some explanation. The composition and procedure for preparing the carbon paste may therefore contribute to heterogenous electron transfer. Due to its configuration, the electrode may be classed as a third generation biosensor as no external reagents were added to the system.

Conducting polymers can be electrodeposited at the working surface of a CPE with great precision. This film functions in retaining both immobilised enzymes in the

bulk of the electrode. Poly(*o*-aminophenol) was stable for a considerable length of time when stored in background electrolye at room temperature. Physical characteristics such film thickness could be easily controlled thus regulating the rate of analyte diffusion to the immobilised biocatalytic layer. Sensor response times could also be controlled by regulating the polymer thickness. Amperometric biosensors are in general limited by interferents present in complex physiological matrices such as serum. Poly(*o*-aminophenol) appeared to adequately protect the electrode from interferences and fouling.

The HRP-uricase based sensor was successfully used to determine the concentration of uric acid in human serum with great accuracy. Results compared favourably with a standard UV spectrophotometric method, regardless of the fact samples were simply diluted in buffer rather than pursuing arduous extraction techniques.

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CHAPTER 5

CONCLUSIONS

Native HRP is a moderately stable enzyme. The initial aim of this project was to investigate the effects of chemical crosslinkers and non-crosslinkers on HRP activity, and to determine the effects of these reagents on HRP thermostability. A range of amino-specific reagents were used. Reaction of HRP's lysine amino acids with both bifunctional and monofunctional succinimides did not result in any loss in catalytic activity; also, the treated HRP fractions were more heat-stable and these stabilisations persisted on storage at a low temperature (4^0C).

All chemical derivatives of HRP displayed a greater level of thermostability when exposed to temperatures of at least 55-60^0C for extended periods. Modified HRPs also resisted solvent-induced activity loss much more successfully than did native HRP. The solvents used in this investigation (MeOH, DMF and THF) may act in a similar fashion to destabilising ions, in that they disrupt the water "shell" surrounding the HRP molecule (which is thought to be critical for catalysis), leading to unfolding and loss of function. Derivatives also possessed greater stability in the presence of denaturing, reducing and chelating agents.

The number of free amino groups in all HRP derivatives decreased after reaction with succinimide compounds. Bifunctional reagents (SA-NHS and EG-NHS) modified 4-5 of HRP's lysines whereas the acetylating agent (acetic acid N-hydroxysuccinimide ester) modified 3 residues. These results implied that two crosslinks at most could have formed in the case of bifunctional HRP derivatives. From both experimental data and the literature, it could be concluded that the degree of stabilisation obtained is related to the molecular length of the chemical crosslinker. Increased stability in adverse conditions (with AA-NHS HRP) was thought to be due to charge neutralisation, as the acetylating reagent is unable to form molecular

crosslinks in proteins. Fluorescence and UV/Visible spectroscopy have also pointed to changes in the unfolding characteristics of protein derivatives after bis-succinimide modification.

Succinimide-treated HRPs have also been shown to increase the amount of phenols removed from aqueous systems at high temperatures. Overall, acetylated HRP had a marginally better ability to catalyse the removal of toxic aromatics; this may be attributed to the absence of intramolecular crosslinks, the presence of which may have in some way interfered with the efficiency of the enzyme's catalytic cycle. Crosslinks may slow down or even block the diffusion of aromatic molecules to the active site, thus limiting the degree of free radical formation. It is therefore possible that the restriction on free radical formation is related to the frequency and length (Å) of such bonds. Nevertheless, the difference in phenol oxidation between acetylated and bifunctional HRPs was relatively minor.

Native HRP was shown to be an effective component in a biosensor for detecting uric acid (an end product of purine metabolism). The sensor had the advantage over more conventional enzyme-based devices in that it operated efficiently without the necessity for an electron transfer mediator (cofactor). The exact nature of this mechanism is unclear, but oxygen-containing functionalities on the working surface area of the electrode are thought to play an important role in electron mediation. Heterogeneous electron transfer between the electrode and the Compound I form of HRP is thought to depend on the exact procedure for preparing the carbon paste (the temperature of the graphite prior to adding paraffin oil is important). As the biosensor was observed to operate in this fashion, it can be classed as a third generation biosensor, i.e. no external reagents were required for its operation. The

electrodeposition of *o*-aminophenol at the surface of the carbon paste electrode (as opposed to employing more conventional membranes) appeared to protect the biocomponent from interferences and fouling

The commercial potential of biosensors varies greatly from one application to another. However, the entire biosensor concept has been slow to be accepted, mainly due to non-acceptance in clinical circles A number of problems may exist in developing a market for biosensing devices, such as product recalls, entrenched competition, legislative problems, poor marketing or distribution, and especially poor production design. However, the potential of these devices could be very great indeed if good marketing strategies were put in place and also if all technical difficulties were overcome.

Chemical methods employed in protein stabilisation could greatly benefit biosensor development. Preventing the unfolding of the protein chain is of great importance in attempting to improve the stability of designated enzymes. A coordinated effort involving both specific chemical alteration and immobilisation on solid supports (or within a matrix) could be beneficial in improving the overall performance of a biosensor. A modified enzyme is considerably less prone to unfolding under adverse conditions even though it is capable of a certain degree of movement necessary for efficient biocatalysis However, gross conformational changes are prevented by covalent attachment to an insoluble, inflexible solid support. The chemical modification strategy described above could be used in conjunction with an immobilisation technique, as succinimides do not react with HRP's carbohydrate side chains (18% of total enzyme). A dual approach could therefore be advantageous, in that an improvement in storage (working lifetime) and operational stability could be

attained, i.e. the production of a robust and reusable device capable of operating under field conditions. The future direction of research will no doubt be concentrated on non-invasive self-contained biosensors where physiologically important metabolites such as uric acid could be quantified through direct analysis of saliva or sweat.

Similar dual stabilisation strategies could be beneficial in treating phenol effluents. As described in Chapter 3, a chemically stabilised and/or immobilised peroxidase could be applied to a continuous flow reactor where effluent is constantly pumped through the system. In this way, maximum utilisation could be obtained from the biocatalyst. Careful consideration of the stabilisation strategies to be undertaken could eventually produce peroxidases with bioactivity and tolerance levels as good as (if not better than) bacterial or fungal cells. A HRP system for purifying phenol effluent could be complemented by a stabilised peroxidase-based biosensor for selectively detecting individual phenols or a group of phenols.

It is likely that combining different stabilisation strategies (chemical modification, immobilisation, use of additives, protein engineering etc.) will become increasingly important in biotechnology processes. Stabilisation can be implemented based on one or a combination of procedures. Manufacturing processes may eventually require specially stabilised biocomponents for designated tasks. Specific chemical modification may not alone alter the protein's conformation but also its active centre, resulting in an enzyme derivative with greater biocatalysis or even multifunctional capabilities.

The specific chemical modification of HRP described here has yielded derivatives with very high thermostability and tolerance of water-miscible organic solvents. These HRP derivatives have demonstrated the feasibility of employing HRP

in the removal of phenols from aqueous solutions. The potential also exists for applications in bioorganic biosynthesis (in the presence of high concentrations of water-miscible organic solvents, for example, the production of phenolic polymers, by the HRP system, may be useful as thermoresistant resins or as conductive polymers). Stabilisations (such as these) would certainly be beneficial to areas such as those mentioned above and others including neurohistochemistry, DNA labelling and immunology.

Appendix

List of Publications

1. Increased thermal and solvent tolerance of acetylated Horseradish peroxidase.
   Enda Miland, Malcolm R. Smyth and Ciarán Ó Fágáin
   Enz. Microb. Technol. (1996) in press

2. Modification of Horseradish peroxidase with bifunctional N-hydroxysuccinimide esters: effects on molecular stability.
   Enda Miland, Malcolm R. Smyth and Ciarán Ó Fágáin
   Enz. Microb. Technol. (1996) in press

3. Poly(*o*-aminophenol) bienzyme carbon paste electrode for the detection of uric acid.
   Enda Miland, Arturo J. Miranda-Ordieres, Paulino Tuñón-Blanco, Malcolm R. Smyth and Ciarán Ó Fágáin
   Talanta (1996) in press

4. Phenol removal from aqueous systems at high temperatures by chemically modified Horseradish peroxidases.
   Enda Miland, Malcolm R. Smyth and Ciarán Ó Fágáin
   J. Chem. Tech. Biotechnol. submitted for publication